Autoregulatory properties of 5-HT neurons and their modification by antidepressant treatments: focus on 5-HT release and uptake

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a la memoria de Angela...

... y de Joaquín

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Article VI.

Piñeyro, G. and Blier, P. (1996) Regulation of [^{3}H]5-HT release from rat midbrain raphe nuclei by 5-HT_{1D} receptors: Effect of tetrodoxin, G protein inactivation and long-term antidepressant administration. *Journal of Pharmacology and Experimental Therapeutics* **276**:697-707.

Article VII.

Piñeyro, G., Castanon, N., Hen, R. and Blier, P. (1996) Regulation of 5-HT release in raphe, frontal cortex and hippocampus of 5-HT_{1B} knock-out mice. *Neuroreport* 7:353-359.

SUMMARY

The present research project was designed to analyse some of the mechanisms implicated in the autoregulation of serotonin (5-HT) function in rats and mice. It focuses on i) the study of 5-HT reuptake activity following the long-term administration of the selective 5-HT reuptake inhibitor (SSRI) paroxetine and the tricyclic drug tianeptine, and ii) autoreceptor-mediated control of 5-HT release at the cell body level and its modifications following long-term antidepressant treatments.

The adaptative properties of the 5-HT transporter were assessed using a combined methodological approach of *in vivo* binding studies as well as [^{3}H]5-HT uptake assays. Such an approach indicated that long-term administration of an SSRI: a) reduced the efficacy of paroxetine to prolong the time for recovery of firing activity of CA_3 pyramidal neurons following the suppression induced on this parameter by microiontophoretic application of 5-HT, b) reduced the amount of [^{3}H]5-HT captured by hippocampal and midbrain raphe slices, and iii) reduced the number of 5-HT transporters in hippocampus and cortex. From these results, it was concluded that prolonged administration of paroxetine down-regulated 5-HT transporters. On the other hand, the sustained administration of the tricyclic drug tianeptine, which enhances 5-HT uptake activity, did not induce any long-lasting changes either in hippocampal 5-HT uptake activity, or in the efficacy of 5-HT synaptic transmission in this terminal projection field.

A second series of studies was then undertaken to assess how is extracellular availability of somatodendritic 5-HT regulated in *in vivo* electrophysiological and voltammetric experiments. The non-selective 5-HT agonist TFMPP inhibited 5-HT release in the dorsal raphe nucleus, independently of the firing activity of 5-HT neurons. *In vitro* superfusion experiments, using midbrain raphe slices from rats and mice (wild type and 5-HT_{1B} knock-out) were then used to assess pharmacological and functional properties of this non-5-HT_{1A} receptor. It was concluded that $G_{i/o}$-coupled 5-HT_{1D} autoreceptors negatively regulate 5-HT release in midbrain raphe nuclei of rats and mice, and that these receptors desensitise following prolonged administration of SSRI's and monoamine oxidase inhibitors (MAOI's).

Résumé

Le présent projet de recherche fut conçu pour étudier certains mécanismes impliqués dans l'autorégulation de la fonction des neurones sérotoninergiques (5-HT) chez le rat et la souris. L'étude s'est concentrée sur l'évaluation i) des effets produits par l'administration prolongée d'un inhibiteur sélectif de la recapture de 5-HT, la paroxétine, et d'une molécule tricyclique, la tianeptine, sur la recapture de la 5-HT, et ii) l'autorégulation de la libération de 5-HT au niveau des corps cellulaires et ses modifications suivant l'administration soutenue de différents antidépresseurs.

La capacité d'adaptation du transporteur 5-HT fut aussi explorée à l'aide d'une combinaison de techniques électrophysiologiques *in vivo*, d'expériences de capture, et de radioliaison *in vitro*. Cette stratégie a permis de démontrer que l'administration à long-terme d'un inhibiteur de la recapture de la 5-HT: a) produit une diminution de l'efficacité de la paroxétine à prolonger le temps de récupération de la fréquence de décharge des neurones pyramidaux de l'hippocampe suite à l'application microiontophorétique de 5-HT, b) réduit la quantité de [^{3}H]5-HT captée par des coupes d'hippocampe et mésencéphale ainsi que le nombre de sites marqués par la [^{3}H]paroxétine dans des membranes d'hippocampe et cortex frontal. À partir de ces résultats, nous concluons que la réduction de la fonction de la récapture de la 5-HT est due à une diminution du nombre de sites de recapture. Par ailleurs, l'administration prolongée de tianeptine, une substance qui augmente l'activité de la recapture de la 5-HT, n'a pas produit de modifications fonctionnelles soit de la recapture de 5-HT ou de l'efficacité de la neurotransmission sérotoninergique dans l'hippocampe.

Une deuxième série d'études fut réalisée afin d'évaluer la modulation de la disponibilité extracellulaire de la 5-HT au niveau somatodendritique. Des expériences électrophysiologiques et voltammétriques *in vivo* ont indiqué l'activation des récepteurs non-5-HT$_{1A}$ par un agoniste 5-HT non-sélectif inhibe la libération de 5-HT dans le raphé dorsal sans modifier la fréquence de décharge des neurones 5-HT. Des études de superfusion de tranches de mésencéphale de rats et de souris (normaux et transgeniques manquant le récepteur 5-HT$_{1D}$) ont été utilisées pour évaluer les caractéristiques fonctionnelles et pharmacologiques de ce récepteur. Nous avons constaté que ce sont des autorécepteurs de type 5-HT$_{1D}$ qui contrôlent négativement la libération

somatodendritique de 5-HT sans changer la fréquence de décharge des neurones 5-HT. Ces récepteurs se désensibilisent après l'administration soutenue d'un bloqueur de la recapture 5-HT où d'un inhibiteur de la monoamine oxydase de type A.

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AUTHORSHIP

I actively participated in the intellectual conception of the research projects, obtainment and processing of results, and production of all manuscripts included in this Thesis.

Drs. de Montigny and Blier supervised electrophysiological approaches. Dr. Blier also supervised superfusion and voltammetry experiments, and Dr. Dennis was in charge of radioligand-binding procedures. Dr. Weiss contributed with the results to produce Figure 1 in manuscript number four.

Dr. Hen provided the transgenic 5-HT_{1B} knock-out mice, and Nathalie Castanon did their genotyping. Lynne Deveault produced part of the experimental results included in manuscripts two and three.

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INTRODUCTION

The general working hypothesis proposed by the Neurobiological Psychiatry Unit states that different antidepressant treatments enhance the efficacy of 5-HT neurotransmission (see Blier and de Montigny; 1992). According to this hypothesis, tricyclic antidepressant drugs and electroconvulsive shocks do so by increasing the sensitivity of postsynaptic 5-HT_{1A} receptors (the predominating subtype in limbic areas such as the hippocampus, see Jacobs and Azmitia; 1992), without inducing any changes at the presynaptic level (de Montigny and Aghajanian, 1978; Blier and de Montigny, 1990; Blier and Bouchard, 1992). On the other hand, SSRIs and MAOIs enhance 5-HT transmission by modifying presynaptic modulatory factors. Recovery from the initial decrease in normal firing rate of 5-HT neurons produced by SSRIs and MAOIs together with an increased 5-HT release in terminal projection areas (Artigas, 1993; Bel and Artigas, 1993; Ferrer and Artigas, 1994) are the main changes that have been implicated in the antidepressant action of both of the latter drugs (Blier and de Montigny; 1994). The mechanisms by which they may induce an increase in 5-HT released per action potential are different for both groups: SSRIs decrease the responsiveness of terminal 5-HT autoreceptors (Blier et al., 1988; Chaput et al., 1988) and MAOIs induce a desensitization of release-inhibiting α_2-adrenergic heteroreceptors on 5-HT terminals as well as increasing the tissue content of 5-HT (Mongeau et al., 1994). 5-HT_{1A} agonists, like buspirone and gepirone have also been claimed to possess antidepressant activity (Robinson et al., 1990; Jenkins et al., 1990) and their antidepressant properties have been attributed to somatodendritic 5-HT_{1A} autoreceptor desensitization and recovery of 5-HT neuron firing activity following long-term administration of 5-HT_{1A} agonists (Blier and de Montigny, 1987). Unlike somatodendritic 5-HT_{1A} autoreceptors, postsynaptic 5-HT_{1A} receptors in the hippocampus do not desensitize following long-term activation (Blier and de Montigny, 1987). Therefore, 5-HT_{1A} agonists further increase the efficacy of 5-HT neurotransmission via an enhanced activation of postsynaptic 5-HT_{1A} receptors located in limbic areas, such

as the hippocampus (Blier and de Montigny, 1994). This enhanced activation is achieved by the stimulating effect of the exogenous 5-HT_{1A} agonist acting together with endogenously released 5-HT following normalization of the firing activity of 5-HT neurons.

The goal of the present research project was two-fold: i) to describe alternative ways in which antidepressant treatments might modify classically described autoregulatory properties of 5-HT neurons (see articles I, II, and III) and ii) to investigate new autoregulatory processes of 5-HT neurotransmission as well as the way in which they might be modified following prolonged antidepressant administration (see articles IV, V, VI and VII).

To attain the first goal our strategy was to investigate whether apart from inducing autoreceptor desensitization prolonged modification of 5-HT uptake activity could induce adaptive changes of the 5-HT transporter. The drugs used were the SSRI paroxetine and the tricyclic drug tianeptine. Paroxetine and tianeptine were chosen because they modify the 5-HT uptake activity in opposite directions. These studies are described in articles I and II. Article III was devoted to study the effect of prolonged tianeptine administration on the efficacy of 5-HT synaptic transmission in the rat hippocampus. Pre- and postsynaptic components of this limbic 5-HT synapse were explored using the electrophysiological paradigm proposed by Blier and de Montigny as an experimental approach for identifying the mechanism of action of antidepressant drugs.

The other subject of interest in the present research project was the characterization of new presynaptic determinants of the efficacy of 5-HT synaptic transmission. As previously described, 5-HT neurons are under potent autoregulatory control. Specifically, at the somatodendritic level, as a result of their role in inhibiting 5-HT neuronal firing rate, local activation of 5-HT_{1A} autoreceptors by endogeneous 5-HT influences the efficacy of 5-HT neurotransmission in forebrain projection areas (Artigas, 1993). Furthermore, drugs like SSRIs or MAOIs only enhance 5-HT synaptic transmission once these autoreceptors become desensitized, allowing for autoregulatory negative feedback to be overcome. In spite of the crucial role played by the somatodendritic 5-HT_{1A} autoinhibitory feedback in the physiology and pharmacology of the 5-HT system, the regulation of extracellular levels of 5-HT in the midbrain raphe nuclei is not well known.

The first series of experiments was devoted to clarify whether somatodendritic availability of 5-HT could be regulated independently of 5-HT_{1A} receptors controlling 5-HT

neuron firing activity (see article IV). The aim of the next studies was to determine the pharmacology of the non-5-HT_{1A} receptors controlling extracellular somatodendritic concentrations of 5-HT in the rat midbrain raphe nuclei (V) and to characterize their functional properties (VI). In the last part of the present research endeavour, 5-HT_{1B} knock-out mice were used to investigate whether in mice, as we had found in rats, 5-HT_{1D} receptors are involved in the regulation of [^{3}H]5-HT release from midbrain raphe nuclei (VII). Additional experiments in terminal projection areas also suggested that terminal 5-HT release could be regulated by multiple 5-HT receptor subtypes.

REVIEW OF THE LITERATURE

Introduction

The existence of an endogeneous vasoconstrictor in blood serum (Stevens and Lee, 1884; Brodie 1900) and of a substance in the gut, enteramine, that increases intestinal motility (Vialli and Erspamer, 1933) had been known by scientists since the beginning of the century. However, it was not until the serum vasotonic substance was identified as 5-hydroxytryptamine (5-HT; Rapport et al., 1948) that the it became evident that 5-HT was also present in the mammalian CNS (Twarog and Page, 1953). Shortly after its discovery in the CNS, and based on the observation that it was heterogeneously distributed in dog brain (Bogdansky et al., 1956), it was proposed that 5-HT might act as a neurotransmitter in the brain. A major turning point in 5-HT neurotransmission research then followed: Fuxe and Dahlström employing Falck-Hillarp histochemical fluorescence provided the first description of 5-HT neurons (Dahlström and Fuxe, 1964) and their projections (Fuxe, 1965). Today, it is an established fact that no region in the mammalian CNS appears to lack 5-HT innervation (Dalhström and Fuxe, 1964; Steinbusch, 1981; 1984; see Azmitia, 1986; Jacobs and Azmitia, 1992; Whitaker and Azmitia, 1995).

The ubiquity of 5-HT as a neurotransmitter is not only anatomical but also phylogenetic. Having been identified in neurons of the cnidarian *Renilla koellikeri* (Umbriaco et al., 1990), 5-HT could be one of the most ancient of currently known transmitters. From primates (see Azmitia and Gannon, 1986; Törk, 1990) to *Chondrichtyes* (Stuesse and Cruce, 1992), the adult 5-HT system is organized into two subsystems: a rostral division with cell bodies localized in the midbrain and rostral pons, providing projections to the forebrain, and a caudal division located primarily in the medulla oblongata with descending projections to the spinal cord and brainstem nuclei. The similarities found among such divergent vertebrate brains indicate that the major nuclear organization and the 5-HT projection network have remained remarkably stable across phylogeny. It would thus appear that results from anatomical and physiological studies performed in different subprimate species could be easily extrapolated to the primate brain. However, though evolutionary stability of the 5-HT system (and of the other monamine systems; Jacobowitz and MacLean, 1978) is indeed remarkable, careful scrutiny of comparative evidence indicates that differences are as important as similarities. The phylogenetic differences have been summarized in the following way: in higher mammals

the system has evolved towards a fast, precise type of neurotransmission in which 5-HT neurons give rise to few collaterals (Fallon and Loughlin; 1982), they have a high proportion of myelinated axons and due to the existence of a higher proportion of junctional synapses, terminal field innervation is localized. In lower mammals the system is diffuse, highly branched, unmyelinated and non-junctional innervation predominates in terminal fields (see Azmitia 1986; Descarries et al., 1990; Törk, 1990; Jacobs and Azmitia, 1992; Azmitia and Whitaker-Azmitia, 1995).

Because 5-HT neurons are amongst the first neuroblasts to differentiate, the 5-HT system is also ontogenically ancient. Indeed, in the rat brain 5-HT-immunoreactivity appears as early as gestational day twelve (Olsen and Seiger, 1972; Lidov and Molliver, 1982), tryptophan hydroxylase activity is already present since fertilization and nearly every embryonic cell contains 5-HT until the gastrula stage (Harris, 1981). Furthermore, early developmental events such as differentiation, migration and outgrowth of 5-HT neurons establish the foundations for the adult organization of the serotonergic system. The first 5-HT neurons to appear are located rostrally, within the mesencephalon (Lidov and Molliver, 1982) and it is only two days after that a more caudal rhombencephalic group appears (Wallace and Lauder, 1983). In the next sections focus will be set on the rostral group.

A. Morphological aspects of the 5-HT rostral system

i) Rostral 5-HT nuclei

Dahlström and Fuxe (1964) divided 5-HT cell clusters into nine groups (B_{1-9}), B_1 being the most caudal group of cells. Today, however, the nomenclature most frequently used refers to 5-HT cells contained within cytoarchitectonic brainstem entities known as the raphe nuclei. The correspondence between the B group classification and the current anatomical designation of rostral serotonergic nuclei in the brainstem (according to Törk, 1990 and Jacobs and Azmitia, 1992) are shown in Table 1. Rostral 5-HT neurons are not confined to midline (raphe) nuclei, they are also present in more lateralized sites of the reticular formation, especially: i) dorsal to the medial lemniscus (rat: Dalström and Fuxe, 1964; Parent et al., 1981; squirrel monkey: Hubbard and DiCarlo, 1974; humans: Baker et al., 1990a) and ii) dorsal to the nucleus raphe pontis oralis (rat: Lidov and Molliver, 1982; humans: Baker et al., 1991).

TABLE 1

Designation of serotonergic cell groups in the raphe nuclei and brainstem reticular formation and the corresponding classification into the B groups

Cytoarchitectonic structure containing 5-HT neurons	B group classification
Median raphe nucleus, caudal part	B_5*
Median raphe nucleus, rostral main part	B_8*
Dorsal raphe nucleus, caudal part	B_6
Dorsal raphe nucleus principal, rostral part	B_7*
Caudal linear nucleus	B_8
Nucleus pontis oralis	B_8/B_9*
Supralemniscal region	B_9

The structures marked * form part of the Nucleus Centralis Superior as defined by Olzewki and Baxter in 1954.

Modified from Törk, 1990

The *caudal linear nucleus (CLN)*, described in primates by Törk (1990) and Azmitia (see Azmitia, 1986; Azmitia and Gannon, 1986; Jacobs and Azmitia, 1992 and Azmitia and Whitaker-Azmitia, 1995) is the most rostral group of serotonergic neurons in the mesencephalic midline, extending along the rostral boundary of the superior cerebellar decussation (Figure 1). Its ventral limit is defined by the interpeduncular nucleus and dorsally it comes in contact with the dorsal raphe nucleus (DRN) through the gap left between the two medial longitudinal fasciculi. In rats, 5-HT neurons located rostral to the decussation of the superior cerebellar peduncle and above the interpeduncular nucleus have been considered in some cases a rostral extension of the median raphe nucleus (MRN; Lorez et al., 1978; Parent et al., 1981). However, CLN and MRN neurons do not share common terminal projection fields (Imai et al., 1986) and display dissimilar dendritic morphology (Hornung and Fristchy, 1988). On the other hand, since DRN and CLN 5-HT neurons innervate similar terminal fields (e.g. caudate-putamen in adult rat brain; Imai et al., 1986) and share a common developmental origin (Wallace and Lauder; 1983), another approach proposes that CLN neurons should be considered similar to those in DRN (see Jacobs and Azmitia, 1992).

The *dorsal raphe nucleus (DRN)* is the largest of the brainstem serotonergic nuclei containing about 50% of the total 5-HT neurons in rat CNS (Wiklund and Björklund, 1980; Descarries et al., 1982), 40% in the cat CNS (Wiklund et al., 1981) and 50%-60% in humans (Baker et al., 1990a). Rostrally, the DR is bound by the Edingher Westphal nucleus (III) and caudally it extends just ventral to the confluence of the fourth ventricle and the cerebral aqueduct (Steinbusch, 1981; Descarries et al., 1982; Imai et al., 1986; Törk, 1990 and Jacobs and Azmitia; 1992; Figure 1). In most species the DRN is composed of several subregions distinguished by their different cell density, morphology and projections (Azmitia and Gannon, 1986; Baker et al., 1990b; Johnson and Ma, 1993): i) a medial portion, subdivided in turn into dorsomedial and ventromedial components just below the cerebral aqueduct and surrounding the medial longitudinal fasciculus (MLF), respectively ii) lateral portions or wings (much more prominent in primates than other mammals, due to a lateralization process through phylogeny; Descarries et al., 1982; Azmitia and Gannon, 1986; Baker et al., 1991) and iii) a caudal component (Figure 1). During development (at 15 days gestation) 5-HT immunoreactive cells group themselves into two different clusters, dorsolateral and ventrolateral to the MLF (Wallace and Lauder,

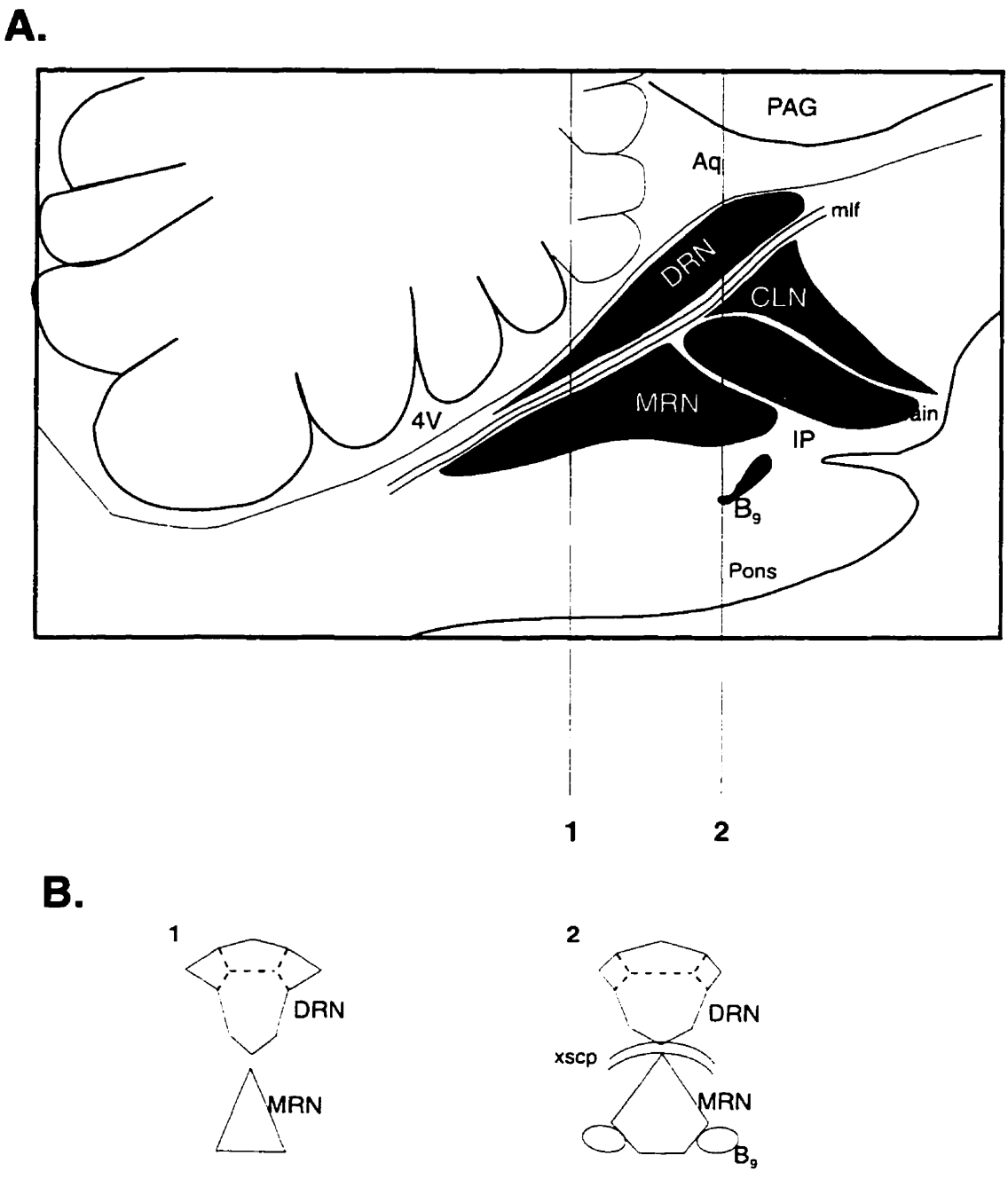


Figure 1. Schematic representation of midbrain serotonergic cell groups in rat raphe nuclei and reticular formation. **A.** Sagital section (modified from Törk, 1990). B. Coronal sections at the corresponding 1 and 2 anteroposterior levels (modified from Johnson and Ma, 1993). 4V: fourth ventricle, Aq: aqueduct, mlf: midbrain longitudinal fasciculus, PAG: periaqueductal grey; xscp: superior cerebellar peduncle decussation..

1983). The dorsolateral portion will give rise to the lateral wings of the DRN while the ventrolateral group will split to form the interfascicular portion of DRN and MRN. For this reason, Azmitia proposes that in the primate CNS (see Jacobs and Azmitia, 1992; Azmitia and Whitaker-Azmitia, 1995) the interfascicular subregion of the DRN should be best considered an entity together with the MRN. Olzewski and Baxter (1954) had previously defined an anatomical entity, nucleus centralis superior (NCS), consisting of three groups of cells: i) a dorsalis component situated between MLF, ii) a medialis component or MRN and iii) a lateralis component which includes the 5-HT cells that form the ring around the nucleus reticularis pontis oralis. The absence of anatomical boundary between B_7 (the main rostral portion of the DRN) and B_8 (the main rostral portion of the MRN) has also been observed in rats (Descarries et al., 1982) and cats (Wiklund et al., 1981). The main rostral component of the DRN (B_7) also merges, in its rear end, with the caudal component (B_6) of this same nucleus (see Jacobs and Azmitia, 1992; Azmitia and Whitaker, 1995). Furthermore, B_6 is in continuity with the dorsocaudal portion of the MRN (see Jacobs and Azmitia, 1992; Azmitia and Whitaker-Azmitia, 1995) and the lateral wings of the DRN (Lidov and Moliver, 1982). This anatomical proximity between different 5-HT cell clusters provides the ground for a network of homotypic interconnections among 5-HT neurons. These interconnections constitute in turn the morphological basis for the high degree of collaboration existing at the somatodendritic level. The major implication of this arrangement is that the most closely-knit and the larger the number of cells in a functional cluster, the more powerful it will be the effect of the group on the target structure towards which it projects (Azmitia, 1986).

The *median raphe nucleus (MRN)* consists of two distinct parts: one group of densely packed cells, vertically oriented and concentrated in the midline, and the paramedian columns: a group of cells scattered in the periphery of the midline cluster (Köhler and Steinbusch, 1982). The nucleus is situated ventral to the MLF and has a rostrocaudal oblique orientation (Figure 1; Azmitia, 1981; Lidov and Molliver, 1982). It is separated from the DRN in its rostral end by the superior cerebellar peduncle decussation (xscp). Its medial portion extends from the mesencephalic interpeduncular nucleus to the trapezoid body in the pons. Laterally the limits of the nucleus are poorly defined towards the reticular formation (Köhler and Steinbusch, 1982), and in the rat the MRN merges laterally with the 5-HT group in *nucleus raphe pontis oralis* (Lidov and Molliver, 1982). The

MRN forms the second largest cluster of 5-HT neurons in the mammalian CNS (Baker et al., 1990a).

The last group of 5-HT neurons to be considered corresponds to 5-HT cells in the *supralemniscal region*. In the rat, 5-HT cells belonging to this group are scattered along the dorsal border of the medial lemniscus and extend their dendrites in between the fibre bundles of the latter (Parent et al., 1981; Steinbusch, 1981). A similarity between supralemniscal 5-HT cells and those of the median raphe nucleus was first noted by Dahlström and Fuxe (1964) in the rat CNS, and has been confirmed for the squirrel monkey (Hubbard and DiCarlo, 1974). In fact the cells in the supralemniscal region may be continuous with those of the paramedian columns of the MRN (Azmitia and Gannon, 1986). Unlike rodents in which the supralemniscal cell cluster is predominantly mesencephalic, in humans it is entirely located in the pons (Baker et al., 1991).

ii) Ultrastructure

In all species studied thus far 5-HT-containing neurons have been found to be a morphologically heterogeneous population (Steinbusch, 1981; Jacobs et al., 1984; Azmitia and Gannon, 1986; Törk and Homung, 1990). The average cell diameter varies from 15-25 µm (Azmitia, 1978; Descarries et al., 1982) and ultrastructurally "5-HT specificity does not closely correlate with any particular neuronal configuration and/or intracellular build-up" (Descarries et al., 1982). No unique element allows to separate 5-HT from non-5-HT surrounding neurons (Azmitia, 1978).

The average number of axosomatic boutons received by 5-HT neuron perikarya in the DRN has been quantified by Descarries et al. (1982). According to his group, 100 µm of somatic membrane receive seven axonic boutons. The average number of spines on the same membrane length is 2.7 (Park et al., 1982). On the dendrites, the number of axonic boutons/100 µm membrane length is roughly 60% higher than on the soma (Descarries et al., 1982). In cats and rats the fibres contributing to these axosomatic or axodendritic contacts are non-5-HT fibres (Descarries et al., 1982; Chazal and Ralston, 1987). In primates, on the other hand, Kapadia et al. (1985) have described 5-HT fibres impinging on 5-HT dendrites.

Sources of extracellular 5-HT in rostral raphe nuclei.- The existence of serotonergic axon terminals in the raphe nuclei of cats and rats has been repeatedly reported (Baraban and Aghajanian, 1981; Chan-Palay, 1982; Descarries et al., 1982; Chazal and Ralston,

1987) and the number of them endowed with synaptic specializations has been found to be consistently low, ranging from nihil (Baraban and Aghajanian, 1981), "exceedingly small number" (Descarries et al., 1982) to "a few" (Chazal and Ralston, 1987). Furthermore, Baraban and Aghajanian (1981) found 5-HT fibres exclusively in axon bundles rather than in proximity to dendrites or cell bodies, and when 5-HT terminals were observed to make somatodendritic synaptic contacts these were on non-5-HT neurons (Descarries et al., 1982; Chazal and Ralston, 1987). In the rare cases in which a 5-HT axon terminal was observed in close apposition to a 5-HT cell body no demonstrable synaptic contact was present (Chazal and Ralston, 1987). Non-synaptic 5-HT axon terminals are not exclusive to the raphe nuclei, they also exist in other structures of the CNS such as cortex, striatum and hippocampus (Descarries et al., 1990; Törk et al., 1990) and it has been suggested that 5-HT release could occur not only from junctional but also from non-junctional sites (Descarries et al., 1975; 1982; 1990). It may be also possible however, that the non-junctional fibre profiles are simply vesicle containing dilations that do not release any transmitter at all.

The total number of 5-HT axons that reach the rostral raphe nuclei (independent on whether they are endowed or not with junctional specializations) as well as their origin is still controversial in the literature. In autoradiographic studies, Descarries et. al. (1982) reported that "only few" [^{3}H]5-HT labelled axon terminals reached the DRN, Chan-Palay (1982) found "numerous" such terminals, while Baraban and Aghajanian (1981) reported prominent labelling of unmyelinated, 5-HT axons. On the other hand, using an immunohistochemical procedure Brusco et al., (1983) found only few 5-HT axon terminals in rat DRN while Chazal and Ralston (1987) concluded that, in the cat, the latter are "widely distributed and not uncommon," though "not numerous as compared to non-5-HT terminals". Furthermore, the fine structure of fibres impinging on the DRN was similar to that of 5-HT fibres found in terminal projection areas (Beaudet and Descarries, 1981), a fact that should be bared in mind when considering whether 5-HT fibres that impinge on the rostral 5-HT nuclei are collaterals or afferents from other 5-HT nuclei. In primates, using immunocytochemistry, Kapadia et al. (1985) have also reported few 5-HT axon terminals in the DRN.

In four out of the five abovementioned studies, the number of 5-HT fibres impinging on the raphe nuclei was found to be scarce. However, the extracellular concentration of

5-HT at the somatodendritic level is twice as much as that observed in projection areas such as the cerebral cortex (5.5 nM and 2.3 nM, respectively; Bel and Artigas, 1992), where the amount of axon terminals is of 5.8 x 10^6 varicosities/mm^3 (Descarries et al., 1990). It is then difficult to conceive that 5-HT fibres (independent of whether they are collaterals or afferents from other nuclei) would be the only source of extracellular 5-HT in the dorsal raphe. An alternative source of extracellular 5-HT in the raphe area might be the soma and dendrites of 5-HT neurons. Indeed, Chazal and Ralston (1987) have reported the existence of 5-HT neurons with vesicle-containing dendrites in the cat DRN. In turn, they divided these dendrites into two different types: a) dendrites in which vesicles were never found to be associated with any membrane specialization, and b) vesicle-containing dendrites which had synaptic membrane specializations. It was not determined whether the two types of dendrites belonged to the same neuron. It is possible that the different types of dendrites could serve different functions: the ones lacking any junctional specialization could contribute to maintain high extracellular-extrasynaptic somatodendritic concentrations of 5-HT, while those bearing specializations could constitute the anatomical basis for dendrodendritic homotypic interactions between 5-HT neurons. In fact, though sometimes vesicle-containing dendrites were found to contact non-5-HT elements, most frequently they were presynaptic to other 5-HT dendrites (Chazal and Ralston; 1987). Apposition between 5-HT dendrites has also been described in rats (Descarries et al., 1982) and rabbits (Felten and Harrigan, 1980), as well as in primates (Kapadia et al., 1985). Synaptic specializations on dendrodendritic contacts were recognized in cats (Chazal and Ralston, 1987) as well as in the primate brain (Kapadia et al., 1985) although 5-HT dendrites containing vesicles were found only in the cat CNS. Despite these vesicle-containing dendrites being present throughout the DRN, they were infrequent as compared to dendrites that did not contain vesicles. Furthermore, 5-HT dendrodendritic synapses constituted a small portion of the total DRN synapses (Chazal and Ralston, 1987). Similarly, it has been observed that in the substantia nigra only few of the total dendrodendritic contacts were interconnections between dopaminergic neurons (Wilson et al., 1977). Still, K^+-induced depolarization evokes significant dopamine release from these dendrites (Chéramy et al., 1983). A neurotransmitter-releasing role for 5-HT dendrites was initially proposed by Wang and Aghajanian (1977a; 1978) who observed that inhibition of 5-HT neuron firing activity following the stimulation of the ascending 5-HT

pathway in the ventrotegmental area (VTA) was abolished by the 5-HT synthesis inhibitor p-chlorophenylalanine and restored by 5-hydroxytrypyophan. It was also proposed that the latter inhibitory effect could be mediated by recurrent 5-HT axon collaterals. Since their initial reports (Wang and Aghajanian, 1977a; 1978), recurrent inhibition of 5-HT neuron firing activity has been confirmed using intracellular (Park et al., 1982) and extracellular (Piñeyro at al., 1996) recordings. In the former study, the injection of horseradish peroxidase into a representative 5-HT neuron that showed recurrent inhibition following VTA stimulation, revealed a single collateral arising from the neuron's axon. Although this observation supports the existence of axon collaterals, it is not evident that they constitute the only or even an important anatomic substrate for recurrent inhibition of 5-HT neuron firing activity. Furthermore, two major Golgi studies in rat and rabbit (Felten and Cummings, 1979; Díaz-Cintra et al., 1981) described "only a few" axon collaterals.

Cell bodies found in the 5-HT nuclei.- Quantitative studies of the total number of 5-HT neurons located in the ascending raphe nuclei indicate that there are about 288,000 in human brain (Törk et al., 1990), 33,000 in the cat brain (Wiklund et al., 1981) and 15,200 in the rat brain (see Jacobs and Azmitia, 1992). These 5-HT neurons represent however, but a percentage of the total neuronal population in the raphe nuclei. Using histofluorescence imaging techniques, Wiklund et al. (1981) reported that in cat brainstem 5-HT neurons constitute 70% of medium-sized cells in the dorsal raphe and 35% of medium-sized neurons of the median raphe nucleus. This percentage could be lower, serotonergic cells constituting 25-50% of the total DRN neuronal population and 20 -30% of the MRN's. With respect to the supralemniscal 5-HT cell group (B_9), the percentage of 5-HT neurons appears much lower than in the other two nuclei (O'Hearn and Molliver, 1984).

Non-5-HT cells reported in the mesencephalic raphe nuclei include peptidergic and non-peptidergic neurons. The most numerous non-5-HT perikarya are the peptidergic enkephalin-immunoreactive cells (Uhl et al., 1979; Moss et al, 1981; 1983). Tanaka et al. (1993) have shown that most of the enkephalin and 5-HT immunoreactivity in dorsal and median raphe nuclei do not colocalize, thus indicating that enkephalin and 5-HT immunopositive cells should be best considered distinct neuronal populations. Other peptides contained in DRN somata include: i) substance P, abundant in lateral wings (Moss, 1983, Magoul et al., 1986) and rostral portion of the nucleus (Ljungdahl, 1978). It

is worth noting that in bulbospinal neurons substance P reduces the affinity and increases the density of [^{3}H]5-HT binding sites (Agnati et al., 1983), indicating that the neuropeptide released from 5-HT neurons directly modulates the effectors of 5-HT responses, ii) neurotensin is found in cells dorsal to the MLF at mid- and caudal levels of the DRN (Beitz, 1982), iii) neurons positive for VIP are few and located just ventral to the aqueduct (Moss et al., 1983; Sims et al., 1980), iv) somatostatin- and CCK-positive cells are both found within the periaqueductal grey, but few of them lie within the DRN (Vanderhaeghen et al., 1980).

There is also consistent evidence indicating the existence of dopamine (DA) positive cells in the midbrain raphe nuclei (Hökfelt et al., 1976; Ochi and Shimizu, 1978; Miachon et al., 1984; Trulson et al., 1985; Descarries et al., 1986). Except for Miachon et al. (1984) who reported "few tyrosine hydroxylase-positive ß-hydroxylase-negative neurons," there is general agreement that DA cell bodies in the midbrain raphe are numerous. Two primary subpopulations of DA neurons have been described: i) A_{10dc} lying on the extreme dorsal border of the rostral half of the DRN, ventral to the cerebral aqueduct and ii) A_{10c} occupying the medial aspect of the DRN and extending dorsocaudaly from the ventrorostral border of this nucleus, where the cells appear contiguous with those of A_{10} (Hökfelt et al., 1984). Similar to A_{10} DA neurons, DA-containing cells in rat DRN project to nucleus accumbens (Stratford and Wirtshafter, 1989) and neostriatum (Descarries et al., 1986) with a low degree of collateralization. This projection pattern differs from that of surrounding 5-HT immunoreactive neurons which show profuse collateralization, and innervate other structures such as septum (de Olmos and Heimer, 1980) prefrontal cortex (O'Hearn and Molliver, 1984; Waterhouse et al., 1986; Imai et al., 1986) as well as neostriatum (Jacobs et al., 1978; Imai et al., 1986). Stratford and Wirtshafter (1989) have thus suggested that DRN DA cells represent a caudal extension of A_{10} and hence a different population from 5-HT neurons. Furthermore, no colocalization of 5-HT and tyrosine-hydroxylase was observed in the raphe nuclei (Vanhalato et al., 1995). Norepinephrine-positive cells have not been found within the DR, but are located just caudal to the lateral wing groups at the limits of the locus coeruleus (Grzanna and Molliver, 1980).

The existence of GABA in midbrain raphe nuclei has been repeatedly reported (Massari et al., 1976; Belin et al., 1979; Gamrani et al., 1979; Nanopoulos et al., 1982;

Harandi et al., 1987). In fact, Massari et al. (1976) have shown that there is as much glutamate decarboxylase activity (GAD, which represents a specific marker for GABAergic neurons; Fonnum and Walberg, 1973; Riback et al., 1976) in DRN as in cerebellar nuclei (the projection from Purkinje cells to deep cerebellar nuclei being one of the first documented GABAergic pathways; see Storm-Mathisen; 1974). The failure to obtain important changes in GAD activity after lesions of the afferents to the DRN, and the presence in this nucleus of terminals as well as dendrites and nerve cell bodies accumulating [^{3}H]GABA (Belin et al., 1979), support the notion that the GABAergic network is predominantly intrinsic to the raphe. GABA-positive cell bodies are less numerous than those for 5-HT (Belin et al., 1979; Harandi et al., 1987). In one of the abovementioned studies, 40% of the GABA-positive somata were found to contain 5-HT and 30% of the 5-HT-containing neurons immunostained for GABA (Harandi et al., 1987). Hence, it was proposed that some neurons could be both GABAergic and serotonergic. Coexistence of 5-HT and GABA in midbrain raphe neurons has been repeatedly reported (Nanopoulous, 1982; Gamrani et al., 1984; Harandi et al., 1987; Gao et al., 1993), however, the proportion and the nuclei in which colocalization occurs varied among different studies. For example, electron microscopy studies revealed coexistence of both neurotransmitters in the somata of DRN neurons (Harandi et al., 1987). On the other hand, confocal microscopy studies revealed that in DRN and MRN 5-HT and GABAergic neurons constitute two largely distinct populations, double-labelled neurons being observed only within the raphe magnus, raphe obscurus and raphe pallidus nuclei (Gao et al., 1993). The latter results have been confirmed by Stamp and Semba (1995) who also observed that in midbrain raphe sections only a very small percentage of 5-HT neurons in the medullary raphe nuclei also contain GABA (raphe magnus is the nucleus where the percentage of colocalization is highest and it reaches only 3.6%). In the latter study, double-labelled cells were virtually absent in the midbrain raphe nuclei constituting 0.1-0.7% of the total number of cells in DRN, MRN and supralemniscal region. Low occurrence of colocalization of 5-HT and GABA has also been found in cultured 5-HT neurons obtained from the ponto-mesencephalic region of neonatal rats (Johnson, 1994a). The use of postnatal rat midbrain-pontine 5-HT-neuron culture has also provided evidence for co-release, and hence colocalization, of 5-HT and glutamate from single 5-HT neurons (Johnson, 1994b). However, the major drawback of the latter technique, is that the

possibility of glutamatergic function having developed in culture may not be ruled out. Coexistence of 5-HT and glutamate in rat and primate brain has been observed in medullo-spinal pathways (Nicholas et al., 1992). 5-HT has also been found to colocalize with the trace amine tryptamine (Dabadie and Geffard, 1993) and with basic fibroblast nerve growth factor (bFGF) in DRN and MRN (Chadi et al., 1993). It is well known, however that bFGF lacks the signal peptide to be released by the classical exocytotic pathway used by neuropeptides (Mignatti et al., 1992). Thus, bFGF could be released from 5-HT cells by a non-exocytotic pathway or when the cell is injured. Rather than a neurotransmitter, bFGF would then act as an autocrine and paracrine factor which elicits trophic responses. Indeed, a neurotrophic role has been postulated for 5-HT itself (see Jacobs and Azmitia, 1992; Azmitia and Whitaker Azmitia, 1995; for review). Finally colocalization of 5-HT and NADPH diaphorase in mesopontine neurons suggest that 5-HT neurons and nitric oxide may be used as neurotransmitters by the same neuron (Johnson and Ma, 1993; Wotherspoon et al., 1994).

iii) Afferents to the midbrain 5-HT nuclei

Afferent connections of the raphe nuclei have been studied using multiple techniques: lesion and axon degeneration, histofluorescence, anterograde/retrograde tracer injection, autoradiography and immunohistochemistry (Brodal et al., 1960; Fuxe, 1965; Aghajanian and Wang, 1977b; Mosko et al., 1977; Sakai et al., 1977; Baraban and Aghajanian, 1981; Kalen et al., 1985; Stratford and Wirtshafter, 1988; Marzienkiewicz et al., 1989; Behzadi et al., 1990). Results from such studies have been summarized in Table 2.

Interconnections among raphe nuclei using retrograde tracing techniques suggest a moderate to high density 5-HT input. These observations contrast with those from most of the immunocytochemical or radioautographic ultrastructural studies indicating a low number of 5-HT fibres in the rostral raphe. Mosko et al. (1977) have shown that the main source of 5-HT fibres reaching the DRN arise either from the DRN itself or the MRN. The latter input has been confirmed by other groups (Sakai et al., 1977, Aghajanian and Wang, 1977b and Kalen et al., 1985; Vertes and Kocsis, 1994). However, given the proximity between injection and labelled sites it cannot be ruled out that tissue damage and tracer diffusion may account for part of the retrograde staining observed in the MRN following tracer injection into the DRN. Furthermore, since retrograde transport of HRP by dendrites

TABLE 2

Afferents to the Dorsal Raphe nucleus

Structure of origin	Projection density	Neurotransmitter implied	References
Telencephalic afferents			
Retina			Foote et al., 1978; Shen and Semba, 1994
Prefrontal cortex	scarce	possibly Ach	Arnsten and Goldman-Rakic, 1984
Subcortical forebrain afferents			Aghajanian and Wang, 1977
Diagonal Band of Brocca	moderate - high low	possibly Ach	Aghajanian and Wang, 1977 Kalen et al., 1985
Stria terminalis	scarce	EAA	Aghajanian and Wang, 1977 Kalen et al., 1985
Diencephalic afferents			
Hypothalamic areas			
lateral area	moderate high	EAA	Sakai et al., 1977, Berk and Finkenstein, 1982 Kalen et al., 1985
dorsomedial area	high moderate	EAA	Sakai et al., 1977 Kalen et al., 1985
parvocellular nucleus	moderate - low		Kalen et al., 1985
magnocellular nucleus	moderate - low		Kalen et al., 1985
perifornical area	moderate moderate - low		Sakai et al., 1977 Kalen et al., 1985
preoptic area	low moderate - low		Aghajanian and Wang, 1977 Kalen et al., 1985
arcuate nucleus	low		Sakai et al., 1977; Saper et al., 1979
submammillothalamic nucleus	low		Kalen et al., 1985
N. Habenularis			
lateralis	high	EAA, GABA, Substance P	Aghajanian and Wang, 1977, Sakai et al., 1977
medialis	–		Herkenham and Nauta, 1977; Neckers et al., 1979; Stern et al., 1981; Kalen et al., 1985; Nishikawa and Scatton, 1985; Park, 1987; Kalen et al., 1989
Brainstem afferents			
periaqueductal periventricular grey	low	EAA Neuropeptides	Kalen et al., 1985
Laterodorsal Tegmental Nucleus	low		Sakai et al., 1977
VTA Sustantia Nigra	low - moderate moderate - low	DA, EAA	Aghajanian and Wang, 1977; Sakai et al., 1977; Kalen et al., 1985
Locus Coeruleus and subcoeruleus	high	NE	Roizen and Jacobowitz, 1976; Sakai et al., 1977; Grzanna and Molliver, 1980; Baraban and Aghajanian, 1981; Kalen et al., 1985
Nucleus Tractus Solitarius / A_2	moderate	NE	Aghajanian and Wang, 1977; Sim and Joseph, 1989; Hebert and Saper, 1992
Paragigantocellular nucleus / C_1, C_2 - C_1		E	Sim and Joseph, 1989; Hebert and Saper, 1992
Parabrachial nuclei	moderate - high		Sakai et al., 1977; Kalen et al., 1985
Raphe nuclei			
caudalis linearis	low	5-HT	Sakai et al., 1977
dorsalis	moderate	5-HT	Mosko et al., 1977
medianus centralis superior	moderate - high	5-HT EAA	Mosko et al., 1977; Sakai et al., 1977; Aghajanian and Wang, 1977; Kalen et al., 1985; Kalen et al., 1988; Vertez and Martin, 1988
magnus	moderate - high	5-HT	Sakai et al., 1977 Kalen et al., 1985
Vestibular nuclei	moderate - low		Sakai et al., 1977 Kalen et al., 1985
prepositus hypoglossy	moderate - low		Kalen et al., 1985
Nucleus principalis trigemini	moderate - low		Kalen et al., 1985
Area dorsolateral to inferior olive	moderate - low		Sakai et al., 1977; Kalen et al., 1985

TABLE 2 (cont)

Afferents to the Median Raphe nucleus

Structure of origin	Projection density	Neurotransmitter implied	References
Cortical afferents			
Prefrontal cortex	moderate - high	possibly Ach, EAA	Aghajanian and Wang, 1977; Marzienkiewicz et al., 1989; Behzadi et al., 1990
Subcortical forebrain afferents			
Lateral Septum	scarce	EAA	Marzienkiewicz et al., 1989; Behzadi et al., 1990
Diagonal Band of Brocca	moderate - high low	possibly Ach non-cholinergic	Marzienkiewicz et al., 1989; Kalen and Wiklund, 1989 Behzadi et al., 1990
Stria terminalis	scarce	EAA	Marzienkiewicz et al., 1989; Behzadi et al., 1990
Amygdala	moderate - low		Hopkins and Holstege, 1878; Marzienkiewicz et al., 1989; Behzadi et al., 1990
Nucleus accumbens	low	EAA	Marzienkiewicz et al., 1989; Behzadi et al., 1990
Ventral pallidum	moderate		Behzadi et al., 1990
Diencephalic afferents			
Hypothalamic areas			
dorsomedial area	high	EAA	Marzienkiewicz et al., 1989; Behzadi et al., 1990
perifornical area	high	EAA	Marzienkiewicz et al., 1989; Behzadi et al., 1990
preoptic area	high	EAA	Aghajanian and Wang, 1977; Villalobos and Ferssiwi, 1987; Marzienkiewicz et al., 1989; Behzadi et al., 1990
arcuate nucleus ventromedial nuclei tuber cinereum	low		Marzienkewicz et al., 1989; Behzadi et al., 1990
premammillary, tuberomammillary supramammillary, submammillo- thalamic nuclei	high		Marzienkewicz et al., 1989; Behzadi et al., 1990
N. Habenularis			
lateralis	high	EAA	Aghajanian and Wang, 1977; Herkenham and Nauta, 1977; Marzienkiewicz et al., 1989; Behzadi et al., 1990
medialis	low		
Brainstem afferents			
periaqueductal periventricular grey	moderate - high	EAA	Marzienkiewicz et al., 1989; Behzadi et al., 1990
Laterodorsal Tegmental Nucleus	high		Marzienkiewicz et al., 1989
VTA Sustantia Nigra	moderate - low moderate - low	DA	Aghajanian and Wang, 1977; Marzienkiewicz et al., 1989; Behzadi et al., 1990
interpeduncular nucleus	high	EAA	Marzienkiewicz et al., 1989; Behzadi et al., 1990
Locus Coeruleus and subcoeruleus	high	NE	Marzienkiewicz et al., 1989
Parabrachial nuclei	moderate		Marzienkiewicz et al., 1989; Behzadi et al., 1990
Raphe nuclei			
caudalis linearis rostralis linearis	low moderate	5-HT 5-HT	Marzienkiewicz et al., 1989
B_6	moderate - high	5-HT / other	Stratford and Wirthshafter, 1988 Marzienkiewicz et al., 1989
dorsalis	high	5-HT	Marzienkiewicz et al., 1989; Behzadi et al., 1990; Vertes and Kocsis, 1994
pontis, magnus	moderate	5-HT	Marzienkiewicz et al., 1989; Behzadi et al., 1990
obscurus	low	5-HT	Marzienkiewicz et al., 1989; Behzadi et al., 1990
Vestibular nuclei	low		Marzienkiewicz et al., 1989
Motor nuclei: oculomotor, trochlear	moderate - low	5-HT	Marzienkiewicz et al., 1989
prepositus hypoglossy	moderate - low		Marzienkiewicz et al., 1989; Behzadi et al., 1990

has been reported (Smith et al., 1974), this raises the possibility that the accumulation of tracer by dorsal and median raphe perikarya resulted from retrograde transport by dendrites, rather than the axon terminals of these neurons. On the other hand, tracer diffusion or retrograde dendrite transport cannot account for the projections arising from more distant nuclei (Sakai et al., 1977; Kalen et al., 1985; Marzinkiewicz et al., 1989; Behzadi et al., 1990). Histochemical confirmation of the neurotransmitter contained in connections among 5-HT nuclei has been performed in rare occasions (Stratford and Wirtshafter, 1988), and when done "numerous non-serotonergic B_9 cells" were retrogradely labelled following tracer injection into the MRN. It is then possible that not all of the retrogradely labelled fibres are serotonergic. The general impression would be that 5-HT axons connecting 5-HT nuclei are "diluted" within the dorsal and median raphe neuropil.

An important afferent area to the raphe both in terms of selectivity and density, is the lateral habenula (Aghajanian and Wang, 1977; Wang and Aghajanian, 1977b). This habenular circuit appears to be comprised of both a monosynaptic GABAergic pathway (Wang and Aghajanian, 1977b; Stern et al., 1981; Park, 1987) and a polysynaptic pathway in which GABA, substance P (Neckers et al., 1979; Nishikawa and Scatton, 1985) and excitatory amino acids serve as components (Kalen et al., 1985; Kalen et al., 1989). Noradrenergic fibres impinge directly onto the dendrites of 5-HT neurons (Baraban and Aghajanian, 1981) producing an excitatory input on the firing activity of 5-HT neurons (Baraban and Aghajanian, 1980). The lateral hypothalamus also gives rise to a monosynaptic excitatory input to the DRN (Aghajanian et al., 1987) though the transmitter is unknown. Multiple afferent fibres immunoreactive for different neuropeptides such as: ß-endorphin (Bloom et al., 1978), substance P (Ljungdahl et al., 1978; Shults et al., 1984), neurotensin fibres (Uhl et al., 1979), CCK fibres (Vanderhaeghen et al., 1980), CLIP/ACTH fibres (Romagnano and Joseph, 1983; Zheng et al., 1991; Léger et al., 1994) and VIP fibres (El Kafi et al., 1994) have also been described.

iv) Efferents and terminal projection areas

Efferent pathways.- The rostral 5-HT nuclei are the main source of 5-HT fibres projecting to telencephalon and diencephalon (Fuxe, 1965; Azmitia and Segal, 1978; Parent et al., 1981; Villar et al., 1987). Though the main contingent of fibres from these nuclei is ascending, they also innervate though more sparely, numerous brainstem

structures (Vertes and Kocsis, 1994), the cerebellar cortex (Waterhouse et al., 1986; Zimny et al., 1988) and the spinal cord (Skagerberg and Bjorklund, 1985). In non-human primate brain two main ascending bundles have been described: dorsal bundle (immediately ventral to the MLF) which receives fibres mainly from lateral wings and ventromedial portion of the DRN and a ventral bundle which receives fibres from the midline DRN and MRN (Schofield and Everitt, 1981, Azmitia and Gannon 1986). In the human fetus, ascending axon bundles were observed: i) in the central grey, ventral to the fourth ventricle and the aqueduct ependyma and ii) between ventromedial and ventrolateral 5-HT groups (Nobin and Bjorklund, 1973). In the rat CNS, two major projection systems to the forebrain have also been described: i) a transtegmental system which probably corresponds to the above-mentioned ventral bundle, courses through the median forebrain bundle (MFB) and is the most prominent of the two systems described by Descarries in the rat brain and ii) a periventricular system dorsally located along the longitudinal fasciculus of Schütz (Parent et al., 1981). Unlike rats, in primates the dorsal component (dorsal raphe cortical tract), is much more developed than the MFB system, presumably due to an increase in fibres projecting to the cortex through this pathway. Moreover, the percentage of myelinated 5-HT fibres in rat MFB is 0.7% of the total immunoreactive 5-HT fibres while it is as much as 25% in primate MFB (Azmitia and Gannon, 1983). The explanation given for the observed increase in myelinization is evolution towards a more rapid, independent and precise form of 5-HT transmission in humans (Azmitia; 1988).

Terminal projection areas.- Dorsal and median raphe nuclei each innervate specific terminal areas (Bobillier et al., 1975; Azmita and Segal, 1978; Jacobs et al., 1978) and in turn each terminal projection area has its unique topographic representation within the respective nucleus. Labelling studies using wheat germ agglutinin, horseradish peroxidase or fluorescent dyes have been used to unveil the midbrain raphe projection network. The more rostral portions of the midbrain raphe relate to basal-ganglia-motor system and caudal areas are more related to the limbic system. Neurons projecting to the striatum occupy the caudal linear nucleus and a rostral portion of the DRN (Jacobs et al., 1978; Imai et al., 1986), those projecting to substantia nigra (Imai et al., 1986) and the motor cortex (O'Hearn and Molliver, 1984; Waterhouse et al., 1986) reside within the rostral portions of the dorsal raphe (Imai et al., 1986). Hippocampus projecting neurons are

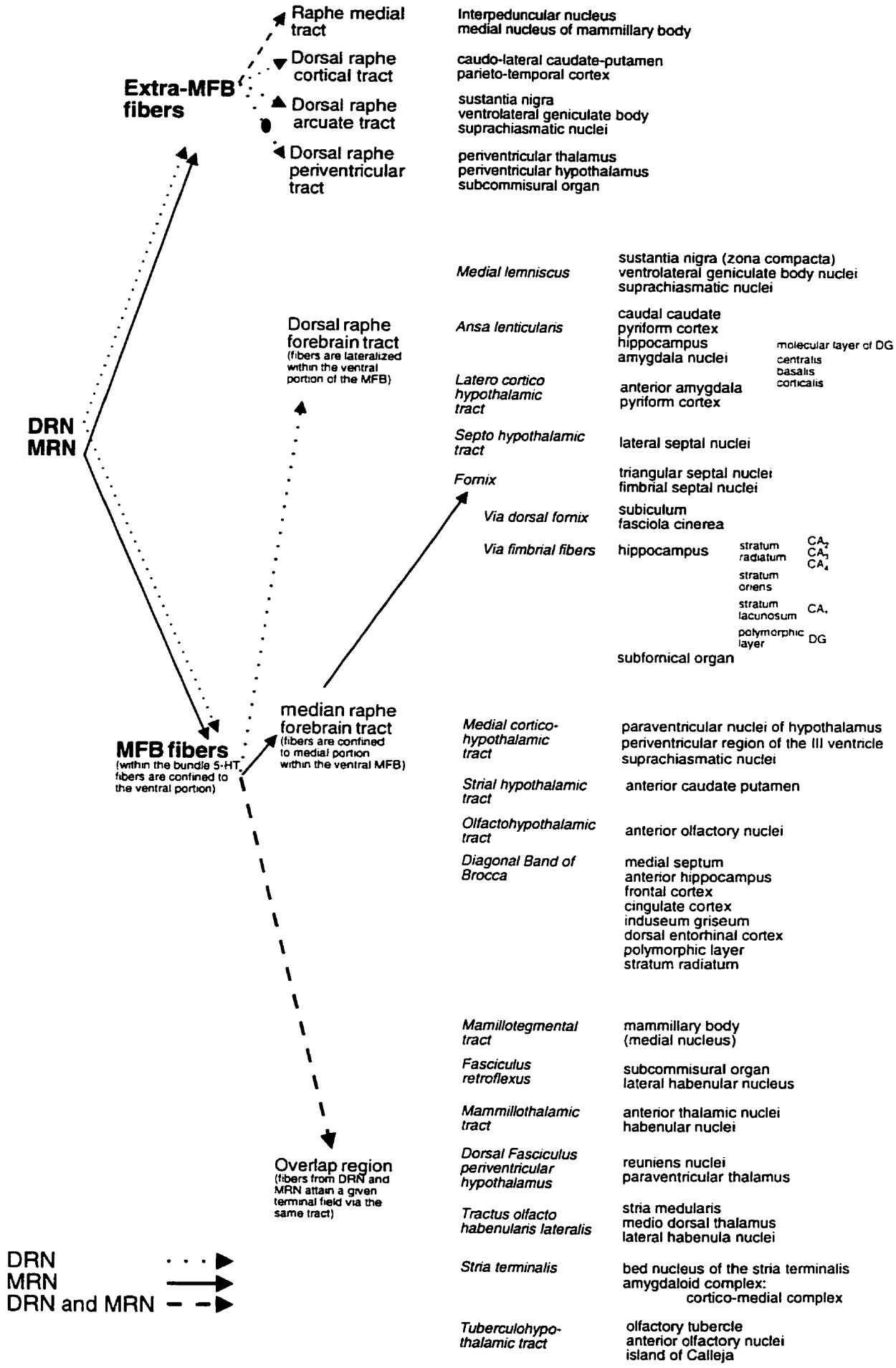


Figure 2. Differential projections from dorsal (DRN) and median (MRN) median raphe nuclei (see Azmitia and Segal, 1978).

situated caudally in the DRN (caudal ventromedial portion and B_6), the MRN and B_9 (Jacobs et al., 1978; Köhler and Steinbusch, 1982; Imai et al., 1986), similar to neurons projecting to the locus coeruleus (Imai et al., 1986) and to the entorhinal cortex (Köhler and Steinbusch, 1982). The raphe representation of the amygdala, on the other hand, bridges the "basal-ganglia-motor system" and the "limbic system representation" (Jacobs et al., 1978; Imai et al., 1986), possibly as a reflection of the functional differences of the amygdaloid nuclei. The consequence of this topographic arrangement is that the selective activation of a given functional group would simultaneously influence interconnected brain circuits. Neurons projecting to interrelated brain circuits such as sensorymotor cortex and cerebellar crus II, the visual cortex and the cerebellar paraflocculus (Waterhouse et al., 1986), or to substantia nigra and caudate-putamen (Imai et al., 1986), entorhinal cortex and hippocampus (Köhler and Steinbusch, 1982), trigeminal sensory complex and nucleus accumbens or the amygdala (Li et al., 1993) arise from overlapping areas within the different nuclei. Moreover, in these regions a single neuron may provide a common input to two different, but functionally interrelated terminal areas. In the ventromedial portion of the DRN a same neuron was found to project to at least three different forebrain structures related with the limbic system: septum, medial thalamus and olfactory cortex (de Olmos and Heimer, 1980). Collateralization provides not only a means for producing concurrent influences on numerous functionally related circuits but it is also a way of achieving extensive serotonergic innervation of most of the CNS from a small number of raphe neurons.

In projection areas, serotonergic axons arising from DRN and MRN raphe have been distinguished on a morphological basis: dorsal raphe fibres are extremely fine with minute varicosities (less than 1 µm in diameter) while those arising from median raphe are distinguished by large spherical varicosities (2-5 µm in diameter; Kosofsky and Molliver, 1987; Mulligan and Törk, 1990). In neocortical areas, dorsal raphe fine axons have been found to be far more numerous than beaded axons (Kosofsky and Molliver, 1987; Mamounas et al., 1991) and to follow a rostrocaudal pattern of distribution with a greater concentration in more frontal regions. These findings are in agreement with those obtained in retrograde labelling experiments in which the frontal cortex was found to receive twice as many projections from cells in the DRN than those in the parietal and occipital cortex (O'Hearn and Molliver, 1984) This morphological duality has also been reported in cat

cortex (Mulligan and Törk, 1990), but such results were not replicated in rat brain where immunostained varicosities exhibited similar shape and size irrespective of the cortical region or sector examined (Séguéla et al., 1989).

Over a wide range of doses (2.5 - 40 mg/kg) and survival times (1 week to 2 months), neurotoxic amphetamine derivatives such as 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymetamphetamine (MDMA) and p-chloroamphetamine (PCA) produce degeneration of fine 5-HT axon terminals while sparing beaded 5-HT axons (Mamounas and Molliver, 1988; O'Hearn et al., 1988; Wilson et al., 1989; Mamounas et al.,1991) as well as raphe cell bodies (O'Hearn et al., 1988; Mamounas et al., 1991). The 5-HT releasing agent, fenfluramine, produces similar effects (Molliver and Molliver, 1990). These neurotoxins have been used to determine the differential distribution of beaded and fine axons, and hence the contribution of DRN and MRN in different projection areas. In hippocampus and neocortex, different neurotoxins have been reported to produce a regional axon loss/sparing pattern which is coincident with fine and beaded axon distribution. PCPA or MDA administration caused marked denervation in parietal and occipital cortices and a moderate number of axons was spared in frontal cortex (Mamounas and Molliver, 1988; Mamounas et al., 1991). In hippocampus there was a greater density of spared axons, most probably due to the fact that in the hippocampal formation in comparison with other cortical areas, beaded axons are especially prevalent (Mamounas et al., 1991). An exceptionally large number of axons was spared in stratum radiatum of CA_3, intact axons were also found in stratum oriens of CA_3, stratum lacunosum of CA_1 and flanking the granular layer in the dentate gyrus. In contrast few 5-HT axons remained in the outer molecular layer of the dentate gyrus and stratum oriens and radiatum of CA_1, areas that normally receive DRN innervation (Mamounas et al., 1991). On the other hand, Oleskevich and Descarries (1990) described 5-HT fibres projecting to the same layers but no distinction between fine or beaded fibres was made. This same group has reported however, that 5-HT varicosities with synaptic membrane specializations are slightly larger than their non-junctional counterparts (Oleskevich et al., 1991). Törk (1990) proposes that together with dual morphology and distinct sensitivity to neurotoxins, the fact that fine axons rarely make synaptic contacts while larger varicosities tend to show distinct synaptic specializations, supports the idea that the ascending raphe projections form a dual system from the anatomical and morphological view point. It

should be noted that the reported percentages of synaptic incidence in terminal areas are very variable: 30% - 80% (out of a total of 5.8 x 10^6 varicosities/mm^3) in rat cortex (Papadopoulos et al., 1987; Séguéla et al., 1989), 3% in monkey cortex (De Felipe and Jones, 1988), 10% - 15% (out of a total of 2.6 x 10^6 varicosities/mm^3) in rat neostriatum (Soghomonian et al., 1987) and 18% - 24% in hippocampus (out of a total of 2.7 x 10^6 varicosities/mm^3; Oleskevich et al., 1991).

B. Physiological aspects of the 5-HT system

i) Firing activity of 5-HT neurons

Midbrain raphe 5-HT neurons exhibit a spontaneous, slow (1 - 5 spikes/s), regular discharge pattern (Aghajanian and VanderMaelen, 1982; VanderMaelen and Aghajanian, 1983). Intracellular recordings from dorsal raphe neurons reveal that 5-HT cells undergo repetitive cycles of interspike hyperpolarization and depolarization, spikes arising from depolarizing ramps rather than from excitatory postsynaptic potentials (Aghajanian and VanderMaelen, 1982; VanderMaelen and Aghajanian, 1983). The ionic basis for this electrical activity is summarized in Figure 3.

In freely moving cats, the regular, stereotyped intrinsic activity of 5-HT neurons remains unchanged over multiple physiological and behavioral processes such as exposure to a hot environment or the administration of a pyrogen, increase in blood pressure, insulin-induced hypoglycaemia, administration of painful stimuli, physical restraint or exposure to aversive stimuli such as a natural enemy (see Jacobs and Fornal., 1993). However, the basic pattern of activity is not fixed, and it has been shown to dramatically change during the sleep-wake-arousal cycle: firing activity progressively slows down from an aroused state through quiet waking and slow wave sleep (SWS), to become silent during rapid eye movement sleep (REM; McGinty and Harper, 1976; see Jacobs and Fornal, 1993 for review). The suppression of firing of 5-HT neuron during REM sleep correlates well with the production of muscle atonia secondary to inhibition of motoneurons controlling antigravity muscles (Trulson et al., 1981; Steinfels et al., 1983). More recently another relationship has been observed between motor output and 5-HT neuron activity. During quiet wakefulness, when cats engage in various types of stereotyped oral-buccal activities such as chewing and biting, licking or grooming with the tongue, approximately 25% of DRN and MRN increase their firing activity 2-5 fold (see Jacobs and Azmitia,

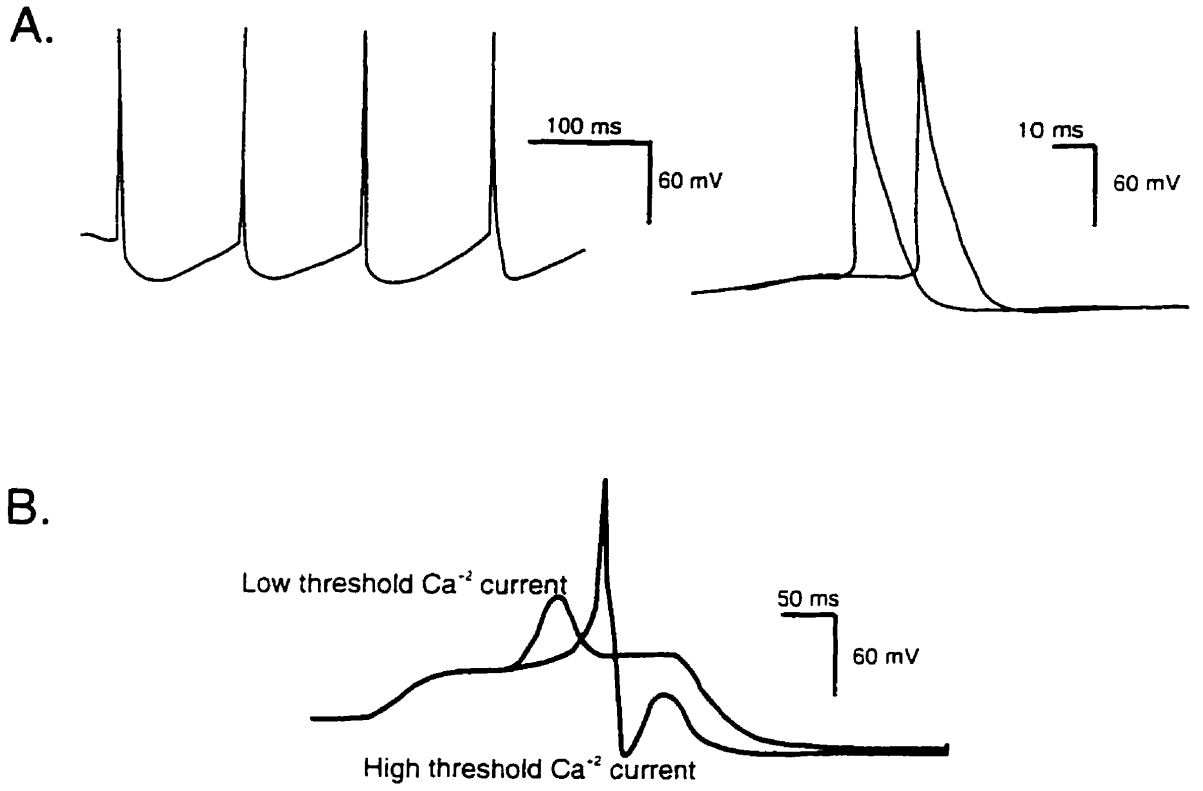


Figure 3. Representative voltage tracings obtained from acutely isolated DRN neurons under current clamp (see Pennington et al, 1991). (**A**) Spontaneous activity exhibited by the cell at resting membrane potential (zero current potential). Typical action potentials consist of an initiating ramp of depolarization, spike, shoulder upon repolarization and an afterhyperpolarization. (**B**) Depolarization of a cell bathed with the Na^+ channel blocker tetrodotoxin (TTX). TTX abolished the fast component of the action potential and uncovered, low- and high-threshold Ca^{+2} components of the action potential. While T-channels seem responsible for the low-threshold current, at least three different channel types (including N- and L-type) underlie the high-threshold current (Pennington and Kelly, 1990; Pennington et al., 1991). The afterhyperpolarization that follows, as in many other vertebrate and invertebrate neurons, is mediated by a Ca^{+2}-activated K^+ outward current (VanderMaelen and Aghajanian, 1982; Aghajanian 1985; Aghajanian et al., 1987). This afterhyperpolarization is responsible for a long-lasting refractory period and the slow firing rate of 5-HT neurons. As Ca^{+2} that entered during the action potential is sequestrated/extruded, the Ca^{+2}-dependent K^+ current and the afterhyperpolarization diminish. When the membrane potential reaches again the value for the low-threshold Ca^{+2} current (aprox. -60 mV) a new spike will be triggered. As repolarization from membrane potentials below the resting potential takes place, a voltage dependent outward K^+ current, that slows the rate of depolarization, is simultaneously activated, the so called I_A current (Aghajanian, 1985).

1992; Jacobs and Fornal, 1993). This increased neuronal activity precedes the onset of the aforementioned motor behaviours and ends with its offset, it does not occur during purposive episodic movements but some of the neurons may be activated by somatosensory and proprioceptive stimulations of head and neck area. These and other data have given support to the current motor hypothesis of 5-HT function that sustains that the primary function of the 5-HT system in the brain is to facilitate motor output and concurrently inhibit sensory information processing (Jacobs and Fornal, 1993).

The main variables that have been described to control the activity of midbrain 5-HT neurons are: autoregulatory influences from 5-HT neurons themselves and heteroregulation by afferents.

Autoregulation of 5-HT neuron firing activity.- The firing rate of 5-HT neurons is decreased by 5-HT and this effect is mediated by somatodendritic 5-HT_{1A} autoreceptors (Aghajanian et al., 1972; VanderMaelen et al., 1986; Blier and de Montigny, 1987). 5-HT and 5-HT_{1A} agonists inhibit 5-HT firing activity by inducing membrane hyperpolarization which is brought about by a two-fold mechanism: i) by increasing conductance to potassium ions (Aghajanian and Lakoski, 1984; Yoshimura and Higashi 1985) and ii) by reducing the high-threshold Ca^{+2} current (Figure 3; Pennington and Kelly, 1990; Pennington and Fox, 1994). In both cases, the response to 5-HT is G-protein mediated via a direct interaction between G proteins and the respective ion channel (Innis and Aghajanian, 1987; Penington et al., 1991; Penington et al., 1993, Penington and Fox, 1994).

Current knowledge of the pharmacological properties of somatodendritic 5-HT_{1A} autoreceptors is mostly based on electrophysiological studies assessing the effect of different compounds on the firing activity of 5-HT neurons. There is a range of agonists for the 5-HT_{1A} receptors (Table 3), some of which like buspirone, are used for the treatment of anxiety and depression (see Yocca, 1990; Glitz and Pohl, 1991). However, few if any are as potent as the agonist 8-OH-DPAT (Hjorth et al., 1982) and some of them such as buspirone or gepirone have been shown to act as <u>partial agonists</u> at postsynaptic 5-HT_{1A} receptors on CA_1 and CA_3 regions of the hippocampus (Yocca and Maayani, 1985; Yocca et al., 1986; Andrade and Nicoll, 1987a). In electrophysiological experiments tandospirone and flesinoxan have also been shown to interfere with the inhibitory effect caused by the microiontophoretic application of 5-HT onto CA, pyramidal neurons

TABLE 3

Effect of 5-HT$_{1A}$ active drugs on 5-HT neuron firing activity

Drug	Effect on 5-HT neuron firing	Dose	References
In vitro studies			
(-) propranolol	↔	10 µM	Kidd et al., 1993
(±) tertatolol	↔	1 µM	Kidd et al., 1993
(-) tertatolol	↔	10 nM - 10 µM	Jolas et al., 1993
WAY 100635	↔	30 nM	Craven et al., 1994
(+) WAY 100135	↔	10 nM - 1 µM	Lanfumey et al., 1993
In vivo studies			
(-) alprenolol	↔	> 2 mg/kg, i.v.	Millan et al., 1994
spiperone	↔	1 mg/kg, i.v.	Lum and Piercey, 1988; Blier et al., 1988; Blier et al., 1993
S-UH-301	↔↑	< 0.25 mg/kg, i.v.	Arborelius et al., 1995
(-) tertatolol	↔	0.5-4 mg/kg, i.v.	Lejeune et al., 1993
(-) tertatolol	↔	> 2 mg/kg, i.v.	Millan et al., 1994
(-) tertatolol	↔	0.1 - 0.5 mg/kg, i.v.	Jolas et al., 1993
(-) tertatolol	↔	1 mg/kg, i.v.	Prisco et al., 1993
(-) tertatolol	↔	ID_{50} > 4 mg/kg, i.v.	Gobert et al., 1995
(+) WAY 100135	↔	0.1 - 1 mg/kg, i.v.	Lejeune et al., 1993
(+) WAY 100135	↔	0.5 mg/kg, i.v.	Piñeyro et al., 1996
(+) WAY 100635	↔↑	0.1-0.5 mg/kg, i.v.	Fornal et al., 1994
(+) WAY 100135	↔	1 mg/kg, i.v.	Haddjeri and Blier, 1995
In vitro studies			
8-OH-DPAT	↓	3 nM - 1 µM	Lanfumey et al., 1993
8-OH-DPAT	↓	IC_{50} 10 nM	Jolas et al., 1993
8-OH-DPAT	↓	IC_{50} 11 nM	Schechter et al., 1990
CM 57493	↓	5-20 µM	Adnen et al., 1989
ipsapirone	↓	50 nM	Jolas et al., 1993
ipsapirone	↓	60 nM	Schechter et al., 1990
lesopitron	↓	300 nM	Jolas et al., 1993
(+) 20499	↓	IC_{50} 6 nM	Kidd et al., 1993
(-) S20500	↓	IC_{50} 130 nM	Schechter et al., 1990
BMY 7378	↓	100 - 150 nM	VanderMaelen et al., 1987
BMY 7378	↓	IC_{50} 10 nM	Gruel and Glaser, 1991
NAN-190	↓	IC_{50} 20 nM	Gruel and Glaser, 1991
SDZ 216525	↓	100 nM	Hamon et al., 1993
SDZ 216252	↓	100 nM - 1 µM	Lanfumey et al., 1990
(+) WAY 100135	↓	1 µM	Hamon et al., 1993
(+) WAY 100135	↓	> 1 µM	Lanfumey et al., 1993

TABLE 3 (cont)

Effect of 5-HT$_{1A}$ active drugs on 5-HT neuron firing activity

Drug	Effect on 5-HT neuron firing	Dose	References
In vivo studies			
8-OH-DPAT	↓	2 µg/kg, i.v.	Blier and de Montigny, 1987; Blier et al., 1988
8-OH-DPAT	↓	1.5 µg/kg, i.v.	Fornal et al., 1994
8-OH-DPAT	↓	1.5 µg/kg, i.v.	Prisco et al., 1993
8-OH-DPAT	↓	2.2 µg/kg, i.v.	Jolas et al., 1994
8-OH-DPAT	↓	0.6 µg/kg, i.v.	Gobert et al., 1995
8-OH-DPAT	↓	1.6 µg/kg, i.v.	McCall et al., 1994
8-OH-DPAT	↓	0.4 µg/kg, i.v.	Arborelius et al., 1994
8-OH-DPAT	↓	1.5 µg/kg, i.v.	Cox et al., 1993
5MeOMT	↓	45 µg/kg, i.v.	Fornal et al., 1994
Buspirone	↓	75 µg/kg, i.v.	Fornal et al., 1994
Buspirone	↓	15 µg/kg, i.v.	McCall et al., 1994
CM 57493	↓	5-20 mg/kg, i.p.	Adrien et al., 1989
Flesinoxan	↓	108 µg/kg, i.v.	Hadrava et al., 1995
Flesinoxan	↓	21 µg/kg, i.v.	Gobert et al., 1995
Gepirone	↓	10 µg/kg, i.v.	Blier and de Montigny, 1987
Ipsapirone	↓	83 µg/kg, i.v.	Fornal et al., 1994
Ipsapirone	↓	7.5 µg/kg, i.v.	Jolas et al., 1994
Ipsapirone	↓	3.7 µg/kg, i.v.	Gobert et al., 1995
Ipsapirone	↓	30 µg/kg, i.v.	Cox et al., 1993
Ipsapirone	↓	0.1-1 mg/kg, i.p.	Adrien et al., 1993
LSD	↓	5 µg/kg, i.v.	Blier et al., 1991; Godbout et al., 1991
LY 293284	↓	0.08 µg/kg, s.c.	Foreman et al., 1994
S 14671	↓	0.16 µg/kg, i.v.	Gobert et la., 1995
S 14506	↓	0.3 µg/kg, i.v.	Gobert et al., 1995
S 14 671	↓	0.16 µg/kg, i.v.	Lejeune et al., 1993
S 14489	↓	5 µg/kg, i.v.	Millan et al., 1994
S 15535	↓	7 µg/kg, i.v.	Millan et al., 1994
S 15931	↓	30 µg/kg, i.p.	Millan et al., 1994
SR 57746A	↓	90-250 µg/kg, i.v.	Bachy et al., 1993
Tandospirone	↓	3.4 µg/kg, i.v.	Gobert et al., 1995
Tandospirone	↓	9.1 µg/kg, i.v.	Godbout et al., 1991
U 92016A	↓	3.4 µg/kg, i.v.	McCall et al., 1994
WY 48723	↓	0.6 µg/kg, i.v.	Gobert et al., 1995
zalospirone	↓	3.6 µg/kg, i.v.	McCall et al., 1994
BMY 7378	↓	17.5 µg/kg, i.v.	Cox et al., 1993
BMY 7378	↓	1.5 µg/kg, i.v.	Millan et al., 1994
BMY 7378	↓	1.6 µg/kg, i.v.	Gobert et al., 1995
BMY 7378	↓	15.3 µg/kg, i.v.	Fornal et al., 1994
MDL73005EF	↓	4 µg/kg, i.v.	Millan et al., 1994

TABLE 3 (cont)

Effect of 5-HT_{1A} active drugs on 5-HT neuron firing activity

Drug	Effect on 5-HT neuron firing	Dose	References
In vivo studies			
MDL 73005EF	↓	4.4 µg/kg, i.v.	Gobert et al., 1995
NAN 190	↓	4 µg/kg, i.v.	Millan et al., 1994
NAN 190	↓	3.9 µg/kg, i.v.	Gobert et al., 1995
NAN 190	↓	34.2 µg/kg, i.v.	Fornal et al., 1994
SDZ 216525	↓	0.1-0.5 mg/kg, i.v.	Mundey et al., 1994
SDZ 216525	↓	0.4 mg/kg, i.v.	Fornal et al., 1994
SDZ 216525	↓	47.3 µg/kg, i.v.	Gobert et al., 1995
S-UH-301	↓	0.5-4 mg/kg, i.v.	Arboreliusl et al., 1994
(+) WAY 100135	↓	0.4 mg/kg, i.v.	Fletcher et al., 1994
(+) WAY 100135	↓	0.25-0.50 mg/kg, i.p.	Arborelius et al., 1995
(+) WAY 100135	↓	31 µg/kg, i.v.	Escandon et al., 1994
(+) WAY 100135	↓	0.1-1 mg/kg, i.v.	Fornal et al., 1994

↔ no effect

(Godbout et al., 1991; Hadrava et al., 1995), further supporting a partial agonistic effect at postsynaptic sites. An inverse situation holds true for several compounds previously thought to act as 5-HT_{1A} receptor antagonists at postsynaptic sites that have been shown to possess agonistic effects at presynaptic sites thus inhibiting raphe cell firing. These compounds are also best classified as partial agonists. For example, drugs like (+) WAY 100135, NAN-190 or BMY 7378 show antagonistic properties in forskolin-stimulated adenylyl cyclase assays using hippocampal membranes (Rydeleck-Fitzgerald et al., 1990), or block the postsynaptic neurochemical, electrophysiological and behavioral effects of 8-OH-DPAT (Chaput and de Montigny, 1988; Sharp et al., 1990; Routledge et al., 1993; Escandon et al., 1994), but also decrease 5-HT neuron firing activity (VanderMaelen et al., 1987; Chaput et al., 1988; Fletcher et al., 1993, Haddjeri and Blier, 1995). Gruel and Glaser (1992) have shown that the suppression of raphe firing caused NAN-190 and BMY 7378 is blocked by the ß/5-HT_{1A} antagonist (±) propranolol (Middlemiss, 1984; Tricklebank et al., 1985). Similarly, the inhibition induced by SDZ 216-235 on 5-HT neuron firing is blocked by the ß/5-HT_{1A} antagonist tertatolol (Lanfumey et al., 1993; Lajeune et al., 1993), confirming thus that all three "postsynaptic antagonists" act as partial agonists when acting upon presynaptic 5-HT_{1A} receptors. The inhibitory action induced by (+) WAY 100135 on 5-HT neuron firing activity may be overcome by the addition of the α_1-adrenergic agonist phenylephrine but not by (-) tertatolol thus indicating that the latter effect is not due to partial agonism but rather α_1-adrenoceptor blockade (Lanfumey et al., 1993). Since WAY 100635 and (-) tertatolol have been consistently shown to block the effect of 5-HT_{1A} agonists without affecting 5-HT neuron firing (Jolas et al., 1993; Lanfumey et al., 1993; Lejeune et al., 1993; Prisco et al., 1993; Craven et al., 1994; Fletcher et al., 1994; Mundey et al., 1994; Table 3), they have been named "silent" antagonists. Though, (+) WAY 100135 lacks partial agonistic effects on somatodendritic 5-HT_{1A} receptors it is not "silent" due to its α_1-adrenergic blocking properties (Lanfumey et al., 1993).

There are two main explanations for the distinct pharmacological properties observed for the different 5-HT_{1A}-active drugs at pre- and postsynaptic 5-HT_{1A} receptors: i) a greater receptor reserve in somatodendritic than postsynaptic areas (Meller et al., 1990; Yocca, 1990; Greuel and Glaser, 1992; Millan et al., 1992) and ii) different presynaptic and postsynaptic 5-HT_{1A} receptors (see de Montigny and Blier, 1992a; 1992b). The first hypothesis sustains that if no receptor reserve exists, like in the case of

the hippocampus (Yocca, 1990; Gozlan et al., 1994), partial agonists will not produce a maximal effect and may not block the effect of full agonists. Conversely, if spare receptors are present, at an appropriately high dose drugs with low intrinsic activity (i.e.: partial agonists) may still elicit a maximal response and appear as full agonists. On the other hand, the effect of silent antagonists will depend on the tonic activation of 5-HT_{1A} receptors in the preparation. If tonic activation exists, silent antagonists might increase 5-HT neuron firing, if not 5-HT discharge will be left unchanged. Though receptor reserve may explain the differential effect of partial agonists at pre- and postsynaptic sites the following observations remain unexplained under this hypothesis: i) different potencies of full agonists like 8-OH-DPAT, 5-carboxyamidotryptamine (5-CT) and 5-HT to inhibit the firing activity of dorsal raphe and hippocampal pyramidal neurons (Blier and de Montigny, 1987; Chaput and de Montigny, 1988; Blier and de Montigny, 1990); ii) spiperone which at the dose of 1 mg/kg, i.v. has been shown to act as a silent antagonist at presynaptic 5-HT_{1A} receptors (Lum and Piercey, 1988; Blier et al., 1988, Blier et al., 1993a; Fornal et al., 1994a), does not alter the responsiveness to microiontophoretically applied 5-HT or 8-OH-DPAT onto CA_3 pyramidal neurons (Blier et al., 1993a); iii) cholera toxin, which causes a permanent activation of G_s proteins, does not alter the responsiveness of 5-HT neurons to microiontophoretic application of 5-HT or 8-OH-DPAT but reduces that of CA_3 pyramidal neurons by 90% (Blier et al., 1993b). The distinct pre- postsynaptic hypothesis offers a possible explanation for all of the above mentioned observations. Furthermore, such an hypothesis is compatible with the detection of three mRNAs of the rat 5-HT_{1A} receptor (Albert et al., 1990).

The extracellular availability of endogeneous 5-HT at the cell body level and the tonic activation of somatodendritic 5-HT_{1A} autoreceptors determines not only the effect of 5-HT antagonists but modifies also that of agonists. Thus, Fornal et al., (1994a) have shown that the acute intravenous administration of the 5-HT_{1A} agonist 8-OH-DPAT, or of the partial agonists ipsapirone and buspirone was more effective to inhibit 5-HT neuron firing activity when cats were inactive (drowsiness) than during active wakefulness (a period of higher neuronal activity, 5-HT neuron depolarization and hence of 5-HT release). Conversely, spiperone and WAY 100635 induced a dose-dependent increase in the firing rate which was evident during wakefulness but not during sleep (Fornal et al., 1994a; 1994b), when 5-HT neurons are silent and therefore there is no depolarization-mediated

release of 5-HT to activate somatodendritic 5-HT_{1A} receptors. Moreover, in anesthesized rats and guinea pigs, in which 5-HT neuron firing activity resembles that of SWS, WAY 100635 had no effect on dorsal raphe neuronal firing (Fletcher et al., 1994, Mundey et al., 1994). It is also noteworthy that the 5-HT_{1A} antagonistic properties of (+) WAY 100135 were not evident in anesthesized rats (Haddjeri and Blier, 1995), in awake freely moving cats (Fornal et al., 1994a; 1994b), but, at similar doses as the ones used in the previous studies (0.5 - 1 mg/kg, i.v.) (+) WAY 100135 reversed the suppression of 5-HT neuron firing activity induced by the blockade of 5-HT uptake (Arborelius et al., 1995; Hajós et al., 1995). The latter observation further supports the contention that the action of 5-HT_{1A} ligands on 5-HT neuron firing activity is determined in part, by the tonic activation of their somatodendritic autoreceptors. Similarly, S-UH-301 which in the dose range of 0.5 - 4 mg/kg, i.v. reduces 5-HT neuron firing, effectively blocked the suppressant effect induced by the systemic administration of selective 5-HT reuptake blocker citalopram (Arborelius et al., 1994; Arborelius et al., 1995). Moreover, (-) pindolol which by itself induces no change in 5-HT neuron firing activity, prevents the inhibitory effect of paroxetine on this parameter (Romero et al., 1996).

Like spiperone, pindolol is an antagonist which discriminates between presynaptic and postsynaptic hippocampal 5-HT_{1A} receptors. Both, spiperone and (-) pindolol block the effect of endogeneous 5-HT on presynaptic receptors without interfering with the inhibitory effect induced on CA_3 pyramidal neuron firing activity by the microiontophoretically application of 5-HT (Blier et al., 1993a; Romero et al., 1995). WAY 100635 on the other hand, has been shown to block the effect of endogeneous 5-HT on postsynaptic 5-HT_{1A} receptors located on CA_3 pyramidal neurons (Haddjeri et al., unpublished observation). Binding studies are consistent with the latter observation: [^{3}H]WAY 100635 behaves as an antagonist in projection areas like the hippocampus since guanyl nucleotides fail to influence its K_D and B_{MAX} parameters (Khawaja et al., 1995). The presynaptic selectivity of the other "silent" antagonist (-) tertatolol remains yet to be proven, leaving then (-) pindolol as the only clinically useful presynaptic 5-HT_{1A} antagonist. Finally, it is also worth mentioning the case of (-) propranolol which also has moderate affinity for 5-HT_{1A} receptors (Pazos and Palacios, 1985; Hoyer and Schoefter, 1991; Prisco et al., 1993) but unlike (-) pindolol it does not block the inhibitory effect of 5-HT or 8-OH-DPAT on hippocampal pyramidal or 5-HT neuron firing activity (Sprouse and

Aghajanian, 1986; Blier et al., 1988; Fornal et al., 1994a).

The local somatodendritic autoreceptor "short-loop" is only one of the mechanisms involved in regulating 5-HT neuron firing activity. Several observations suggest that, in fact, the systemic administration of low doses of 8-OH-DPAT may regulate 5-HT neuron firing activity by an alternative "long feedback loop", which also entails 5-HT_{1A} receptor activation: i) the effectiveness of microiontophoretic application of 5-HT_{1A} agonists to inhibit 5-HT neuron firing activity, but not that of systemic 8-OH-DPAT administration, is reduced following long-term treatment with the 5-HT_{1A} antagonist gepirone and the SSRI cericlamine (Blier and de Montigny, 1987; Jolas et al., 1994); ii) the effect of intravenous 8-OH-DPAT on 5-HT neuron firing activity, but not that of its microiontophoretic application onto 5-HT neurons, is increased by short-term lithium treatment (Blier et al., 1987); iii) the inhibitory effect of intravenous administration of 8-OH-DPAT on the firing activity of dorsal raphe serotonergic neurons in rats is attenuated by lesion of the frontal cortex (Ceci et al., 1994); iv) the local application of pertussis toxin into the dorsal raphe, (which inactivates $G_{i/o}$-coupled 5-HT_{1A} autoreceptors; Innis and Aghajanian, 1987) reduces the effectiveness of intraraphe but not systemic 8-OH-DPAT administration to reduce 5-HT release in terminal projection areas (Romero et al., 1994). These observations should be bared in mind when interpreting the site of action of 5-HT_{1A} active drugs.

Role of other 5-HT receptors in the regulation of 5-HT neuron firing activity.- Autoradiographic and binding studies have documented the presence of different 5-HT binding sites in the rat raphe nuclei (Waeber et al., 1988; Herrick-Davis and Titeler, 1988; Waeber et al., 1989; Laporte et al., 1992). However, the role of 5-HT receptors other than 5-HT_{1A} in modulating 5-HT neuron firing activity at the cell body level has not been confirmed. The lack of effect of 1-[3-(trifluoromethyl)phenylpiperazine (TFMPP) and *m*-chlorophenylpiperazine (*m*CPP) on the firing activity of 5-HT neurons led Sprouse and Aghajanian (1986, 1987) to conclude that 5-HT_{1B} receptors were not present on the cell body of 5-HT neurons in the rat brain. The systemic and microiontophoretic application of the 5-HT_2 agonist DOI reduce 5-HT neuron firing frequency, however, this effect could not be blocked by the 5-HT_2 antagonist ketanserin nor the 5-$HT_{2C/2}$ antagonist ritanserin (Wright et al., 1990; Garratt et al., 1991). Moreover, the microiontophoretic application of ketanserin does not inhibit the inhibitory effects of 5-HT but reduces basal firing rates in the majority of 5-HT cells tested (Lakoski and Aghajanian, 1985). These observations

together with the fact that the effectiveness of 8-OH-DPAT (administered systemically), but not that of DOI to inhibit the firing activity of 5-HT neurons is decreased following repeated DOI administration (Kidd et al., 1991), suggests that 5-HT_2 receptors are not directly involved in the regulation of 5-HT neuron firing. The role of 5-HT_3 receptors in regulating 5-HT neuron firing activity has also been investigated. The fact that systemic administration of BMY 7378 but not that of the 5-HT_3 antagonist BRL 46470A blocks the reduction of dorsal raphe 5-HT neuron firing rate induced by the microiontophoretic application of the 5-HT_3 agonist 2-methyl-5-HT (Haddjeri and Blier, 1995), and that 5-HT neuron firing activity remains unchanged following systemic administration of three different 5-HT_3 receptor antagonists MDL 72222, ICS-205-930 and ondansetron (Adrien et al., 1992), indicate that 5-HT_3 receptors do not contribute to the regulation of 5-HT neuron firing actvity.

Heteroregulation of 5-HT neuron firing.- NMDA receptors may elicit excitatory postsynaptic potentials (EPSPs; Pan and Williams 1989; Pinnock, 1992; Johnson, 1994) and increase the firing activity of 5-HT neurons (Alojado et al., 1994) *in vitro,* but they do not seem to maintain a tonic activation of 5-HT neuron firing *in vivo* (Levine and Jacobs, 1992). However, glutamate does mediates the phasic increase in firing activity observed following presentation of phasic auditory stimuli (Levine and Jacobs, 1992). In turn, EAA release in the dorsal raphe is negatively regulated by k-opiod receptors (Pinnock, 1992). Rather unexpectedly, the systemic administration of the NMDA channel blocker (+)MK-801 has been shown to facilitate the electrical activity of 5-HT neurons in the DRN. Such an observation could be explained by assuming if the antagonist blocks the facilitation of an inhibitory influence. Microiontophoretic application of GABA onto dorsal raphe 5-HT neurons does indeed produce inhibition of their firing rate (Gallager and Aghajanian, 1976; Levine and Jacobs, 1992). Also, the GABA/Cl^--channel blocker picrotoxin reduces the suppressant effect on 5-HT neuron activity caused by habenula and pontine reticular formation stimulation (Wang et al., 1976; Wang and Aghajanian, 1977; Stern et al., 1981). In freely moving animals, microiontophoretic application of bicuculline produces a significant increase of 5-HT neuron firing activity during slow wave sleep but not REM or quiet waking, indicating that GABAergic input is state dependent and not tonic (Levine and Jacobs, 1992). In anesthesized rats, microiontophoretic and systemic administration of α-adrenoceptor antagonists, as well as 6-hydroxydopamine pretreatment suppress 5-HT

neuron firing activity, suggesting a tonic facilitatory role for noradrenaline on dorsal raphe 5-HT neurons (Baraban and Aghajanian, 1980). Furthermore, activation of α_2-adrenergic autoreceptors by clonidine decreases NE output and suppresses the firing activity of 5-HT neurons (Clement et al., 1992; Hadjjeri et al., 1996). Contrastingly, systemic administration of α-adrenoceptor antagonists in the awake, freely moving animals produced no change in 5-HT neuron firing activity (Heym et al., 1981). Unlike EAAs NA does not elicit EPSPs on 5-HT neurons, rather it suppresses the voltage-dependent K^+ current I_A (Figure 3; Aghajanian, 1985) leading to a more rapid activation of the low-threshold inward calcium current that triggers the spike at the end of the pacemaker cycle of these neurons. Activation of CCK_A receptors located on 5-HT neurons also stimulates their firing activity (Boden et al., 1994), as does bombesin via the stimulation of a neuromedin B receptor (Pinnock et al., 1994).

Effect of antidepressant administration on 5-HT neuron firing activity.- The firing activity of 5-HT neurons is inhibited not only by the acute administration of 5-HT_{1A} agonists (VanderMaelen et al., 1986; Blier and de Montigny, 1987; Godbout et al., 1990; Schechter et al., 1990; Hadrava et al., 1995), but also by drugs like SSRIs (Blier and de Montigny, 1983; Blier et al., 1984; Chaput et al., 1986; Jolas et al., 1994; Arborelius et al., 1995; Hajós et al., 1995) and monoamine oxidase inhibitors (MAOIs; Blier and de Montigny, 1985; Blier et al., 1986), which induce an activation of 5-HT_{1A} autoreceptors due to the immediate increase they produce in extracellular 5-HT at the somatodendritic level (see Sharp and Hjorth, 1990; Artigas; 1993). However, following sustained administration of SSRIs, MAOIs and 5-HT_{1A} agonists a progressive desensitization of somatodendritic 5-HT_{1A} autoreceptors takes place, and the effectiveness of 5-HT_{1A} agonists and antidepressant drugs to inhibit 5-HT neuron firing activity decreases. By 14 - 21 days of treatment 5-HT neurons recover their pretreatment firing frequency (Blier and de Montigny, 1983; Blier et al., 1984; Blier and de Montigny, 1985; Blier et al., 1986; Chaput et al., 1986; Blier and de Montigny, 1987; Godbout et al., 1990; Schechter et al., 1990; Jolas et al., 1994; Arborelius et al., 1995; Hadrava et al., 1995). These multiple *in vivo* and *in vitro* electrophysiological studies have suggested desensitization of 5-HT_{1A} somatodendritic autoreceptors could be a possible explanation for the recovery of 5-HT neuron firing activity. However, despite the fact that considerable functional evidence supports the occurrence of 5-HT_{1A} autoreceptor desensitization, the mechanism underlying this

adaptative process remains unclear. The possibility that down-regulation of 5-HT_{1A} receptors in the midbrain may mediate the observed electrophysiological desensitization has not been confirmed: though long-term treatment with gepirone (Welner et al., 1989), buspirone (Gobbi et al., 1991) and fluoxetine (Li et al., 1994) reduce the total number of [^{3}H]8-OH-DPAT binding sites in midbrain raphe nuclei; citalopram, sertraline (Hensler et al., 1991), paroxetine, fluoxetine (Le Poul et al., 1995) and cericlamine sustained administration (Jolas et al., 1994), as well as that of clorgyline, phenelzine, tranylcypromine (Hensler et al., 1991) and ipsapirone (Schechter et al., 1990) did not modify [^{3}H]8-OH-DPAT binding parameters in the same region (all drugs were administered for a 14 - 21 day time period and at doses that induce functional desensitization). Moreover, though desensitization of 5-HT_{1A}-mediated inhibition of adenylyl-cyclase has been documented following the administration of clorgyline, tranylcypromine, fluoxetine, and buspirone (see Newman et al., 1993), and the use of *in vitro* biochemical techniques has allowed to determine that agonist-induced desensitization of adenylyl cyclase inhibition correlates well with 5-HT_{1A} receptor down-regulation in Swiss 3T3 cells (van Huizen et al., 1993), patterns of desensitization may differ depending on the host cell used to express the receptor. Indeed, desensitization of the inhibitory effect of 5-HT_{1A} receptor activation on cAMP production, is linked to receptor phosphorylation by PKC in CHO cells (Raymond, 1991) or by G protein kinases in insect Sf9 cells (Nebigil et al., 1995). It is unlikely then, that results obtained in these last studies may be directly extrapolated to 5-HT neurons. Furthermore, since 5-HT_{1A} receptors that control the firing activity of these neurons are linked (via a G protein) to K^+ and Ca^{+2} channels (Innis and Aghajanian, 1987; Penington et al., 1991; Penington et al., 1993, Penington and Fox, 1994), it is not guaranteed that they will possess the same desensitization mechanisms as receptors linked to adenylate cyclase. Alternatively, it has been proposed that antidepressant-induced desensitization of 5-HT_{1A} mediated responses could be mediated at the signal transducing (G-protein) level (Lesch et al., 1991; 1992; Lesch and Manji, 1992; Chen and Rasenick, 1995). Sustained fluoxetine and clorgyline administration have been found to respectively decrease $G_{\alpha s}$ and increase $G_{\alpha 12}$ mRNA in rat midbrain (Lesch et al., 1992; Lesch and Manji; 1992). Finally, another possible target for antidepressant-induced desensitization of somatodendritic 5-HT_{1A} receptor-mediated control of 5-HT neuron firing are the effector channels to which the receptor is linked by the G-protein.

The effect of sustained 5-HT_{1A} receptor activation on K^+ channels has been studied on p-neurons of the leech CNS, where they induce phosphorylation of two different types of K^+ channels increasing their open state probability (Goldermann et al., 1994). Similar to abovementioned restrictions, the possibility that such a mechanism might account for desensitization of 5-HT_{1A}-mediated responses in mammalian 5-HT neurons is not at all clear, however this as well as the previous observations open new research avenues that would be worth to explore.

ii) 5-HT release

Cellular and molecular mechanisms of neurotransmitter release and its regulation.- Exocytosis is the main mechanism used by neuronal cells for releasing neurotransmitter molecules. By this process synaptic vesicles fuse with the plasma membrane (Heuser and Reese, 1979) and the neurotransmitter(s) contained within them reaches the synaptic cleft. Exocytosis is triggered by cell depolarization. Depolarization induces opening of voltage sensitive calcium channels and subsequent Ca^{+2} entry. Achieving localized concentrations of 10 - 100 μM concentrations around the open channel (Smith and Augustine, 1988), intracellular Ca^{+2} increase constitutes the major coupling signal that links depolarization and exocytotic secretion (reviewed by Burgoyne and Cheek, 1995). One of the main characteristics of neurotransmitter release is its high speed, the complete cycle being achieved in hundreds of milliseconds. This is apparently due to the fact that secretory vesicles are already docked to the plasma membrane so that when Ca^{+2} entry takes place, vesicles in close vicinity of activated calcium channels will immediately void their contents into the synaptic cleft by formation of a fusion pore (see Burgoyne and Cheek, 1995). Docking and fusing of the vesicles to the plasmalema is achieved by Ca^{+2}-sensitive vesicle membrane proteins (Augustine et al., 1985, Smith and Augustine, 1988; Leveque et al., 1992). One of these proteins, synapsin I, in an unphosphorylated state, fixes secretory granules to the cytoskeleton. Once it undergoes Ca^{+2}-calmodulin/cAMP dependent phosphorylation it releases the vesicles from the cytoskeletal network and allows them to move to the presynaptic membrane (Valtorta et al., 1992), where they will be ultimately docked and voided to the extracellular space. The cytoskeleton is not likely to be involved in a first burst of release, which usually empties already docked vesicles, yet releasing the bound granules from the actin network will facilitate their subsequent recruitment by the

plasma membrane in preparation for the arrival of the next axon potential (see Burgoyne and Cheek, 1992).

The amount of neurotransmitter released is subject to receptor-dependent regulation that may theoretically occur at any stage in the release process described above. However, since most of the exocytotic steps are regulated by Ca^{+2} influx in an exponential manner (fourth power relationship, Augustin and Charlton, 1986), relatively small changes in its influx will be expected to produce profound changes in neurotransmitter release. Serotonin has been shown to enhance release by increasing voltage activated Ca^{+2} currents in postsynaptic neurons in the substantia nigra (*pars* compacta) and spinal cord (Nedegaard et al., 1988; Berger and Takahashi, 1990). In contrast, the main autoregulatory effect of 5-HT on its own release is inhibitory (see below). An inhibition in Ca^{+2} influx may be produced either by a direct effect on Ca^{+2} channels (shift in the voltage of activation of the channel; Bean, 1989) or by reducing the depolarization time during which Ca^{+2} enters the cell. This reduction in depolarization duration is frequently achieved by speeding the activation of K^+ or Cl^- currents that end depolarization linked to the action potential (Berlardetti and Siegelbaum, 1988; Brezina and Erxleben, 1988). Serotonin, acting on $5\text{-}HT_{1A}$ autoreceptors, has been shown not only to reduce a high threshold Ca^{+2} current (Pennington and Kelly, 1990; Pennington et al., 1991;.Pennington and Fox, 1994) but also to increase conductance to potassium ions (Aghajanian and Lakoski, 1984; Yoshimura and Higashi 1985). It is important to realize that the experiments that have assessed the cellular and molecular mechanisms of the regulation of neurotransmitter release have been performed on the cell body and that there is no direct electrophysiological information on the mechanism by which terminal 5-HT autoreceptors modify Ca^{+2} influx. Direct linkage, via G protein, of neurotransmitter receptors to Ca^{+2} or K^+ channels is widely used in nature as a means of inhibiting neurotransmitter release (see Miller, 1990; Anwyl, 1991) and could therefore be a possible mechanism by which 5-HT autoreceptors regulate release from 5-HT terminals. On the other hand, agents like cAMP by promoting Ca^{+2}-independent phosphorylation of synapsin I (Bähler and Greengard, 1987), may induce regulation of neurotransmitter release without modifying Ca^{+2} influx to the cell. Indeed, this is in keeping with the observation not only that adenylate cyclase inhibition by $5\text{-}HT_{1B/1D}$ receptor activation reduces 5-HT release, but also with the fact that cAMP analogues and stimulation of adenylate cyclase by

forskolin increase 5-HT release from neuroectodermal cell cultures (Tamir et al., 1990).

Neurotransmitter release from neuronal structures has been classically studied in terms of exocytosis, and its only recently that carrier-mediated release has been considered as a functionally acceptable mechanism for increasing extracellular concentration of a wide number of neurotransmitters including 5-HT (Levi and Raitieri, 1993). Unlike exocytosis, carrier mediated-release is Na^+ but not Ca^{+2} dependent, does not rely on a vesicular but a cytoplasmic pool of neurotransmitter, is not modulated by presynatic receptors and it is blocked by uptake inhibitors (see Levi and Raitieri, 1993). Indeed, based on the fact that the 5-HT transporter moves 5-HT with Na^+ and Cl^- across the membrane in one step, and K^+ repositions the carrier in a second step (Keyes and Rudnick, 1982; Nelson and Rudnick, 1982; Rudnick, 1986), Rudnick and Wall (1992a, 1992b) have recently shown that purified platelet plasma membrane vesicles, containing the same 5-HT transporter as the one responsible for 5-HT uptake into presynaptic nerve endings (Lesch et al., 1993), may either accumulate or extrude [^{3}H]5-HT when appropriate ionic transmembrane ionic gradients are imposed. If manipulating the Na^+ or K^+ electrochemical gradient changes the direction of neurotransmitter flow, it would then be tempting to assume that 5-HT may be released via this process during depolarization. Such a mechanism could explain observations by McKenna et al., (1991) in which Ca^{+2}-independent 5-HT release from rat brain synaptosomes was induced by high extracellular KCl concentrations. However, K^+-induced, Ca^{+2}-independent 5-HT release was not confirmed by Berger et al., using a similar *in vitro* preparation (1992).

On the other hand, multiple studies have confirmed that the 5-HT transporter mediates 5-HT release induced by substituted amphetamines such as *p*-chloroamphetamine (PCA), the anorexigenic drug fenfluramine, 3,4-methylenedioxyamphetamine (MDA) and the drug of abuse "ecstasy" (3,3-methylenedioxymethamphetamine, MDMA; McKenna et al., 1991; Berger et al., 1992; Rudnick and Wall, 1992a; 1992b; Gu and Azmitia, 1993; Bonanno et al., 1994). Since apart from releasing 5-HT these drugs also cause degeneration of fine terminals (Mamounas and Molliver, 1988; O'Hearn et al., 1988; Molliver and Molliver, 1990), transporter-mediated release of 5-HT has been proposed as a possible neurotoxic mechanism for 5-HT neurons, and different properties of 5-HT carriers in fine and beaded axons have been claimed as a possible explanation for the differential effect of these

drugs on the two types of fibres. More recently, demonstration that non-neurotoxic amphetamine derivatives also induce transporter-mediated 5-HT release, strongly suggests that release through the 5-HT transporter is not sufficient to cause destruction of serotonergic terminals (Rudnick and Wall, 1993). Indeed, several studies indicate that the dopamine releasing property of these amines may be a necessary cofactor for 5-HT fibre destruction (Stone et al., 1988; Schmidt et al., 1990; Johnson and Nichols, 1991).

Release of 5-HT in the midbrain raphe nuclei.- It is important to bear in mind that all of the methods presently used to determine neurotransmitter release such as synaptosomal or slice superfusion, *in vitro* or *in vivo* voltammetry and microdialysis measure not only release but the summatory of neurotransmitter release, uptake, diffusion and metabolism, generally referred to as neurotransmitter output. Hence, whenever the term release is used it is understood that it does not strictly refer to 5-HT release but rather what is detected by the method used. With this in mind, the extracellular concentration of 5-HT in the DRN has been estimated between 3-10 nM using *in vivo* voltammetry and microdialysis experiments (Crespi et al., 1988; Bel and Artigas, 1992; Adell et al., 1993). There seems to be general agreement on the fact that extracellular 5-HT levels may vary according to behavioral state changes (Cespuglio et al., 1990; Houdouin et al., 1991; Portas and McCarley; 1994). It is still controversial however, the direction in which these changes take place. While Jouvet's group has observed the highest 5-hydroxindole peak during sleep (Cespuglio et al., 1990; Houdouin et al., 1991) and supports the idea that enhanced 5-HT_{1A} autoreceptor activation secondary to dendritic 5-HT release triggered by hypnogenic factors such as CLIP (class II associated invariant chain peptides) or VIP (vasoactive intestinal polypeptide; El Kafi et al., 1994) might be responsible for determining the decrease in 5-HT neuron firing activity observed during SWS and REM (see Jacobs and Azmitia, 1992; Jacobs and Fornal, 1993), Portas and McCarley (1994) argue that extracellular 5-HT in the DRN is highest during wakefulness, lowest during REM, and that extracellular somatodendritic 5-HT availability depends directly on the serotonergic action potential activity. The latter observations are in agreement with results from electrophysiological studies in which 5-HT_{1A} antagonists were more effective in increasing 5-HT neuron firing activity during wakefulness than during sleep (Fornal et al., 1994). Furthermore, *in vitro* neurochemical studies indicate that within a range of 5 - 100 Hz, higher stimulation frequencies elicit increasingly higher extracellular

concentrations of 5-HT as measured by fast cyclic voltammetry in rat DRN slices (O'Connor and Kruk, 1991).

The role of firing activity on 5-HT release in the raphe nuclei has also been studied using the Na^+ channel blocker tetrodotoxin (TTX). The local administration of TTX into the DRN or MRN was shown to induce a decrease in spontaneous [^{3}H]5-HT release in non-anesthesized encéphale-isolé cats (Héry et al., 1986) and freely moving rats (Bosker et al., 1994) suggesting that 5-HT release is mainly dependent on firing activity. Other studies do not confirm this view: TTX injection in the immediate vicinity of the DRN (lateral boundaries) induced no change in extracellular concentration of 5-HT (Adell et al., 1993). Neither was the 5-HIAA peak in the DRN altered by intraraphe administration of this Na^+ channel blocker at a concentration that effectively reduced the voltammetric signal in the striatum (Scatton et al., 1985). Though it could be argued that it is possible that TTX injected in the vicinity and not within the raphe nuclei may not reach 5-HT neurons and that extracellular 5-HIAA may not always reflect 5-HT release, results from *in vitro* experiments have also been variable. When TTX was introduced into the perfusion medium spontaneous [^{3}H]5-HT release from midbrain raphe slices was increased (Héry et al., 1986), electrically-evoked release of 5-HT was reduced (Starkey and Skingle, 1994) and K^+-induced release of the neurotransmitter was unaffected (El Mansari and Blier, 1996), Interestingly, Pan et al., (1989) have shown that the electrical stimulation of midbrain raphe slices elicits a multicomponent postsynaptic potential in which a fast EPSP precedes the slow IPSP produced by 5-HT_{1A} receptor activation. This observation was interpreted by the authors as an indication that at least some of the 5-HT is released as a result of synaptically induced excitation of cell bodies, an assumption that seems confirmed by the fact that TTX abolishes not only electrically-evoked 5-HT release (Starkey and Skingle, 1994) but also the multiple PSP (Pinnock, 1992). On the other hand, TTX resistance of K^+-induced 5-HT release may be explained either because i) K^+-induced depolarization triggers a direct activation of voltage-sensitive Ca^{+2} channels and exocytotic release of 5-HT (experiments to determine the effect of Ca^{+2}-channel blockers on K^+ induced 5-HT release in presence and absence of TTX may help to assess this possibility) and/or ii) an increase in extracellular K^+ may induce carrier-mediated 5-HT release. In favour of the latter mechanism is the observation that 5-HT receptor agonists are much less potent in inhibiting K^+- than electrically-induced 5-HT release from raphe nuclei

(Middlemiss, 1987; Starkey and Skingle, 1994; Piñeyro and Blier, 1996). Furthermore, in view of the fact that pretreatment with reserpine does not modify 5-HT release in the DRN of freely moving rats, release from a cytoplasmic pool has been proposed (Adell et al., 1993). However, it is worth noting that Ca^{+2} omission greatly reduces or even abolishes electrically- and K^{+}-induced 5-HT release (Kerwin and Pycock, 1979; Héry et al., 1986; Starkey and Skingle, 1994; Piñeyro et al., 1995a) from midbrain raphe nuclei, indicating hence that exocytosis is the main mechanism involved in neurotransmitter release in this area. Also, in the cat nodose ganglia, which contain cell bodies and dendrites but no axons of 5-HT neurons, K^{+}-induced 5-HT release is totally abolished in a Ca^{+2} free medium (Fueri et al., 1984). Such an observation confirms not only that the soma and dendrites of 5-HT neurons may release 5-HT but also that somatodendritic release of 5-HT is predominantly exocytotic.

Physiological role of 5-HT release in midbrain raphe nuclei.- There is considerable evidence indicating that the activation of somatodendritic 5-HT_{1A} autoreceptors has a major impact in the amount of 5-HT released in forebrain projection areas. For example, infusion of the 5-HT_{1A} agonist 8-OH-DPAT into the DRN results in a decrease of 5-HT release in the striatum (Bonvento et al., 1992) and the hippocampus (Hutson et al., 1989, Sharp et al., 1989, Adell et al., 1993). Similarly, if the extracellular availability of 5-HT in the biophase of 5-HT_{1A} somatodendritic receptors is increased by the direct administration of the neurotransmitter or intraraphe perfusion of SSRIs or MAOIs, 5-HT release in cortex, striatum and hippocampus is markedly reduced (Becquet et al., 1990; Adell and Artigas, 1991; Celada and Artigas, 1993). If the injection volume within a specific nucleus is small enough to ensure no diffusion to the neighbouring dorsal or median raphe nucleus, then reduction of 5-HT release will follow the differential projection pattern of the nucleus into which the agonist was administered: *e.g:* injection of 8-OH-DPAT into the MRN reduces the extracellular 5-HT concentration in the hippocampus but not that of the striatum and the converse is true for intra DRN administration (Bonvento et al., 1992; Kreiss and Lucki, 1994).

The reported effect of 5-HT_{1A} autoreceptor blockade on 5-HT release in projection areas, is variable and may in part depend on the extracellular somatodendritic availability of 5-HT which determines in turn the activation state of 5-HT_{1A} receptors. In unanesthetized cats and freely moving rats, drugs that block 5-HT_{1A} receptors such as

methiothepin, S-UH-301, (+)WAY 100135 and pindolol produce no change in striatal or hippocampal 5-HT release (Becquet et al., 1990; Nomikos et al., 1992; Routledge et al., 1993; Romero et al., 1996). In contrast, the systemic administration of S-UH-301 or pindolol prior to local infusion into the DRN or systemic administration of citalopram blocks the reduction caused by the latter drug in hippocampal and striatal 5-HT release (Hjorth, 1993; Romero et al., 1996). Furthermore, infusion of methiothepin into the DRN blocks the reduction in hippocampal 5-HT release caused by systemic administration of the SSRI sertraline (Invernizzi et al., 1991), and systemic administration of the 5-$HT_{1A/1B}$ antagonist (-)penbutolol (Hjorth and Sharp, 1993) prevents the decrease in hippocampal 5-HT output caused by systemic citalopram administration (Hjorth, 1993). Similar to what has been observed for 5-HT neuron firing frequency (Table 3), 5-HT_{1A} ligands with low intrinsic activity, which had been classified as 5-HT_{1A} receptor antagonists when evaluated on postsynaptic receptors, such as BMY 7378 and NAN-190 have been shown to produce a small decrease in hippocampal 5-HT. This effect has been attributed to 5-HT_{1A} receptor activation (Hjorth and Sharp, 1990; Sharp et al., 1990). Systemic administration of the partial agonist SDZ 216525 also reduces 5-HT release in terminal projection areas. In this case however, the authors did not rule out the possibility that α_1-adrenoreceptor blockade and not partial agonism may explain the observed decrease in release (Sharp et al., 1993a, Gurling et al., 1993). On the other hand, (+) WAY 100135, which has been shown to inhibit 5-HT neuron firing activity by blocking α_1-adrenoceptors, when systemically administered by itself does not modify hippocampal 5-HT release (Routledge et al., 1993).

The idea that apart from somatodendritic autoreceptors 5-HT_{1A} receptors not located on the somata of 5-HT neurons may regulate firing-dependent terminal 5-HT release, has now gained considerable support. In fact, inactivation of autoreceptors by intraraphe infusion of pertussis toxin prevents the reducing effect on striatal 5-HT release caused by direct administration of citalopram into the dorsal raphe but does not interfere with the reducing effect caused by the systemic administration of 8-OH-DPAT (Romero et al., 1994). In another line of evidence, electrophysiological experiments in which 5-HT_{1A} agonists were microiontophoretically applied onto 5-HT neurons in the DRN and MRN indicate that the former are less responsive than the latter to the local application of such drugs (Blier et al., 1990a). Taking the latter observation into consideration it could be possible that Hjorth and Sharp (1991) found no differential effect on 5-HT released in

dorsal and median raphe-innervated areas following systemic administration of 8-OH-DPAT (250 µg/kg) in spite of the fact that 5-HT_{1A} receptors on both nuclei have different sensitivity to 5-HT_{1A} agonists, because post- and not presynaptic 5-HT_{1A} receptors were being stimulated. On the other hand, also using systemic administration Sinton and Fallon (1988) have reported a differential sensitivity of dorsal and median raphe neurons to this 5-HT_{1A} agonist: only 5 µg/kg of 8-OH-DPAT are needed to abolish 5-HT neuron firing activity in the DRN, whereas 30 µg/kg are not enough to induce cessation of firing of 5-HT neurons in the MRN. Whether these different observations following systemic administration of 8-OH-DPAT are due to different doses acting at different 5-HT_{1A} receptors or to the fact that the results from the two different methods used (electrophysiological and neurochemical) is not comparable, is not clear.

Autoregulation of 5-HT release in the raphe nuclei.- The abovementioned evidence indicates that by means of a potent feedback control mechanism on 5-HT neuron firing frequency 5-HT_{1A} somatodendritic autoreceptors constitute a major presynaptic determinant in the efficacy of 5-HT neuron synaptic transmission. Hence, all auto- or heteroregulatory mechanisms that control 5-HT availability in the biophase of 5-HT_{1A} autoreceptors will also contribute to determine the overall efficacy of 5-HT neuron synaptic transmission. The view that 5-HT release in the cell body area may be regulated by firing-controlling 5-HT_{1A} autoreceptors is further supported by evidence obtained in *in vitro* superfusion experiments. The 5-HT_{1A} agonists 8-OH-DPAT, buspirone and ipsapirone have been shown to inhibit electrically-evoked [^{3}H]5-HT release from midbrain raphe slices and this effect may be blocked by 5-HT_{1A} antagonists such as NAN-190, WAY 100135 and S-UH-301 (Starkey and Skingle, 1994; Davidson and Stamford, 1995a; Piñeyro et al., 1995a; Piñeyro et al., 1995b; Piñeyro and Blier 1996). It has also been shown that WAY 100135 increases electrically-evoked 5-HT release in the absence and in the presence of a selective 5-HT reuptake blocker (Starkey and Skingle, 1994; Davidson and Stamford, 1995b), an effect that has been attributed to the blockade by the SSRI, of the tonic activation of 5-HT_{1A} autoreceptors by the endogeneous neurotransmitter. Moreover, the local decrease in extracellular 5-HT observed by Bosker et al., (1994) following 8-OH-DPAT injection into the median raphe region further supports the notion that somatodendritic 5-HT release is subject to local feedback mechanisms through 5-HT_{1A} autoreceptors. Adell et al., (1993) on the other hand observed a decrease in 5-HT release

at the terminal but not at the somatodendritic level, following injection of 8-OH-DPAT in the vicinity of the DRN.

Apart from being regulated by somatodendritic 5-HT_{1A} autoreceptors 5-HT release from 5-HT neurons is under terminal 5-$HT_{1B/1D}$ autoreceptor control (see Starke et al., 1989 and Table 4). The latter, unlike the former can control 5-HT release without altering 5-HT neuron firing activity (*e.g.:* see Crespi et al., 1990). There is now considerable evidence indicating that this firing-independent control of 5-HT release also takes place at the cell body level, and that non-5-HT_{1A} receptors are involved. Using *in vivo* voltammetry, Blier et al. (1990a) have shown that the systemic administration of the 5-HT_1 agonist RU 24969 could reduce extracellular availability of 5-hydroxindoles in the DRN of anesthesized rats without altering 5-HT neuron firing frequency. More recently, these results have been confirmed and extended to TFMPP which following its systemic administration, was also shown to reduce extracellular availability of hydroxindoles in rat DRN without modifying 5-HT neuron firing activity (Piñeyro et al., 1995a; Piñeyro et al., 1996). Results from *in vitro* superfusion studies using midbrain raphe slices obtained from guinea pigs also support the notion that 5-HT release at the cell body level is regulated via non-5-HT_{1A} receptors: i) the 5-$HT_{1B/1D}$ antagonist GR 127935 by itself increases electrically-evoked release of 5-HT in guinea pig (Starkey and Skingle, 1994, El Mansari and Blier, 1996), ii) the 5-HT_{1D} agonist sumatriptan inhibits 5-HT release and this effect is blocked by the 5-$HT_{1B/1D}$ antagonist GR 127935 which also blocks the inhibitory effect of the non-selective agonist 5-methoxy-tryptamine (5-MeOT) but not by WAY 100135 (Starkey and Skingle, 1994; El Mansari and Blier, 1996).

Since in guinea pigs, only 5-HT_{1D} and not 5-HT_{1B} receptors have been detected (Heuring et al., 1986; Bruinvels et al., 1993), the previous evidence indicates that in this species 5-HT_{1D} receptors negatively regulate 5-HT release in the raphe nuclei. This contention is further supported by the fact that the 5-HT_{1B} agonist CP 93129 produced no effect on electrically evoked release from midbrain guinea-pig slices (El Mansari and Blier, 1996). In rodents, where brain 5-HT_{1D} as well as 5-HT_{1B} receptors have been detected (Hoyer et al., 1985a; Waeber et al., 1989a, Bruinvels et al., 1993), Davidson and Stamford (1995a), have reported that CP 93129 and sumatriptan inhibited 5-HT release in rat raphe nuclei. In this same study, the effect of the 5-HT_{1D} agonist sumatriptan was blocked by low concentrations of the 5-$HT_{1B/1D}$ antagonist GR 127935 (50 nM) but that of the 5-HT_{1B}

agonist was blocked at scattered time points during a continuous superfusion with a high concentration (500 nM) of isamoltane the 5-HT_{1B} antagonist isamoltane. The studies of the present research endeavour indicate a role for 5-HT_{1D} receptors in the regulation of the efficacy 5-HT synaptic transmission.

A possible interpretation of the observations mentioned above could be that in species like the guinea pig, that lack 5-HT_{1B} receptors, 5-HT_{1D} receptors would control not only 5-HT release in terminal areas but also in the cell body area. In rats, terminal autoreceptors are predominantly of the 5-HT_{1B} subtype (Table 4) and thus in control of 5-HT release from 5-HT fibres impinging on 5-HT nuclei as well as in terminal projection areas. On the other hand, somatodendritic release of 5-HT in rodents could be controlled by the 5-HT_{1D} receptor subtype.

Comparing the results obtained in microdialysis studies in rats and guinea pigs may help evaluate the role of 5-$HT_{1B/1D}$ receptor subtypes in the regulation of 5-HT release in raphe nuclei (Figure 4). In anesthesized rats pretreated with citalopram, the systemic administration of the ß-antagonist (-)penbutolol (8 mg/kg, s.c), produced an increase in hippocampal 5-HT release (Hjorth, 1993), most possibly by simultaneously blocking activated somatodendritic 5-HT_{1A} and terminal 5-HT_{1B} autoreceptors. This effect was similar to that observed following intrahippocampal perfusion of (-)penbutolol (1 µM in the perfusion medium) in combination with systemic administration of the 5-HT_{1A} antagonist S-UH-301 (3 mg/kg, s.c.). Hence, in anesthesized rats following the systemic administration of citalopram, if 5-HT_{1A} autoreceptor blockade is ensured, (-)pindolol given either systemically or locally through the dialysis probe in the hippocampus induces the same enhancing effect on hippocampal 5-HT release. It seems then that the blockade of 5-HT_{1B} receptors in midbrain raphe nuclei following the systemic administration of (-)penbutolol does not induce an additional increase in terminal 5-HT release as compared to local hippocampal perfusion of (-)penbutolol. In cerebral cortex of freely moving guinea pigs the results observed were quite different (Price et al., 1994). The systemic administration of the 5-$HT_{1B/1D}$ antagonist GR 127935 (0.3 mg/kg) produced a marked <u>decrease</u> in extracellular 5-HT, an unexpected effect since this drug had been shown to block terminal 5-HT_{1D} autoreceptors and increase 5-HT release in cortical slices (Price et al., 1994; Table 6). Though the observed decrease could be due, in theory, to 5-HT_{1D} partial agonistic properties of GR 127935 proposed by Tingley et al., (1994), it is

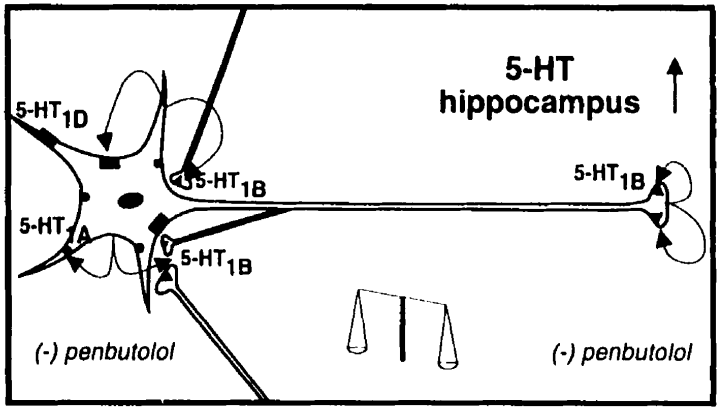
TABLE 4

Pharmacological profile of 5-HT terminal autoreceptors in cortex and hippocampus of different species determined by their effect on 5-HT release

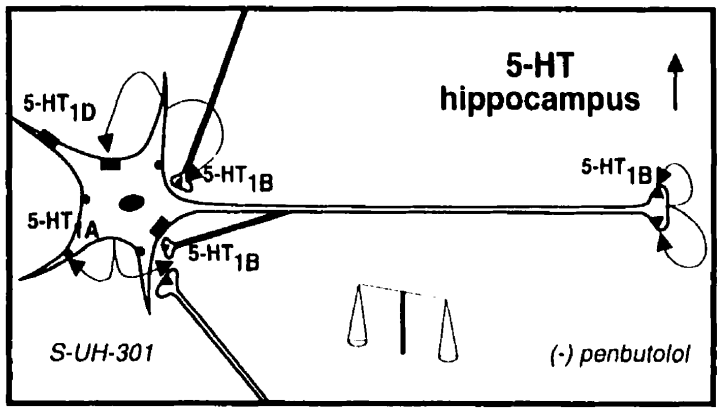
Species	Agonists	Antagonists	References
Cortex			
RAT	5-CT = RU 24969 > 5-HT >>> 8-OH-DPAT	methiothepin > metergoline	Limberger et al., 1991
	5-HT	(±)cyanopindolol < (-)alprenolol < (-)pindolol < (-)oxprenolol	Middlemiss, 1986
		(-)propranolol	Middlemiss, 1984
		(-)penbutolol	Hjorth and Sharp, 1993
	5-CT > 5-HT > 5-MeOT > LSD	(-)cyanop. > (±)cyanopi. > methio. > (+)cyanop. > (±)prop. > (-)pind. > meterg. > quip.	Engel et al., 1986
		(±)cyanopindolol < methiothepin < (-)propranolol < (-)pindolol	Schlicker et al., 1985
PIG	5-HT > 5-MeOT > RU 24969 >> 8-OH-DPAT	methio. > meterg. >> mianserin > prop. > mesuler.	Schlicker et al., 1989
GUINEA PIG	5-CT > 5-HT > RU 24969	methiothepin > metergoline >>> propranolol	Limberger et al., 1991
	sumatriptan		Sleight et al., 1990
	5-CT > 5-HT	metergoline	Ormandy, 1993
	5-MeOT > sumatriptan		
	L694247		Beer et al., 1993
	5-CT > 5-HT > sumatriptan	methiothepin	Wilkinson and Middlemiss, 1992
	5-CT > 5-MeOT		Blier and Bouchard, 1994
	5-MeOT > sumatriptan	methiothepin > metergoline >> methysergide	Middlemiss et al., 1988
		GR 127935	El Mansari and Blier, 1996
		GR 127935 > methiothepin	Price et al., 1994
RABBIT	5-CT > 5-HT > RU 24969	methiothepin > metergoline >>> propranolol	Limberger et al., 1991
HUMAN	RU 24969 > 5-CT	methiothepin > methysergide > metergoline = propranolol	Galzin et al., 1992
	5-HT > sumatriptan	methiothepin > metergoline	Maura et al., 1993
Hippocampus			
RAT	RU 24969 < 5-HT	methiothepin > (-)propranolol	Maura et al., 1986
	RU 24969 > CP 93129	methiothepin	Hjorth and Tao, 1991
GUINEA PIG	5-HT > sumatriptan	methiothepin	Wilkinson et al., 1993
	5-CT > 5-MeOT		Blier and Bouchard, 1994
RABBIT	5-CT > 5-HT > 5-MeOT >>> 8-OH-DPAT	metergoline > cyanopindolol > methiothepin	Feuerstein et al., 1987

Within each species drugs have been placed in a visual analog scale (decreasing affinity to the right)

RAT
(anesthesized rats, pretreated with systemic administration of an SSRI)

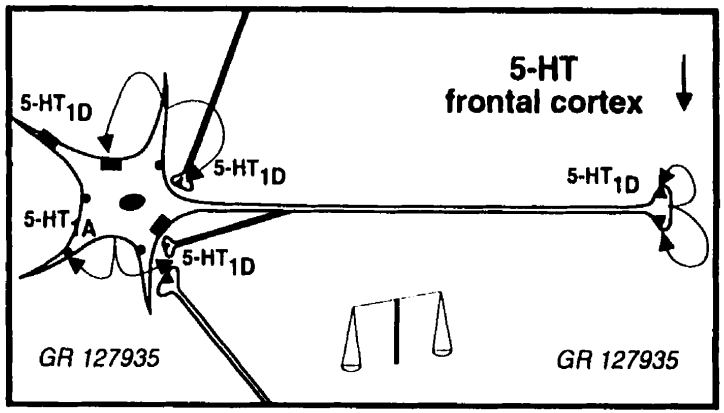


A. (-) penbutolol (8 mg/kg)

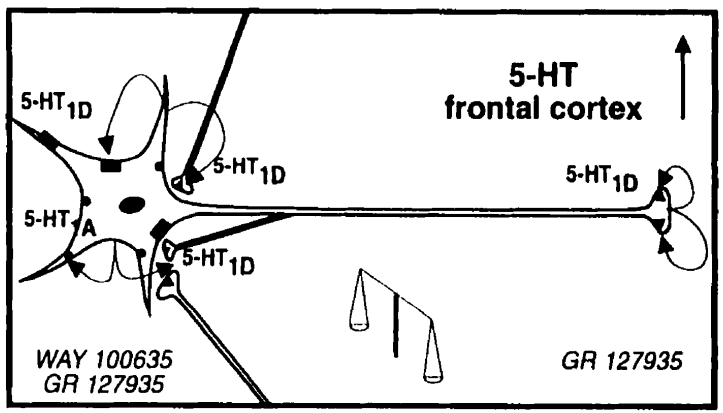


B. local perfusion (-)penbutolol (1 µM) + S-UH-301 (3 mg/kg)

GUINEA PIG
(freely moving guinea pigs)



C. GR 127935 (0.3 mg/kg)



D. WAY 100635 (0.3 mg/kg) + GR 127935 (0.3 mg/kg)

Figure 4

Figure 4. (**A**) Effect of systemic administration of the 5-$HT_{1A/1B}$ antagonist (-)penbutolol on spontaneous hippocampal 5-HT release in anesthesized rats. (**B**) Effect of hippocampal perfusion of the 5-$HT_{1A/1B}$ antagonist (-)penbutolol, and systemic administration of the 5-HT_{1A} antagonist S-UH-301 on spontaneous 5-HT release in rat hippocampus. (**C**) Effect of the systemic administration of the 5-$HT_{1B/1D}$ antagonist GR 127935 on cortical 5-HT release of freely moving guinea-pigs. (**D**) Effect of the combined administration of a 5-HT_{1A} (WAY 100135) and the 5-HT_{1D} antagonist on cortical 5-HT release in freely-moving guinea pigs (see text for details).

improbable that this could be the actual cause for the observed reduction in cortical 5-HT release since systemic WAY 100635 administration (1 mg/kg) not only blocked, but in fact reversed the inhibitory effect of GR 127935, producing thus a marked elevation in cortical 5-HT levels (Price et al., 1994). The reversal by WAY 100635 of the GR 127935 effect can be explained by assuming that GR 127935 blocked 5-HT_{1D} receptors that negatively control 5-HT release at the somatodendritic level. By doing so, 5-HT_{1D} antagonism would promote higher extracellular 5-HT levels in raphe nuclei and greater activation of somatodendritic 5-HT_{1A} autoreceptors which would lead in turn to a reduction in 5-HT neuron firing and thus a reduction in firing-dependent terminal 5-HT release. The administration of WAY 100635 blocks the 5-HT_{1A} autoreceptors, 5-HT neurons maintain their firing activity and the enhancing effect of terminal 5-HT_{1D} receptor blockade (by GR 127935) on terminal 5-HT release is manifested. It would be interesting to prove whether this hypothesis is correct by assessing the effect of systemic administration of GR 127935 in rat brain terminal projection areas. If the effect of GR 127935 is inhibitory like in guinea pigs it would suggest the blockade of a somatodendritic 5-HT_{1D} receptor. In such a case it should be further tested whether WAY 100635 unveils the enhancing effect produced by terminal 5-HT_{1B} receptor blockade by GR 127935 (a 5-$HT_{1B/1D}$ antagonist). Since guinea pig 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors are species homologues of rat 5-HT_{1D} and 5-HT_{1B} receptors, the speculation that 5-$HT_{1D\alpha}$ is in control of somatodendritic 5-HT release while 5-$HT_{1B/1D\beta}$ receptors control terminal release of the neurotransmitter from 5-HT axons seems plausible. Moreover, the facts that GR 127935 has been shown not to block the inhibitory effect of 5-HT on 5-HT neuron firing activity in guinea pig raphe slices (Craven et al., 1994), and that in rat DRN TFMPP as well as RU 24969 reduce somatodendritic availability of 5-hydroxindoles without changing 5-HT neuron firing activity (Blier et al., 1990), suggest that in anesthesized animals non-5-HT_{1A}, possibly 5-HT_{1D} receptors, regulate somatodendritic release of 5-HT without changing 5-HT neuron firing activity. Such an interpretation is further supported by the observation that in anesthesized guinea pigs, systemic administration of GR 127935 left 5-HT neuron firing activity unchanged (Sprouse et al., 1995).

Heteroregulation of neurotransmitter release in midbrain raphe nuclei.- Numerous neurotransmitters and neuromodulators from afferent terminals or intrinsic non-5-HT neurons influence 5-HT release in the raphe nuclei. For example, low frequency (1.5 Hz)

stimulation of the habenulo-raphe pathway elicits a decrease in 5-HT release in the DRN (Reisine et al., 1982). Similar results are obtained by intraraphe or systemic administration of GABA or GABA agonists (Scatton et al., 1985; Becquet et al., 1990). Interestingly, this pharmacological inhibitory effect on DRN 5-HT release is abolished by transection of the habenulo-raphe pathway (Nishikawa and Scatton, 1985). The need for the integrity of the habenulo-raphe connection has thus been interpreted in two alternative ways: i) the effect of GABA or GABA agonists is an indirect one and GABA receptors are located on habenulo-raphe fibres or ii) the inhibitory effect of GABA is made evident only if 5-HT neurons have a certain degree of tonic activation, provided in turn by the habenulo-raphe pathway. The common feature shared by both of these interpretations is that they assume that the habenulo-raphe pathway must contain an excitatory neurotransmitter, an assumption which is in disagreement with the fact that the main observed effect of habenulo-raphe pathway stimulation is depression of 5-HT neuron firing (72-88% of the 5-HT neurons are inhibited; Stern et al., 1981). However, when a high stimulation frequency (15 Hz) is used, the habenulo-raphe pathway induces a marked increase in 5-HT release in projection areas, an effect that is blocked by injection of kynurenic acid into the DRN (Kalen et al., 1989). Such an observation has led Kalen et al., to suggest that EAA are the main neurotransmitter in the habenulo-raphe pathway, and that glutamatergic fibres could have a double effect on 5-HT neurons: i) direct stimulation and ii) indirect inhibition by stimulating GABAergic interneurons. Such an observation is in keeping with the observations that stimulation of midbrain slices with an electrode in the DRN causes fast IPSPs and EPSPs that are respectively blocked by bicuculline or picrotoxin and the NMDA antagonists CNQX and APV (Pan et al., 1989; Pinnock 1992). The EPSP due to electrically-evoked release of EAA from afferent fibres onto 5-HT neurons, may be reduced by activation of presynaptic inhibitory kappa-opiod receptors located on the glutamatergic fibres (Pinnock, 1992). However, the predominant effect of systemically administered morphine is a stimulation of 5-HT release, an effect contrary to the one expected from activating inhibitory receptors on excitatory fibres impinging onto 5-HT neurons. A possible explanation for this observation could be that the effect of activation of kappa-receptors on EAA terminals may be overcome by activation of other opioid receptors, probably also located within the raphe nucleus as suggested by Tao and Auerbach (1994). Apart from eliciting postsynaptic potentials on 5-HT neurons recorded

from midbrain raphe slices, GABA and glutamate have been shown to modulate 5-HT release in rostral rhombencephalic raphe cells in primary cultures (Becquet et al., 1993a). GABA produces its negative modulation predominantly via $GABA_A$ but also $GABA_B$ receptors, while EAA induce 5-HT release by stimulating NMDA receptors (Becquet et al., 1993a; 1993b). Furthermore, *in vivo* application of the $GABA_A$ antagonist picrotoxin into the DRN, locally increased 5-HT release in unanesthetized rats (Becquet et al., 1990), suggesting that the latter receptors might induce a tonic inhibition of 5-HT release in the dorsal raphe. The release of 5-HT in the DRN is also modulated by tachykinins and cathecholamines. Indeed, substance P has been shown to increase 5-HT release *in vitro* in midbrain raphe slices (Kerwin and Pycock, 1979) and *in vivo* following its intraraphe injection (Reisine et al., 1982). The local infusion of amphetamine, apomorphine, and the selective D_2 receptor agonist quinpirole also increases 5-HT release in DRN, the effect of apomorphine being blocked by the selective D_2 receptor antagonist raclopride but not the D_1 antagonist, SCH 23390 (Ferré and Artigas, 1993; Ferre et al., 1994). The latter observations confirm the role of D_2 receptors in modulating extracellular availability of 5-HT in the DRN but whether the source of DA are dopaminergic afferents or DA neurons within the nucleus is still unknown. In the case of NA, this catecholamine inhibited K^+-evoked release of [^{3}H]5-HT from raphe slices. This effect was mimicked by α_2-adrenoceptor agonists clonidine and oxymetazoline but not by the α_1-adrenoceptor agonists phenylephrine and methoxamine. Furthermore, yohimbine and rauwolscine not only blocked the effect of clonidine, but when administered alone they both increased the K^+-induced release of [^{3}H]5-HT (Frankhuijzen et al., 1988). A possible interpretation of the auto- and heteroregulatory connections of 5-HT release in the DRN, is given in Figure 5.

An interesting feature to be noticed is that in some cases heteroregulatory influences may activate autoregulatory mechanisms, *e.g:* D_2 receptor activation induces increase in DRN 5-HT release but a decrease in striatal release, the latter effect being blocked by the administration of (+)WAY 100135 (Ferré et al., 1994). In other cases, heteroregulatory mechanisms may override autoregulation, *e.g.:* local infusion of substance P enhances 5-HT release in the dorsal raphe (Reisine et al., 1982) and in hippocampus (Gradin et al., 1992); or the contrary is observed with GABA which in spite of decreasing somatodendritic availability of 5-HT when injected into the DRN it also decreases striatal 5-HT release (Becquet et al., 1990).

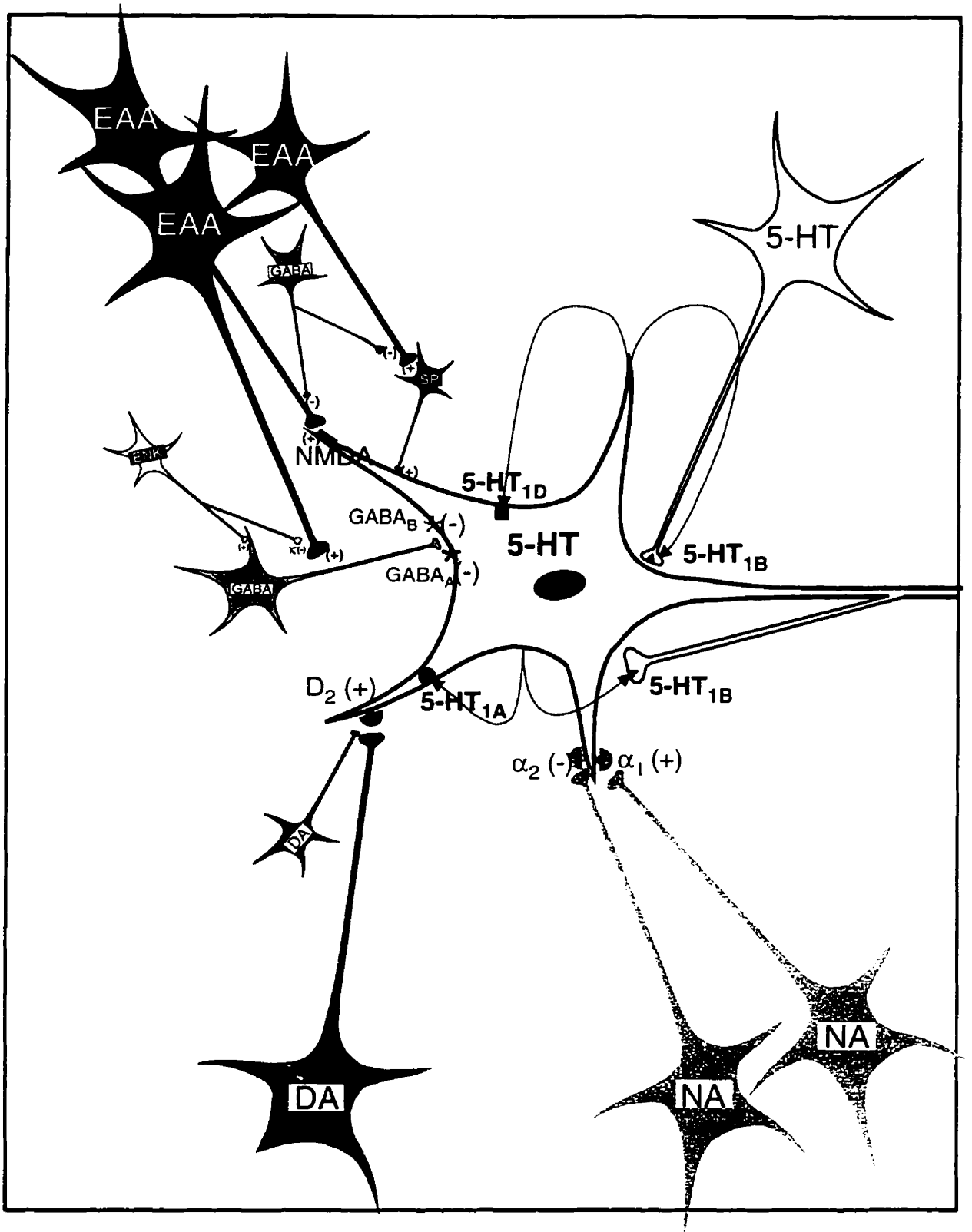


Figure 5. Graphic representation of auto- and heteroregulatory connections of midbrain 5-HT neurons

Autoregulation of 5-HT release in terminal projection areas: cortex and hippocampus.- Evidence indicating that activation of 5-HT_{1B} (in the rat and mouse brain) or 5-HT_{1D} (guinea pig, pig, rabbit, human brain) terminal autoreceptors inhibits 5-HT release is extensive and convincing (Table 4 for pharmacological profiles; Middlemiss and Hoyer, 1989; Starke et al., 1989; Göthert, 1990; Middlemiss and Hutson, 1990). Apart from this well established fact there is also considerable evidence indicating that more than one receptor subtype is involved in controlling release in the same region, and that some of these receptors might not be of the 5-$HT_{1B/1D}$ subtype. In rat cortical slices, 8-OH-DPAT has been shown to inhibit electrically or K^+-evoked 5-HT release (Hamon et al., 1984; Limberger et al., 1991). Since such an effect was not blocked by the 5-HT_{1B} antagonist isamoltane (Waldmeier et al., 1988; Schoefter and Hoyer, 1989), it was suggested that 5-HT_{1D} isamoltane-resistant sites might contribute to regulate 5-HT release in the rat frontal cortex (Limberger et al., 1991). Today we know that 8-OH-DPAT has considerable affinity not only for 5-HT_{1D}, but also 5-HT_5 and 5-HT_7 receptors (Table 5), and also that mRNA for all three receptor subtypes are present in the DRN (Table 5), suggesting that similarly to 5-$HT_{1B/1D}$ subtypes, 5-HT_5 as well as 5-HT_7 receptor subtypes could be expressed as autoreceptors on 5-HT neurons. The concentrations at which 8-OH-DPAT effectively inhibited evoked 5-HT release in the abovementioned studies were higher than 100 nM. Thus it is tempting to speculate that the probability for 5-HT_{1D} or 5-HT_5 receptors to be involved in this effect is greater than for 5-HT_7 receptors, since the affinity of the agonist for the latter receptors is very high (Table 5) and 8-OH-DPAT would have been expected to be effective at lower concentrations. Moreover, it should be noted that at concentrations of up to 1 μM of 8-OH-DPAT some groups were unable to demonstrate an inhibition of cortical evoked release of 5-HT (Middlemiss, 1984; Engel et al., 1986; Maura et al., 1986). Further evidence supporting the fact that 5-HT receptors controlling 5-HT release in rat hippocampus are heterogeneous has been obtained by our group. In superfusion experiments sumatriptan (1-1000 nM) and CP 93129 (1-300 nM) induced a dose-dependent inhibition of electrically-evoked [^{3}H]5-HT release from rat hippocampal slices. While the effects of CP 93129 and sumatriptan were blocked by (±)cyanopindolol (1 μM), only that of sumatriptan was blocked by mianserin (0.3 and 1 μM). On the other hand, the effect of CP 93129 was blocked by (-)propranolol (0.3 μM) which did not block that of sumatriptan. Moreover, incubation of rat hippocampal slices

TABLE 5. Affinity values of G-protein coupled 5-HT receptors for different drugs used in the present research endeavour*

	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{1D}$	5-HT$_{1E}$	5-HT$_{1F}$	5-HT$_{2A}$	5-HT$_{2C}$	5-HT$_{4}$	5-HT$_{5A/B}$	5-HT$_{6}$	5-HT$_{7}$
5-CT	0.2 nM[31]	7 nM[17] 0.35 nM[13]	0.7 nM[19] 0.37 nM[13] 1.5 nM[29]	7875 nM[3]	717 nM[3]	2000 nM[31]	600 nM[31]	1000 nM[8] 3160 nM[45]	16-40 nM[4] 12-235 nM[9]	774 nM[6] 253 nM[8]	0.12 nM[5] 0.93 nM[8]
methiothepin	79 nM[35] 89 nM[36]	5 nM[14] 11 nM[13] 50 nM[35]	0.6 nM[13] 50 nM[35]	194 nM[3]	652 nM[3]	1.58 nM[35]	25 nM[35]	> 10000 nM[8]	16-100 nM[4] 23-145 nM[9]	0.39 nM[6]	3.7 nM[8]
8-OH-DPAT	1.2 nM[24] 0.4 nM[36]	> 10000 nM[14,13]	120 nM[19] 99 nM[13] 975 nM[15]	3333 nM[3]	1772 nM[3]	700 nM[24]	> 10000 nM[44]	> 10000 nM[45]	400 - 1200 nM[4]	–	52 nM[5]
WAY 100135	23 nM[32]	> 10000 nM[31]	–	–	–	6000 nM[31]	> 10000 nM[31]	–	–	–	–
S-UH-301	52 nM[32] 126 nM[34]	> 10000 nM[35]	–	–	–	7200 nM[35]	7150 nM[45]	–	–	–	–
CP 93129	1500 nM[12]	8 nM[12] 55 nM[16]	1100 nM[18] 954 nM[41]	–	–	7200 nM[12]	2900 nM[12]	–	–	–	–
(-) propranolol	52.5 nM[35]	55 nM[35] 5 nM[13] 50 nM[41]	1509 nM[15] 1300 nM[13] 57 nM[16]	> 10000 nM[18]	> 10000 nM[18]	2987 nM[33] 158 nM[43]	200 nM[33]	–	6000 - 20000 nM[4]	–	–
sumatriptan	320 nM[29] 239 nM[39]	465 nM[16] 45 nM[13] 100 nM[14]	9.5 nM[13] 8.9 nM[15]	2520 nM[3]	23 nM[3]	> 10000 nM[22]	> 10000 nM[22]	–	8000 - 15000 nM[4]	–	506 nM[5] 951 nM[8]
GR 127935	125 nM[31]	3 nM[30]	3 nM[30]	3000 - 4000 nM[30]	–	40 nM[30]	630 nM[30]	> 10000 nM[30]	6000 - 20000nM[30]	–	–
mianserin	500 nM[13] 1000 nM[35] 1150 nM[40]	6000 - 10000 nM[13,35]	30 nM[13] 398 nM[35]	–	–	7.9 nM[35]	10 nM[35] 1.9 nM[42]	–	–	38 nM[6]	67 nM[5]
TFMPP	200 nM[35] 300 nM[23] 170 nM[24]	3.3 nM[13] 27 nM[24]	59 nM[13] 690 nM[35]	6293 nM[3]	1002 nM[3]	32 nM[24] 780 nM[35]	13 nM[35] 50 nM[23]	–	3000 - 4000 nM[4]	482 nM[6]	466 nM[8]
RU 24969	7.9 nM[35] 2.5 nM[38]	1.22 nM[14] 0.36 nM[13] 2 nM[17]	65.6 nM[15] 47 nM[13]	–	–	42 nM[35]	316 nM[35] 47.8 nM[39]	> 10000 nM[45]	3000 - 4000 nM[4]	–	–

TABLE 5. (cont.)

	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{1E}	5-HT_{1F}	5-HT_{2A}	5-HT_{2C}	5-HT_{4}	5-$HT_{5A/B}$	5-HT_{6}	5-HT_{7}
selective ligand	8-OH-DPAT WAY100635[25]	CP93129 SDZ21009[28]	L694247[27] GR172935[28]	–	–	ketanserin MDL100907[28]	mesulergine SB200646[28]	renzapride BUIM8 GR113808[28]	–	–	–
mRNA in DRN	YES very high[7]	YES high[2,20]	YES moderate[2]	NO[2]	YES low[2]	–	–	–	YES (5-HT_{5B})[4]	–	YES[5]
binding sites in DRN	YES very high[7]	NO[21] YES/ moderate[26]	YES/ low[26]	–	–	–	–	–	–	–	NO[1] YES/ moderate[11]

* Affinity values are given as KD or Ki values for binding studies performed in brain membranes or heterologous expression systems

TABLE 6

Differential influence of species and stimulation paradigm on the effects of terminal autoreceptor antagonists on 5-HT release in cortex and hippocampus.

Species	Antagonist	Effect observed	Stimulation procedure used	References
Cortex				
RAT	(-)alprenolol (1 μM)	↑	K^+ 25 mM	Middlemiss, 1986
	(-)atenolol (1 μM)	↔	-	-
	(±)cyanopindolol (10 -100 nM)	↑	-	-
	(10 nM)	↓	3 Hz; 360 pulses	Schlicker et al., 1985
	isamoltane (1 μM)	↑	100 Hz; 4 pulses	Limberger et al., 1991
	mesulergine (1 μM)	↔	K^+ 25 mM	Middlemiss et al., 1988
	metergoline (1 μM)	↔	3 Hz; 360 pulses	Göthert, 1980
	(100 nM)	↓	100 Hz; 4 pulses	Limberger et al., 1991
	methiothepin (1 μM)	↑	K^+ 25 mM	Middlemiss, 1984
	(100 nM)	↑	3 Hz; 360 pulses	Göthert, 1980
	(100 nM)	↔	100 Hz; 4 pulses	Limberger et al., 1991
	methysergide (1 μM)	↔	K^+ 25 mM	Middlemiss et al., 1988
	(-)oxprenolol (1 μM)	↑	-	Middlemiss, 1986
	(+)oxprenolol (1 μM)	↑	-	-
	(-)pindolol (1 μM)	↔	-	-
	(-)propranolol (1 μM)	↔	-	Middlemiss, 1984
	(1 μM)	↓	100 Hz; 4 pulses	Limberger et al., 1991
	quipazinel (10 μM)		K^+ 25 mM	Middlemiss, 1984
GUINEA PIG	(±)cyanopindolol (1 μM)	↔	K^+ 25 mM	Middlemisset al., 1988
	GR 127935 (10 μ)	↑	spontaneous release, freely moving	Roberts et al., 1994
	(100 nM)	↑	spontaneous release, anesthesized	Skingle et al., 1995
	isamoltane (1 μM)	↑	100 Hz; 4 pulses	Limberger et al., 1991
	mesulergine (1 μM)	↔	K^+ 25 mM	Middlemiss et al., 1988
	metergoline (300 nM)	↔		
	(1 μM)	↓	100 Hz; 4 pulses	Limberger et al., 1991
	methiothepin (10 nM)	↔	K^+ 25 mM	Middlemiss et al., 1988
	(300 nM)	↑		Wilkinson et al., 1993
	(1 μM)	↔	100 Hz; 4 pulses	Limberger et al., 1991
	methysergide (1 μM)	↓	K^+ 25 mM	Middlemiss et al., 1988
	(-)propranolol (1 μM)	↔	100 Hz; 4 pulses	Limberger et al., 1991
RABBITT	isamoltane (1 μM)	↑	100 Hz; 4 pulses	Limberger et al., 1991
	metergoline (100 nM)	↓		-
	methiothepin (1 μM)	↔		-
	(-)propranolol (1 μM)	↔		-

TABLE 6 (cont)

Species	Antagonist	Effect observed	Stimulation procedure used	References
Hippocampus				
RAT	BMY 7378 (0.25 mg/kg, i.v.)	↑	0.5 or 1 Hz; 60 or 120 pulses anesthesized	Chaput and de Montigny, 1988
	(±)cyanopindolol (10nM - µM)	↓	3Hz; 360 pulses	Feuerstein et al., 1987
	(30 - 300 nM)	↓	K^+ 15 mM	Maura et al., 1987
	(±)cyanopindolol (10 -100 nM) + nitroquipazine (1 µM)	↑	3Hz; 360 pulses	Feuerstein et al., 1987
	(±)cyanopindolol (10 nM- 1 µM) + fluvoxamine (1 µ)	↑	-	-
	mesulergine (300 nM)	↔	K^+ 15 mM	Maura et al., 1986
	metergoline (1 mg/kg, i.v.)	↑	1 Hz; 120 pulses	Haddjeri et al., 1996
	methiothepin (30 - 300 nM)	↔	K^+ 15 mM	Maura et al., 1986
	(1 mg/kg, i.v.)	↑	5 Hz; 600 pulses anesthesized	Chaput et al., 1986
	(1 mg/kg, i.v.)	↑	1 Hz; 120 pulses anesthesized	-
	(3 µM)	↑	K^+ 30 mM	Auerbach et al., 1990
	(10 µM)	↑	spontaneous release anesthesized	Hjórth and Tao; 1991
	methysergide (300 nM)	↔	K^+ 15 mM	Maura et al., 1986
	(-)penbutolol (1 µM) + citalopram (5 mg/kg; s.c.)	↔	spontaneous release anesthesized	Hjórth, 1993
	(-)propranolol (300 nM)	↔	K^+ 15 mM	Maura et al., 1986; 1987
GUINEA PIG	methiothepin (300 nM - 1 µM)	↑	1 Hz.; 120 pulses	Wilkinson and Middlemiss, 1992
RABBIT	(±)cyanopindolol (10 nM - 1 µM)	↓	3 Hz.; 360 pulses	Feuerstein et al., 1987
	(±)cyanopindolol (10 nM - 1 µM) + nitroquipazine (1 µM)	↓	-	-
	(±)cyanopindolol (10 nM - 1 µM) + fluvoxamine (1 µM)	↓	-	-
	metergoline (10 nM - 1 µM)	↔	-	-
	metergoline (10 nM - 1 µM) + nitroquipazine (1 µM)	↑	-	-
	metergoline (10 nM - 1 µM) + fluvoxamine (1 µM)	↑	-	-
	methiothepin (10 nM - 1 µM)	↑	-	-
	methiothepin (10 nM - 1 µM) + nitroquipazine (1 µM)	↑	-	-
	methiothepin (10 nM - 1 µM) + fluvoxamine (1 µM)	↑	-	-
	methysergide (10 nM - 1 µM)	↓	-	-
	(-)propranolol (10 nM - 1 µM)	↔	-	-

with N-ethylmaleimide (NEM), abolished the effect of CP 93129 but not that of sumatriptan (Piñeyro et al., 1996c). Furthermore, the inhibitory effect of 5-MeOT in rat hippocampus has been shown to remain unaffected by NEM, pertussis or cholera toxin-pretreatment, further indicating that in the rat there is an hippocampal 5-HT receptor subpopulation which is indeed resistant to G-protein inactivation (Blier, 1991). *In vivo* experiments in frontal cortex and hippocampus of 5-HT_{1B} knock-out mice also support the idea that non-5-HT_{1D}, non-5-HT_{1B} receptors regulate 5-HT release in mouse frontal cortex and that terminal 5-HT autoreceptor populations in cortex and hippocampus may be different (Trillat et al., 1996).

In species bearing predominantly 5-HT_{1D} terminal autoreceptors, an inhibitory effect of 8-OH-DPAT on terminal 5-HT release (Feuerstein et al., 1987; Schlicker et al., 1989), is not surprising given the affinity of the drug for the 5-HT_{1D} receptor (Table 5). However, until more selective ligands become available it is not possible to rule out an inhibitory autoreceptor role for 5-HT_5, 5-HT_7 receptors or even other 5-HT receptors not as yet characterized. Methiothepin is the only antagonist that effectively blocks the inhibition induced by 8-OH-DPAT (Feuerstein et al., 1987), but since methiothepin binds with high affinity to all 5-HT_{1D}, 5-HT_5 and 5-HT_7 receptor subtypes no further inferences can be made. In guinea pig hippocampus, but not in cortex, methiothepin has been shown to block the inhibitory effect of 5-CT and sumatriptan with less potency than that of 5-HT (Wilkinson and Middlemiss, 1992; Wilkinson et al., 1993), suggesting heterogeneity in the receptor subtypes regulating 5-HT release in the former but not the latter region, where methiothepin equipotently blocks the effects of both 5-HT and sumatriptan. Differences among the 5-HT receptor populations controlling 5-HT release in cortex and hippocamus in guinea-pig brain has also been reported by El Mansari and Blier (1996), who showed that G-protein inactivation with NEM attenuates the inhibitory effect of 5-MeOT in cortex but not in hippocampus though the inhibitory effect of sumatriptan was reduced in NEM-pretreated slices from both regions. The difference between cortical and hippocampal 5-HT autoreceptor populations is further supported by the fact that the inhibitory effect of 5-MeOT on electrically-evoked release of [^{3}H]5-HT, was attenuated in hippocampus but not frontal cortex slices obtained from guinea pigs that had received paroxetine for 21 days (Blier and Bouchard, 1994; El Mansari et al., 1995).

In vitro as well as *in vivo* studies have shown that the amount of 5-HT released per

electrical impulse increases with decreasing frequencies of stimulation (Göthert 1980; Baumann and Waldeier, 1981; Chaput et al., 1986a; Blier et al., 1989), probably due to a lesser degree of activation of the autoreceptors by prolonging the interval between the stimulation pulses. Furthermore, this interpretation is supported by the findings that at higher stimulation frequencies the effectiveness of terminal 5-HT receptor full agonists (5-HT itself, 5-CT, 5-MeOT) to inhibit 5-HT release is reduced and that of the antagonist methiothepin to enhance this parameter is increased (Baumann and Waldeier, 1981; Chaput et al., 1986a; Blier et al., 1989). Another way in which the concentration of endogeneous 5-HT in the biophase of 5-HT autoreceptors may be increased is by inhibition of neuronal 5-HT uptake. Similar to high stimulation frequencies, in the presence of 5-HT reuptake blockers the effectiveness of 5-HT agonists is reduced (Langer and Moret, 1982; Galzin et al., 1985) and that of antagonists is enhanced (Feuerstein et al., 1987). In order to explain SSRI-induced changes in efficacy, two alternative interpretations have been proposed: a) an increase in 5-HT concentration in the biophase of terminal autoreceptors and b) an interaction between neuronal uptake inhibitors and terminal 5-HT agonists (Langer and Moret, 1982; Galzin et al., 1985; Pasarelli et al., 1987; Moret and Briley, 1988). Two important arguments against the "molecular link hypothesis" favour on the other hand, the "5-HT biophase hypothesis": i) in experiments in synaptosomes in which the released transmitter is washed away rapidly, 5-HT in the biophase remains too low for autoreceptor activation and 5-HT receptor agonists produce the same inhibition of synaptosomal [^{3}H]5-HT release in the absence or presence of reuptake blockers (Raiteri et al., 1984; Bonnano and Raiteri, 1987); and ii) when very short trains of high frequency pulses are used in brain slices, release of [^{3}H]5-HT is measurable and yet pulses are too short to generate autoinhibition (Limberger et al., 1991). In the latter circumstances 5-CT and 5-MeOT generate similar concentration-effect curves for the inhibition of evoked [^{3}H]5-HT release in the absence and presence of different reuptake blockers (Limberger et al., 1990). On the other hand, the fact that 5-HT reuptake blockers continue to reduce the effectiveness of 5-HT autoreceptor agonists following 5-HT depletion by the 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA) has been used as a further argument to support the "molecular link hypothesis" (Galzin et al., 1985; Passarelli et al., 1987). It is important to notice however, that even following a 90% depletion of hippocampal 5-HT by PCPA pretreatment, 5-HT pre- and postsynaptic functions were found to be unchanged

(Chaput et al., 1990), suggesting the adaptability of the 5-HT system to maintain its efficiency even following a great reduction of endogeneous 5-HT.

The effect of changes in 5-HT biophase concentrations are particularly evident in the case of partial agonists which can either inhibit or enhance 5-HT release depending on the circumstances. For example, in the absence of nitroquipazine or fluoxetine (1 μM) the 5-HT_{1B} ligand (±)cyanopindolol inhibited electrically-evoked release of 5-HT in rat hippocampus and enhanced the same parameter when the slices were superfused with the uptake inhibitors (Feuerstein et al., 1987). Similarly, in conditions of negligible autoinhibition (4 pulses at 100 Hz), metergoline inhibited [^{3}H]5-HT release from rabbit frontal cortex slices (Limberger et al., 1991), had no effect when 5-HT biophase was higher (360 pulses at 3 Hz), and enhanced release when 360 pulses at 3 Hz were delivered in the presence of reuptake inhibitors (Feuerstein et al., 1987). Table six summarizes the effects on evoked 5-HT release in cortex and hippocampus obtained with different terminal autoreceptor blockers in different species and using different stimulation paradigms. Methiothepin has been shown to consistently enhance evoked release of 5-HT in conditions where autoinhibition exists, suggesting that it might be a pure antagonist. Furthermore, under the same conditions, (360 pulses at 3 Hz; rat hypothalamic slices) in which antagonists like metergoline and alprenolol had no effect by themselves but blocked the effect of LSD, methiothepin induced opposite effects to those of the terminal autoreceptor agonist. This observation has prompted the suggestion that methiothepin could possess inverse agonistic properties (Moret and Briley, 1993). More recently methiothepin was found to behave as an inverse agonist in [^{35}S]GTPγS binding to human $5\text{-HT}_{1D\alpha}$ and $5\text{-HT}_{1D\beta}$ receptors (Jones et al., 1995). This observation suggests that methiothepin binding to the 5-HT_{1D} receptors favours the uncoupling of the receptor-G-protein complex, and might explain why the drug appears more potent antagonist in functional assays in comparison to its binding affinity. An inverse agonist at the human terminal 5-HT_{1D} autoreceptor that would enhance 5-HT release in terminal projection areas, might prove an effective antidepressant with quick onset of action.

5-HT release in guinea pig and rat cortex and hippocampus is also modulated by 5-HT_3 receptors (Galzin et al., 1990; Barnes et al., 1992; Blier and Bouchard, 1993; Martin et al., 1993). Unlike $5\text{-HT}_{1B/1D}$, 5-HT_3 receptors are not localized on 5-HT terminals (Chen et al., 1991; Blier et al., 1993) instead of inhibiting, they enhance 5-HT release (Galzin et

al., 1990; Barnes et al., 1992; Blier and Bouchard, 1993; Martin et al., 1993), and desensitize within minutes of agonist exposure (see Hoyer 1990; Blier and Bouchard, 1993). They share with terminal autoreceptors their frequency dependence, being more effective to enhance 5-HT release at lower than higher frequencies (Blier and Bouchard, 1993).

Heteroregulation of neurotransmitter release from 5-HT fibres in cortex and hippocampus.- Several *in vivo* and *in vitro* studies, using brain slices or synaptosomes have provided evidence for the involvement of multiple neurotransmitters in the local regulation of 5-HT release in terminal projection areas. α_2-Adrenergic heteroreceptors on 5-HT terminals in the brain of different species have long been known to inhibit 5-HT release (Gobbi et al., 1990; Raiteri et al., 1990; see Göthert and Schlicker, 1991; Maura et al., 1992a; Blier et al., 1993). Several findings support the idea that the α_2-heteroreceptors differ from α_2-adrenergic autoreceptors regulating NA release in terminal projection areas: i) they have different pharmacological profiles (Raiteri et al. 1983a; 1983b; Maura et al., 1992b) ii) α_2-auto and heteroreceptors are differentially modulated by neuropeptides such as NPY (Martire et al., 1989) and iii) only the former desensitize following long-term treatment with befloxatone, a selective MAO-A inhibitor (see Blier et al., 1993). The idea of different adrenoceptors regulating 5-HT and NE release in terminal projection areas has been exploited in the development of new antidepressant drugs. Interestingly, selective α_2-heteroreceptor antagonists even given acutely, may have the capacity to enhance 5-HT neurotransmission (Haddjeri et al., 1996a). Like NE, histamine also exerts a negative regulation of 5-HT release, probably via the activation of H_3 heterotoreceptors (Fink et al., 1990). On the other hand, acetylcholine enhances forebrain 5-HT release by activating nicotinic receptors (Toth et al., 1992; Summers and Giacobini, 1995). The fact that kynurenic acid almost completely prevents the enhancing effect on neurotransmitter release induced by the local administration of nicotine, suggests that its effect on 5-HT release is indirect, mediated via glutamic acid release (Toth et al., 1992). Inhibitory amino acids are also involved in the regulation of 5-HT release in cortex and hippocampus. Following systemic administration of benzodiazepine agonists spontaneous or evoked release of 5-HT in either region has been shown to decrease (Hitchcott et al., 1990; Broderick, 1991; Cheng et al., 1993). Local effects are more ambiguous, in the hippocampal formation for example, the activation of the $GABA_A$ receptor complex has

been shown to produce a decrease, no effect or increase in 5-HT release (Pei et al., 1989; Lista et al., 1990). The fact that the local application of the Cl^--channel blocker picrotoxin, induced a robust increase in hippocampal 5-HT of freely moving rats support the idea that GABA exerts a tonic inhibition of 5-HT release in this region (Pei et al., 1989). Numerous polypeptides have also been found to locally modulate 5-HT release in projection areas. For example, PYY and pancreatic polypeptide inhibit cortical 5-HT release via the activation of the same presynaptic G-protein coupled receptor as NPY (Schlicker et al., 1991). On the other hand, substance P and neurokinin A, two coexisting neuropeptides of the tachykinin family, stimulate 5-HT release in this same area (Iverfeldt et al., 1990). Pharmacological activation of local opioid receptors (delta, kappa and mu) suggests that endogenous hippocampal opiates may also be involved in the negative regulation of 5-HT release in this area (Passarelli and Costa, 1989; Cui et al., 1994).

5-HT$_{1D}$ versus 5-HT$_{1B}$ receptors.- 5-HT$_{1B}$ binding sites, as opposed to 5-HT$_{1A}$ sites, were initially described as the sites that bind spiperone and 8-OH-DPAT with low affinity (Pedigo et al., 1981; Middlemiss and Fozard, 1983) but present high affinity for [I^{125}]cyanopindolol (Hoyer et al., 1985a, 1985b). They have also been defined by exclusion as [^{3}H]5-HT binding sites which are neither of the 5-HT$_{1A}$ nor of the 5-HT$_{1C}$ subtype (Blurton and Wood, 1986; Peroutka, 1986; Alexander and Wood, 1987). Until today 5-HT$_{1B}$ sites have been described in rodents (Hoyer et al., 1985a, Waeber et al., 1989a; Waeber and Palacios, 1992) but not in guinea pig, pig, cow or human brain (Heuring et al., 1986; see Bruinvels et al., 1993 and references within). In species in which 5-HT$_{1B}$ receptors have not been found, the sites visualized in the presence of saturating concentrations of 8-OHDPAT and mesulergine (Heuring and Peroutka, 1987; Waeber et al., 1988), have been named 5-HT$_{1D}$. However, it has been established that the sites labelled in this way represent an heterogeneous receptor population composed at least of 5-HT$_{1D}$ (5-CT sensitive) and 5-HT$_{1E}$ (5-CT insensitive) receptors (Leonhardt et al., 1989; Sumner and Humphrey, 1989; Beer et al., 1992, Lowther et al., 1992; Miller and Teitler, 1992). Furthermore, [^{3}H]5-CT has been shown to label two different subpopulations of 5-HT$_{1D}$ high affinity sites in guinea pig cortex and striatum (Mahle et al., 1991). It was the development of an iodinated radioligand, serotonin-5-O-carboxymethyl-glycyl-[^{125}I]tyrosinamide ([^{125}I]GTI; Boulengez et al., 1991; 1992) that allowed 5-HT$_{1D}$ binding sites to be directly labelled in human, non-human primate and guinea pig brain, among others

(Bruinvels et al., 1991; 1992).

5-HT_{1B} and 5-HT_{1D} binding sites are pharmacologically distinct; compounds that show at least 1.5 log units difference in their affinities for 5-HT_{1B} versus 5-HT_{1D} binding sites in mammalian brain membranes are: (-)SDZ 21009 (> 3; Hoyer et al., 1985a; Hoyer and Schoeffter, 1991), CP 93129 (> 2; Bruinvels et al., 1991; Koe et al., 1992), (-)pindolol (> 1.9; Hoyer et al., 1985a; Heuring et al., 1987; Hoyer and Schoeffter, 1991), cyanopindolol (> 1.8; Engel et al., 1986; Hoyer and Schoeffter, 1991) and (-)propranolol (> 1.8; Hoyer et al., 1985; Heuring et al., 1987; Hoyer and Schoeffter, 1991); or with greater affinity for 5-HT_{1D} vs 5-HT_{1B} binding sites are: yohimbine (> 1.8; Heuring et al., 1987; Bruinvels et al., 1991; Hoyer and Schoeffter, 1991), sumatriptan (> 1.5; Van Wijngaarden et al., 1990; Bruinvels et al., 1991), mianserin (> 1.5; Hoyer et al., 1985a; 1985b, Peroutka, 1986; Heuring et al., 1987; Bruinvels et al., 1991; Hoyer and Schoeffter, 1991) and 8-OH-DPAT (> 1.5 Heuring et al., 1987; Van Wijngaarden et al., 1990; Bruinvels et al., 1991; Hoyer and Schoeffter, 1991).

Recently, human 5-HT_{1D} (Hamblin et al., 1991; Adham et al., 1992; Demchyshyn et al., 1992; Hamblin et al., 1992a, Jin et al., 1992; Levy et al., 1992; Weinshank et al., 1992) and rat 5-HT_{1B} receptors (Voigt et al., 1991; Adham et al., 1992; Hamblin et al., 1992b, Maroteaux et al., 1992) have been cloned. From these studies it has become clear that the 5-HT_{1D} pharmacological phenotype, as described above, is conferred by two separate genes: a 5-$HT_{1D\beta}$ gene cloned in humans (Adham et al., 1992; Demchyshyn et al., 1992; Hamblin et al., 1992a, Jin et al., 1992; Levy et al., 1992; Weinshank et al., 1992) and a 5-$HT_{1D\alpha}$ gene cloned in rodents and humans (Hamblin et al, 1992b, Weinshank et al., 1992). While the overall similarity between 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors, which share an almost identical pharmacology, is 61-63% (Hamblin et al., 1992b; Weinshank et al., 1992), human 5-$HT_{1D\beta}$ and rat 5-HT_{1B} receptors share a 93% identity in their deduced amino acid sequence but have different pharmacological profiles. When expressed in heterologous systems 5-HT_{1D} and 5-HT_{1B} receptors maintain the same pharmacological profile as in brain membranes: the former have a higher affinity for sumatriptan, 8-OHDPAT and α_2-adrenergic antagonists and bind CP 93129 and arylalkylamine compounds such as propranolol and pindolol with lower affinity than the latter (Metcalf et al., 1992; Oksenberg et al., 1992; Parker et al., 1993; Adham et al., 1994). Interestingly, a single amino acid difference (asparagine vs threonine at position

355) has been shown to be responsible for the distinct pharmacological profiles, and 5-$HT_{1B/1D}$ receptor pharmacological phenotypes may be interconverted by a point mutation at amino acid 355 (Metcalf et al., 1992; Oksenberg et al., 1992; Parker et al., 1993; Adham et al., 1994). The relationship between 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$ and 5-HT_{1B} receptors has been summarized by Hartig et al. (1992): given the 70-80% identity in their transmembrane domains human 5-$HT_{1D\alpha/\beta}$ and rat 5-$HT_{1B/1D}$ receptors should be considered pairs of intraspecies receptor subtypes and members of the same gene product subfamily. Furthermore, given the high (95%) transmembrane homology between human 5-$HT_{1D\alpha}$ and rat 5-HT_{1D} receptors on the one hand and 5-$HT_{1D\beta}$ and 5-HT_{1B} receptors on the other, these two pairs should be considered as species homologues. As members of the same subfamily, apart from sequence identity receptors may share a common second messenger and similar pharmacological profile. Indeed, it was not until very recently that the 5-HT_2 receptor antagonists ritanserin and ketanserin were found to discriminate between 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$. These receptors were initially thought to be pharmacologically indistinguishable (1.1 - 1.8 log unit difference in K_D values, 5-$HT_{1D\alpha}$ receptors having a higher affinity than 5-$HT_{1D\beta}$ receptors for the 5-HT_2 antagonists; Doménech et al., 1994; Zgombick et al., 1995). Though less selective, GR 127935 and metergoline also distinguish between the two receptors with higher affinity for the 5-$HT_{1D\beta}$ subtype (1 and 0.6 log units, respectively; Skingle et al., 1991; Doménech et al., 1994). Moreover, in transfected cells, GR 127935 further distinguishes 5-$HT_{1D\alpha}$ from 5-$HT_{1D\beta}$ receptors, by eliciting a full agonistic response in the former and behaving as a silent antagonist for the latter receptor (Pauwels and Colpaert., 1995; Pauwels and Palmier, 1995). However, though transfected systems constitute a useful first approach for studying receptor pharmacology, interpretation of functional data from these studies should be cautious since drug activity may vary according to the level of receptor expression. For example, the aroalkylamines propranolol and pindolol, or the ergot derivative metergoline which have been described as antagonists or partial agonists in *in vivo* or *in vitro* studies of terminal 5-HT autoreceptors, were found to behave as full agonists in cells expressing the rat 5-HT_{1B} or human 5-$HT_{1D\beta}$ receptor, respectively (Miller et al., 1992; Adham et al., 1993a). Hence, further development of selective 5-$HT_{1D\alpha/\beta}$ compounds will depend on the availability of naturally occurring systems expressing the different receptors to allow drug evaluation. Hamel and coworkers have demonstrated that 5-$HT_{1D\beta}$ receptors mediate 5-

HT-induced contractions in cerebral arteries, establishing this tissue as an appropriate model of 5-$HT_{1D\beta}$ receptor function (Hamel and Bouchard, 1991; Hamel et al., 1993). On the other hand, the low expression of 5-$HT_{1D\alpha}$ relative to 5-$HT_{1D\beta}$ receptors in mammalian brain (Beer and Middlemiss, 1993; Bruinvels et al., 1993; 1994a; Doménech et al., 1994) has precluded identification of potentially 5-$HT_{1D\alpha}$-selective compounds using native systems. In the present research endeavour, the regulation of 5-HT release from murine raphe nuclei is shown to be regulated by 5-HT_{1D} murine receptors (Piñeyro et al., 1995a; Piñeyro et al., 1995b). Since murine 5-HT_{1D} receptors are of the 5-$HT_{1D\alpha}$ subtype, it is possible then, that the inhibitory effect of sumatriptan on electrically evoked release of 5-HT from midbrain raphe slices of 5-HT_{1B} knock-out mice, may prove a useful model for 5-$HT_{1D\alpha}$ activity.

The use of autoradiographic and *in situ* hybridization techniques, by respectively comparing distributions of 5-$HT_{1B/1D}$ binding sites and 5-$HT_{1B/1D}$ mRNAs have allowed to trace not only anatomical, but also cellular distribution of 5-$HT_{1B/1D}$ receptors (Bruinvels et al., 1994b; Doucet et al., 1995). [^{125}I]Cyanopindolol (Pazos et al., 1985) and more recently [^{125}I]GTI (Bruinvels et al., 1993) autoradiographic studies in rat brain have revealed a particularly high concentration of 5-HT_{1B} binding sites in striatum, substantia nigra and dorsal subiculum, and yet no mRNA for 5-HT_{1B} receptors was found in the latter two regions (Voigt et al., 1991; Bruinvels et al., 1993; Doucet et al., 1995). Conversely, the dorsal raphe nucleus which exhibited an intense mRNA hybridization signal displayed low or no 5-HT_{1B} binding at all (Vergé et al., 1986; Voigt et al., 1991; Bruinvels et al., 1993; Doucet et al., 1995). Mismatches between 5-HT receptor protein and mRNA previously observed in mice have been explained by assuming that 5-HT_{1B} receptors are transported along fibres far from somas where they are synthesized (Boschert et al., 1994). It is possible, for example, that 5-HT_{1B} receptors synthesized in the soma of CA_1 pyramidal neurons (CA_1 pyramidal neurons are intensely labelled for mRNA; Doucet et al., 1995) are transported via their glutamatergic projections to reach the dorsal subiculum as heteroreceptors. Similarly, 5-HT_{1B} heteroreceptors on striatal projections to the substantia nigra have been proposed to explain the high binding and low hybridization observed in this region (Bruinvels et al., 1994a). Moreover, 5-HT_{1B} receptors synthesized in the midbrain raphe could be transported via axons of the MFB to reach terminal areas where they have been described as autoreceptors.

Autoradiographic studies in human brain indicate that very high densities of 5-HT_{1D} receptors labelled by [^{125}I]GTI, are present in the basal ganglia, especially substantia nigra (Bruinvels et al., 1991; Palacios et al., 1992). A similar population of [^{125}I]GTI labelled sites have also been identified in human cortical membranes (Beer and Middlemiss, 1993) and guinea pig basal ganglia (Bruinvels et al., 1994a). In rat brain, [^{125}I]GTI labels 5-HT_{1B} as well as 5-$HT_{1D\alpha}$ sites. The latter have been defined as the non-5-HT_{1B}-[^{125}I]GTI binding displaced by the 4[2-[4-[3-(trifluoromethyl)phenyl]1-piperazinyl]ethyl]benzeneamine (PAPP; which has more than 2 log units higher affinity for 5-HT_{1D} than 5-HT_{1B} receptors; Schoeffter and Hoyer, 1989). These PAPP displaceable sites represent 15% and 11% of cortical and striatal iodine labelled sites, in rat brain (Bruinvels et al., 1993). In rat cortex the percentage of [^{125}I]GTI sites (5-HT_{1B} + 5-HT_{1D}) displaced by PAPP (designed 5-HT_{1D} sites; 15%) are in agreement with the percentage of total [^{125}I]GTI (5-HT_{1B} + 5-HT_{1D}) minus [^{125}I]CYP (5-HT_{1B}) in the same region (19%), and with the percentage of total 5-HT_1 binding represented by non-5-$HT_{1A/1B/1C}$ (5-HT_{1D}) cortical sites reported by Herrick-Davis and Titeler (18%; 1988). Contrastingly, in striatum the respective [^{125}I]GTI (5-HT_{1B} + 5-HT_{1D}) minus [^{125}I]CYP (5-HT_{1B}) and non-5-$HT_{1A/1B/1C}$ (5-HT_{1D}) sites where 20% and 30% (Herrick-Davis and Titeler, 1988; Bruinvels et al., 1993), representing more than double of the PAPP displacement sites (11%) in this same region. In the same study Herrick-Davis and Titeler (1988), reported more than 40% of non-5-$HT_{1A/1B/1C}$ sites with 5-HT_{1D} receptor pharmacology (5-HT>5-CT>>TFMPP>8-OH-DPAT>>(±)pindolol) in midbrain membranes. On the other hand, Bruinvels et al., (1993) reported an average of 8% PAPP-defined 5-HT_{1D} sites in the same area, while the 5-HT_{1B} sites labelled by [^{125}I]CYP in dorsal raphe and central grey represented approximately 60% of the 5-$HT_{1B/1D}$ sites labelled by [^{125}I]GTI). Such observations may indicate that PAPP does not identify all of 5-HT_{1D} sites or that [^{125}I]GTI labels other non-5-$HT_{1B/1D}$ sites. Given that the non-5-$HT_{1A/1B/1C}$ population described by Herrick-Davis and Titeler (1988) showed high affinity for 5-CT it is improbable that they are 5-$HT_{1E/1F}$ sites. However, the presence of multiple receptors within this population cannot be ruled out since Hill's coefficients for sites that recognized 5-CT ranged from 0.62 to 0.86 (Herrick-Davis and Titeler, 1988). Hence, the above-mentioned observations suggest that [^{125}I]GTI binding displaced by PAPP may not represent all of the 5-HT_{1D} receptors in rat raphe area. The midbrain, and especially the nuclei raphe are one of the areas which express the highest levels of 5-$HT_{1D\alpha}$ mRNA

(Hamblin et al., 1992b; Bruinvels et al., 1994a). Hence the high co-expression of 5-HT_{1D} sites (according to Herrick-Davis and Titeler; 1988) and mRNA could be interpreted as further support of the role of 5-$HT_{1D\alpha}$ as somatodendritic autoreceptors in the rat brain. Conversely, results from Bruinvels and co-workers (1993, 1994a; 1994b) would support the idea that most of the 5-HT_{1D} receptors are transported to terminal areas. On the other hand, there is also evidence for a matching distribution of 5-HT_{1B} sites and mRNA in the rat midbrain; Bruinvels et al., 1993; 1994b; Doucet et al., 1995). However, when tested in functional studies, at concentrations that do not alter basal outflow, the 5-HT_{1B} agonist CP 93129 did not modify 5-HT release (Piñeyro et al., 1995a; 1995b). A possible interpretation for this set of observations would be that 5-HT_{1B} receptors may contribute to regulate 5-HT release from 5-HT terminals in the raphe nuclei, though the latter do not seem as a major source of released 5-HT. Pharmacological properties, mRNA distribution and second messenger system of 5-$HT_{1B/1D}$ receptors as compared to other metabotropic 5-HT receptors are summarized in Table 5.

Effect of antidepressant administration on 5-HT release.- The reported effect of acute administration of 5-HT reuptake blockers depends on the dose used and the region examined. At high doses (10 mg/kg i.p or s.c) the acute administration of fluoxetine, citalopram or sertraline has been shown to induce an increase in extracellular 5-HT in terminal projection areas such as cortex, striatum or diencephalon (Dailey et al., 1992; Invernizzi et al., 1992; Perry and Fuller, 1992; Rutter and Auerbach, 1993, see Fuller 1994). The increase in extracellular 5-HT is dependent on neuronal firing since it is blocked by TTX (Perry and Fuller, 1992) or 8-OH-DPAT (Rutter and Auerbach, 1993). The latter observation is somewhat puzzling given the fact that ED_{50} i.v. doses of different SSRIs to inhibit 5-HT neuron firing are within the 0.1 and 0.5 mg/kg range (i.v.; Blier and de Montigny, 1980; Blier et al., 1984; Chaput et al., 1986b; Gartside et al., 1995; Hajos et al., 1995; Maudhuit et al., 1995; Kasamo et al., 1996), suggesting that at a dose of 10 mg/kg, no matter which SSRI is given, it would result in total shut down of 5-HT neuron firing activity. Even if rats are much more rapid metabolizers than humans, a dose of 10 mg/kg is 15 - 35 times the therapeutic dose. Hence, other studies have been performed using much lower doses of SSRIs. In such cases the systemic administration, 1 mg/kg of citalopram or 32 µmol/kg of sertraline (Invernizzi et al., 1991; Invernizzi et al., 1992) produced no increase in extracellular 5-HT in cortical projection areas, and experiments

in which extracellular 5-HT was simultaneously measured in cortex and raphe nuclei reveal that following systemic administration of these reuptake inhibitors, there is a preferential increase in extracellular 5-HT in the raphe region (Adell and Artigas, 1991; Bel and Artigas, 1992). It is this increase in somatodendritic extracellular 5-HT that activates the powerful 5-HT_{1A} autoreceptor feedback leading 5-HT neurons to establish their own "ceiling" to the extent to which uptake inhibitors increase extracellular 5-HT in terminal projection areas. In the cases in which an increase in terminal 5-HT availability has been documented, the magnitude of the response is not as great as it would have been if the adaptative response did not occur, mostly taking into account the fact that if extracellular concentration of 5-HT is high enough, terminal autoreceptors will also be activated. Reasoning that long-term reuptake blockade induces a desensitization of somatodendritic 5-HT_{1A} autoreceptors, Bel and Artigas (1993) proposed that it could be possible to overcome negative feedback and increase extracellular availability of 5-HT in terminal projection areas even using low doses of SSRIs. Indeed they treated rats with 1 mg/kg of fluvoxamine (s.c.) for two weeks and at the end of this time period the increase in extracellular concentration of 5-HT in frontal cortex of treated rats, still carrying the osmotic minipump, was similar to that observed following a 10 mg/kg acute i.v. dose (Bel and Artigas, 1992; 1993). These results have been confirmed in a study by Invernizzi et al., (1994) in which sustained treatment with citalopram (10 mg/kg/day x 14 days) facilitated the enhancing effect on terminal 5-HT release produced by a dose of 1 mg/kg of the SSRI administered 24 hs after the end of the treatment. Moreover, it was also shown in this study, that the reducing effect of a systemic dose of 25 μg/kg of 8-OH-DPAT on terminal 5-HT release was abolished following long-term citalopram administration, confirming a desensitization of 5-HT_{1A} autoreceptors (Invernizzi et al., 1994). On the other hand, Bosker et al., (1995a; 1995b) reported that 14 or 21-day treatment with oral or s.c. fluvoxamine (3 mg/kg or 6.5 mg/kg, at study outset, respectively) did not produce desensitization of 5-HT_{1A} autoreceptors, facilitation of a further dose of fluvoxamine nor increase in hippocampal 5-HT output. If indeed 5-HT_{1A} autoreceptor desensitization may account for antidepressant effect of prolonged SSRI administration, acutely reducing the activation of the feedback mechanism should produce a similar effect as sustained SSRI treatment. This assumption has been tested in two clinical trials that showed that in most drug-resistant depressed patients or in untreated unipolar depressed patients the

combination of (-)pindolol and an SSRI produced at least a 50% improvement in their depressive symptoms during the first week of treatment (Artigas et al., 1994; Blier and Bergeron, 1995). These results are in agreement with results referred in a previous section, in which blockade of 5-HT_{1A} autoreceptors was shown to increase extracellular availability of 5-HT in terminal projection areas following the acute administration of an SSRI.

Apart from inducing desensitization of somatodendritic 5-HT_{1A} autoreceptors, sustained paroxetine (10 mg/kg/day, s.c. x 21 days) administration has been reported to reduce the effectiveness of 5-HT_{1D} receptor activation to reduce electrically evoked release of 5-HT from midbrain guinea pig raphe slices (El Mansari and Blier, 1996). This same treatment as well as fluoxetine (5 mg/kg/day; i.p. x 21 days) induced an increase in electrically evoked 5-HT release from raphe slices (O'Connor and Kruk, 1994; El Mansari and Blier, 1996), indicating that 5-HT receptors that negatively control 5-HT release in the somatodendritic area are less sensitive to activation by the endogenous neurotransmitter following sustained 5-HT uptake inhibition. In contrast, Bel and Artigas (1993) found no increase in basal extracellular 5-HT levels in the dorsal raphe of rats treated with fluvoxamine (1 mg/kg/day, s.c. x 21 days).

Terminal 5-$HT_{1B/1D}$ autoreceptors in different projection areas including hippocampus, hypothalamus, orbito-frontal cortex have also been found to desensitize following sustained administration of SSRIs. Desensitization was demonstrated by a reduced efficacy of 5-$HT_{1B/1D}$ agonists to inhibit evoked 5-HT release (Blier and de Montigny, 1983; Blier et al., 1984; Chaput et al., 1986; Blier et al., 1988; Moret and Briley, 1990; O'Connor and Kruk, 1994, Blier and Bouchard, 1994, El Mansari et al., 1995). Such a desensitization results in a greater release of 5-HT per action potential, as indicated by a greater inhibition of the firing activity of CA_3 pyramidal neurons following 5-HT pathway stimulation (in the absence of changes in postsynaptic receptor sensitivity; Blier and de Montigny, 1983; Blier et al., 1984; Chaput et al., 1986; Blier et al., 1988) and an increase in electrically evoked release of [^{3}H]5-HT from preloaded slices of different projection areas, following a 48 h washout period after SSRI administration (Blier and Bouchard, 1994, El Mansari et al., 1995). Interestingly, not all terminal regions respond in a similar way to the same SSRI treatment, *e.g.:* following a 21 day treatment with paroxetine (10 mg/kg/day, s.c.) the inhibitory effect of the terminal autoreceptor agonist 5-

methoxytryptamine on the evoked release of 5-HT, was attenuated in slices of hippocampus and hypothalamus but not frontal or orbito-frontal cortex (Blier and Bouchard, 1994, El Mansari et al., 1995). In turn, of the two latter regions, only in the orbito-frontal cortex did desensitization occur after an eight-week treatment with paroxetine (10 mg/kg/day, s.c.; El Mansari et al., 1995). It is still unclear whether this difference results from 5-HT neurons being endowed with different autoreceptor populations depending on the terminal region to which they project, or whether it is the local influences that determine the different adaptative properties of the same autoreceptor subtype. Sustained administration of a low dose of fluvoxamine was found to induce desensitization of somatodendritic 5-HT_{1A} receptors but not of 5-$HT_{1B/1D}$ terminal autoreceptors the sensitivity of which remained unchanged following fluoxetine (5 mg/kg/d x 21 days) or fluvoxamine (6.7 mg/kg/d x 28 days; Bel and Artigas, 1993; Bosker et al., 1995; El Mansari et al., 1995). Furthermore, chlomipramine (10 mg/kg/day, s.c. x 21 days) did not produce a desensitization of 5-HT_{1D} terminal autoreceptors in rabbit hypothalamus (Schoups and Potter, 1988), probably due to the fact that though *in vitro* this drug is a potent and highly selective 5-HT reuptake blocker, *in vivo* it loses its selectivity as soon as it is degraded to chlordesipramine. It is thus possible that a high percentage or complete blockade of 5-HT reuptake should be achieved for terminal autoreceptor desensitization to occur.

The importance of autoreceptor plasticity in ensuring an enhanced 5-HT transmission is supported by the observation that prolonged administration of SSRIs reduces brain 5-HT (Hrdina, 1987; Caccia et al., 1992; Trouvin et al., 1993), indicating that following prolonged reuptake blockade increase in extracellular 5-HT availability is occurring in face of a reduction in the total 5-HT tissue content.

In the case of prolonged administration of MAOIs the situation is inverse, brain 5-HT being actually increased following sustained blockade of MAO-A (Blier et al., 1986a; 1986b; Celada and Artigas, 1993; Ferrer and Artigas, 1994). On the other hand, similar to SSRIs, the acute systemic administration of a selective MAO-A inhibitor, or non-selective MAO-A MAO-B inhibitors produces an immediate inhibition of 5-HT metabolism and a reduction in 5-HT neuron firing activity (Blier and de Montigny, 1985; Blier et al., 1986a; 1986b). It is not surprising then that when given acutely, MAOIs as SSRIs, produce a preferential increase in extracellular 5-HT in midbrain raphe nuclei as compared

to terminal projection areas. In contrast, prolonged MAOI administration increases extracellular availability of 5-HT to a similar extent in in pre- and postsynaptic projection areas (Celada and Artigas, 1993; Ferrer and Artigas, 1994; Bel and Artigas, 1995) with timing comparable to that of the desensitization of somatodendritic 5-HT_{1A} autoreceptors (Blier and de Montigny, 1985; Blier et al., 1986a, 1986b). In long-term experiments, tranylcypromine given at a dose that had no effect in acute experiments (0,5 mg/kg/day, s.c. x 14 days), produced a greater increase in extracellular cortical availability of 5-HT than the acute administration of a dose six times higher, even if the increases in tissue 5-HT concentrations were of 40% and 700%, following the long-term/low dose and acute high/dose treatments, respectively. These observations suggest that even if the increase in intracellular 5-HT is almost 20-fold higher following an acute high dose of tranylcypromine, the neurotransmitter is trapped within 5-HT terminals, only a small part of it being acutely releasable (intracellular/extracellular ratio of 5-HT increase in frontal cortex: 11.6 and 5.5 for acute/high dose and prolonged/low dose treatments respectively). In part, increased extracellular availability of 5-HT following long-term MAOI administration is due to desensitization of 5-HT_{1A} autoreceptors and recovery of 5-HT neuron firing frequency. However, an increase in terminal 5-HT release (hippocampus, cortex and hypothalamus) is seen not only *in vivo* in the whole animal, but also *in vitro* in slices containing only 5-HT terminals (Blier and de Montigny, 1985; Blier et al., 1986a; 1986b; Blier and Bouchard, 1994; Mongeau et al., 1994a), indicating that there is a 5-HT_{1A}-independent enhancement of neurotransmitter release. Unlike long-term treatment with SSRIs, the sensitivity of 5-HT autoreceptors remains unchanged (Blier et al., 1986a; 1986b; Blier and Bouchard, 1994). The question arising then is what is the mechanism involved in increasing the releasable amount of 5-HT following long-term MAOI administration? It has long been known that the A form of MAO catalyses the oxidative deamination not only of 5-HT but also of NE (Hall et al., 1969, Yang and Neff, 1973) and that 5-HT terminals are endowed with inhibitory α_2-heteroreceptors (Göthert and Huth, 1980; Göthert et al., 1981; Maura et al., 1982). More recently, our laboratory has shown that enhanced 5-HT release following sustained MAO-A inhibition correlates with the production of α_2-heteroreceptor desensitization by these treatments (Blier et al., 1986a; 1986b; Blier and Bouchard, 1994; Mongeau et al., 1994b) Moreover, destruction of the NE system impairs the heteroreceptor desensitization caused by the prolonged administration

of the reversible MAO-A inhibitor befloxatone (Mongeau et al., 1994b).

Antidepressants with α_2-adrenoceptor antagonistic properties like mianserin and (±)mirtazapine, whose long-term administration increases the duration of suppression of firing of CA_3 pyramidal neurons produced by 5-HT pathway stimulation, have also been shown to desensitize α_2-heteroreceptors on 5-HT neurons (Mongeau et al., 1994b; Haddjeri et al., 1996b). Other studies of the neurochemical effects of long-term mianserin administration (Raiteri et al., 1983a; Schoups and Potter, 1988) reported no change in the sensitivity of these receptors. A likely explanation for this discrepancy could be that in the latter studies mianserin was injected i.p. whereas in the studies in which they induced α_2-heteroreceptor desensitization the former antidepressant drugs were delivered continuously via osmotic minipumps implanted s.c.

If acute administration of MAOIs induces a preferential increase of 5-HT release in midbrain raphe nuclei, then like in the case of SSRIs, the combined administration of a 5-HT_{1A} antagonist together with a MAOI should produce a greater enhancement of 5-HT availability in the extracellular space of projection areas than the MAOI by itself. Indeed, depressed patients treated with moclobemide or phenelzine and (-)pindolol showed a reduction in the HDRS score within the first week of combined treatment (Artigas et al., 1994; Blier and Bergeron, 1995). On the other hand, in microdialysis studies in freely moving rats, the acute administration of supramaximal doses of the non-selective inhibitor tranylcypromine (15 mg/kg, i.p.) which increase motor activity; Celada and Artigas, 1993; Ferrer and Artigas., 1994) have been shown to produce a considerable increase (500% - 1100%) in extracellular cortical 5-HT, two to four fold higher than the long-term administration of 0.5 mg/kg, s.c. x 14 days (224% increase). Furthermore, the ratio of DRN/frontal cortex extracellular 5-HT was of six following 15 mg/kg, and one following 0.5 mg/kg, indicating that the observed 500%-1100% increase in extracellular cortical 5-HT following the supramaximal dose of tranylcypromine takes place even with a full activation of the negative somatodendritic autoregulatory feedback. Similar results were observed with the acute administration of the non-selective MAOI pargyline (75 mg/kg, i.p.) which elicited 10 - 14 fold increases in caudate-putamen and frontal cortex with the same time course as that seen with tranylcypromine (Kalen et al., 1988; Carboni and Di Chiara, 1989). In the tranylcypromine series of experiments, tissue concentrations of 5-HT were found to reach a plateau at an acute dose of 3 mg/kg, with no further change at 15 mg/kg,

while the cortical extracellular concentration of neurotransmitter increased 4-fold with this dose increase (Ferrer and Artigas, 1994). The latter observation indicates that even if MAO-A activity is completely blocked at the low dose of tranylcypromine, it is not the only factor determining extracellular 5-HT availability. The latter interpretation is supported by the fact that the concurrent administration of brofaromine (10 mg/kg, s.c.) or clorgyline (5 mg/kg, i.p.) with deprenyl (2.5 mg/kg, i.p; Bel and Artigas, 1995) also increase the extracellular concentration of cortical 5-HT to a much greater extent than the MAO-A inhibitor by itself. This group of observations may be interpreted as an indication that extracellular changes in 5-HT availability are determined not only by MAO-A activity, but also by 5-HT deamination by MAO-B as well as the capacity of the brain to store 5-HT.

The previous experiments also indicate that it is possible to induce an acute 250% - 400% increase in extracellular 5-HT concentration in projection areas by the concurrent inhibition of MAO-A and MAO-B. If such a strategy were to be applied to achieve a faster therapeutic response or as a potentiation in patients treated with but not responding to MAOIs (*ie:* used to induce a sufficient increase in extracellular 5-HT availability in projection areas without inducing dose-related side effects), various facts should be taken into account: i) MAO-A (contained in cathecolaminergic neurons and terminals; Westlund et al., 1985; 1988) is only 1.4 times more concentrated in DRN than cortex or hippocampus while the concentration of MAO-B (contained in 5-HT neurons; Levitt et al., 1982; Westlund et al., 1985; 1988) in the midbrain raphe is three times higher than in cortico-hippocampal terminal areas (Saura et al; 1992) ii) that MAO-B inhibition has no antidepressant effect by itself (Mann et al., 1984) but it may contribute to increase terminal 5-HT release if a high proportion, if not all, MAO-A activity is blocked; iii) that it is possible to produce an almost complete MAO-A blockade without an excessive increase of DRN extracellular concentration of 5-HT (Ferrer and Artigas, 1994); iv) that the doses of MAO-A/MAO-B inhibitors chosen should favour a terminal versus raphe nuclei blockade of the enzymes and v) at the doses that non-selective MAOI produce a significant acute increase in extracellular 5-HT in projection areas they might also induce collateral side effects. Hence, if the minimal dose of selective or non-selective MAO-A inhibitor that completely blocks MAO-A activity (*e.g:* 3 mg/kg tranylcypromine) is systemically administered in combination with one third of the 2.5 mg/kg deprenyl dose used by Artigas' group (in order to avoid the disproportionate increase in DRN 5-HT), an immediate and significant

increase in extracellular 5-HT in projection areas and hence a more efficient therapeutic could be expected.

One further question, that immediately arises is how the "speeding up strategies" of SSRI/pindolol, MAOI/pindolol or the latter proposed one, if successful, may manage to overcome inhibitory terminal autoregulation by 5-HT autoreceptors? Given that supramaximal doses of chlomipramine and tranylcypromine produce 300% - 1100% increase of the basal 2 nM cortical 5-HT concentration (Bel and Artigas, 1992) and that 5-HT concentrations between 10 - 100 nM inhibit 5-HT release from rat frontal cortex (Middlemiss, 1986; Limberger et al., 1991), an insufficient increase in extracellular concentration of neurotransmitter does not seem an appropriate explanation. The possibility that terminal autoinhibition could in fact be activated but 5-HT still released is not in agreement with the observation that 100 nM 5-HT has been shown to produce 90% inhibition of electrically evoked release of 5-HT in frontal cortex slices (Limberger et al., 1991). Further speculating, it is also possible that there is so much releasable 5-HT within the terminal vicinity that even if autoinhibition is activated by the first bursts of arriving action potentials the amount of released 5-HT is already high enough to increase its extracellular availability. Such an explanation would fit only in the case of MAOIs but not of SSRIs (which do not increase intracellular 5-HT availability). *In vivo* pharmacological studies of terminal autoreceptor-mediated regulation of 5-HT release in rats and guinea pigs treated with SSRI/pindolol or MAOI/pindolol combinations should help clarify this issue. Of course that even if in rats pindolol could block the terminal 5-HT_{1B} autoreceptor, this would not explain the improvement observed in patients where terminal autoreceptors seem to be predominantly of the 5-HT_{1D} type.

Moreover, another interesting point to be considered is why if both prolonged SSRI and MAOI administration increase *extracellular availability of 5-HT* only following the former are terminal autoreceptors desensitized? A possible answer to this question could be that by prolonging reuptake time, SSRIs produce a sustained increase in *intrasynaptic* 5-HT availability. On the other hand, the increase produced by MAOIs occurs following the arrival of action potentials to the terminal and hence extracellular 5-HT concentration increases in an "on-off" manner, probably not suitable for producing desensitization.

Direct activation of presynaptic 5-HT_{1A} receptors by 5-HT_{1A} agonists causes inhibition of 5-HT cell firing, synthesis, and release in forebrain areas (Blier and de

Montigny, 1987; Hjorth and Margusson, 1988; Sharp et al., 1989; Schechter et al., 1990; Godbout et al., 1991). At postsynaptic receptors biochemical and electrophysiological experiments in hippocampus (Yocca and Maayani, 1985; Yocca et al., 1986; Andrade and Nicoll, 1987) have shown that 5-HT_{1A} agonists act as partial agonists inhibiting cAMP formation and pyramidal neuron firing activity. Since upon acute administration there is net decrease in forebrain extracellular availability of 5-HT and their beneficial clinical antidepressant and anxiolytic effects are usually not observed until several weeks of administration (see Charney et al., 1990), it appears that the presynaptic effects of 5-HT_{1A} agonists override their postsynaptic actions and overall serotonergic tone falls. Based on electrophysiological results, it has been proposed that tolerance develops to the autoreceptor-mediated effects (Blier and de Montigny, 1987; Schechter et al., 1990; Godbout et al., 1991) and a combination of normal 5-HT tone together with simultaneous activation of postsynaptic normosensitive receptors by the drug relate to their therapeutic actions (see Blier and de Montigny, 1994). Neurochemical studies on the incidence of 5-HT_{1A} autoreceptor desensitization following sustained 5-HT_{1A} agonist administration have been less consistent with positive (Kreiss and Lucki; 1992) and negative results (Sharp et al., 1993b; Söderpalm et al., 1993). A possible explanation for the discrepancy between electrophysiological and neurochemical studies could be that the sensitivity of 5-HT_{1A} autoreceptors was tested directly on 5-HT neurons in the former and systemically in the latter. As previously discussed, systemic administration of 5-HT_{1A} agonists may modify 5-HT neuron electrophysiological and neurochemical activities acting not only at pre- but also postsynaptic 5-HT_{1A} receptors. Furthermore, the hypothesis generated by electrophysiological data predicts that concurrent administration of a 5-HT_{1A} agonist with a selective 5-HT_{1A} autoreceptor blocker should produce an immediate enhancement of 5-HT neurotransmission (Blier and de Montigny, 1994) and indeed the administration of buspirone (20 mg/day) with pindolol (2.5 mg/kg thrice daily) produced quick reductions of HRDS.

iii) 5-HT Reuptake

Following release, 5-HT is actively cleared from the synaptic cleft by a high affinity, transporter located on presynaptic neuronal membranes (Kuhar et al., 1972; see Kanner and Schuldiner, 1987; O'Reilly and Reith, 1988). Monoamine neuronal transporters function in series with another type of carrier, the vesicular transporter, that sequesters

intracellular 5-HT within secretory vesicles. The carriers taking up neurotransmitter from the extracellular space into the neuron are integral membrane proteins with twelve transmembrane spanning domains, they couple reuptake to Na^+ and Cl^- displacement across the plasma membrane and are encoded by a closely related gene family: Type I or plasma membrane Na^+/Cl^- coupled transporter family which includes GABA, catecholamine and 5-HT transporters. The vesicular transporter belongs to a different gene superfamily as do the glutamate transporters and will not be further considered (for reviews see Uhl and Hartig, 1992; Amara and Kuhar, 1993; Lester et al., 1994).

Molecular aspects of the 5-HT transporter (5-HTT).- Two main strategies have been used in an attempt to identify the molecular characteristics of the 5-HTT: i) biochemical purification and ii) cloning of cDNA coding the transporter. Using digitonin as a detergent the 5-HTT in rat brain and human platelets has been solubilized and purified in a conformational state that retained almost an identical pharmacological profile to that observed in native membrane preparations (Biessen et al., 1990; Graham et al., 1991; Launay et al., 1992). In a further step, the human placental 5-HTT has been reconstituted after purification displaying not only the same antidepressant binding profile of the native carrier but also NaCl-dependent 5-HT transport (Ramamoorthy et al., 1993a). The molecular weight of this functional protein isolated from human placenta is 300,000 (Ramamoorthy et al., 1993a). On the other hand, the other purified proteins have a molecular mass ranging between 55,000 and 78,000 (Biessen et al., 1990; Launay et al., 1992) and that of the cloned 5-HTT itself is about 70,000 (Blakely et al., 1991; Lesch et al., 1993a; Ramamoorthy et al., 1993b). Thus, in analogy with the Na^+/glucose transporter (Stevens et al., 1990), it has been proposed that the 5-HTT exists as an homotetramer (Ramamoorthy et al., 1993b). However, though dimeric concatenated constructs of the transporter have been shown to possess similar 5-HT transport activity as the monomer, concatenated tetramers have substantially lower activity (Chang et al., 1994). The latter observation does not preclude the existence of functional tetramers since simple functional monomers or dimers could associate and transport 5-HT.

The recent development of site-specific antibodies has allowed further characterization of the 5-HTT (Lawrence et al., 1995a; 1995b; Quian et al., 1995; Ovalle et al., 1995; Wade et al., 1996). The molecular weight of immunoprecipitates was found to vary according to the structure of origin (rat platelets 94,000; rat pulmonary membranes

80,000; rat brain 71,000 - 76,000, Hela cells: 90,000 - 200,000; Ovalle et al., 1995; Wade et al., 1996), and differential N-linked glycosylation, has been claimed to account for different molecular mass of CNS and peripheral 5-HTT units. In transfected Hela cells, inhibition of glycosylation changes the molecular mass of the transporter shifting the 90 kDa 5-HTT immunoreactivity band, to its presumably unglycosylated state of 56 kDa, In contrast, the mobility of a 200 kDa form of the transporter remained unchanged following glycosylation inhibition. Since unglycosylated monomers have less tendency to aggregate, it has been suggested that the high weight slow mobility form may represent a transporter aggregate, and that glycosylation may be involved in multimerization (Qian et al., 1995). If this interpretation is true the molecular weight of the placental purified transporter (Ramamoorthy et al., 1993a) also suggests the possibility of a tetrameric organization of the transporter (300.000/ 55.000 - 78.000 = 5.5 - 3.8).

Despite the differences in molecular weight, human CNS and peripheral 5-HTTs are polypeptides encoded by a single gene located on chromosome 17 (Lesch et al., 1993b; Ramamoorthy et al., 1993b). The existence of a single hybridizing mRNA, as well as the identity of the cDNAs cloning brain and peripheral rat 5-HTTs suggest that this is also the case for rodents (Blakely et al., 1991; Hoffman et al., 1991; Blakely et al., 1993). Though encoded by a single gene, in humans unlike rodents, there are different types of 5-HTT mRNA have been found in placenta, lung (Ramamoorthy et al., 1993b) and brain (Austin et al., 1994), abundance of each mRNA species depending on the tissue of origin. The factors determining expression of different 5-HTT transcripts is presently unknown. In contrast with these results, Lesch et al. (1993b) have reported a single hybridizing transcript for human mRNA. These dissenting observations that could be explained by differences in probes used in the latter and former studies.

Analysis of the amino acid sequence of mammalian transporters for 5-HT, NE and DA shows that 41% of their amino acid residues are identical, homology being highest at the twelve hydrophobic membrane-spanning domain level, lower in the intracytoplasmic carboxi- and amino-termini and particularly low in the large extracellular loop connecting TM3 and TM4, where the 5-HT carrier has two potential N-linked glycosylation sites (see Amara and Kuhar, 1993; Rudnick and Clark, 1993). Multiple putative phosphorylation sites by PKC and PKA, consistent with rapid postranslational regulation of the 5-HTT are predominantly found in carboxy- and aminoterminus (Blakely et al., 1991; Hoffman et al.,

1991; Lesch et al., 1993, Corey et al., 1994; Demchyshyn et al., 1994). Moreover, recent reports on the organization of the human 5-HTT gene indicate that the latter is endowed with an upstream combination of positive and negative cis-acting elements, including the cAMP response element (CRE), that may regulate transcription activity via a basal promoter unit (Lesch et al., 1994; Heils et al., 1995).

The mechanism of 5-HT uptake has been thoroughly studied in platelets (Rudnick, 1977; Nelson and Rudnick, 1979; Nelson and Rudnick 1982) mouse brain plasma membrane vesicles (O'Reilly and Reith, 1988; Reith et al., 1989), human placenta brush-border membranes vesicles (Balkovetz et al., 1989; Cool et al., 1990) and more recently following stable expression of cloned 5-HTTs in different expression systems (Blakely et al., 1991; Hoffman et al., 1991; Ramamoorthy et al., 1993b; Corey et al., 1994; Demchyshyn et al., 1994; Gu et al., 1994; Mager et al., 1994). 5-HT is the specific substrate for the transporter, with the following K_m values reported across different studies: 600 nM for h5-HTT in platelets, 500 - 890 nM for r5-HTT expressed in xenopus oocytes (Mager et al., 1994), 530 nM for r5-HTT expressed in CV-1 cells (Hoffman et al., 1991), 460 nM h5-HTT expressed in HeLa fibroblasts (Ramamoorthy et al., 1993b), 320 nM for r5-HTT in HeLa fibroblasts (Blakely et al., 1991), 220 nM for r5-HTT in basophilic leukaemia cells (Kanner and Bendahan, 1985), 60 - 180 nM for mouse 5-HTT brain plasma membranes (O'Reilly and Reith, 1988), 150 nM for r5-HTT expressed in parental LLC-PK_1 cells (Gu et al., 1994), 60 - 80 nM for r5-HTT in brain synaptosomes (Ross and Renyi, 1975; Angel and Paul, 1984; Wood et al., 1986), and 50 - 60 nM for h5-HTT in placental brush-border vesicles (Balkovetz et al., 1989). Reported values for the tum-over number (maximal number of 5-HT molecules carried by one transporter molecule in a given time unit) in different systems have similar variability: 500 5-HT molecules/porcine 5-HTT in platelet membrane vesicles/min (Talvenheimo et al., 1979), 110 5-HT molecules/r5-HTT expressed in parental LLC-PK_1 cells/min (Gu et al., 1994) or 30 5-HT molecules/r5-HTT expressed in xenopus oocytes/min (Mager et al., 1994). Tryptamine and its derivatives as well as 5-HT derivatives and phenylethyamines like (+)amphetamine and PCA are additional substrates for the 5-HTT (Segonzac et al., 1984, Wöfel and Graefe 1992; Magel et al., 1994). On the other hand, tryptophan, 5-hydroxytryptophan, 5-HIAA, histamine and the catecholamines NE and DA, at concentrations as high as 10 µM do not significantly bind to this carrier (Balkovetz et al., 1989; Hoffman et al., 1991; Wöfel and

Graefe, 1992; Corey et al., 1994; Barker and Blakely, 1995). However, higher concentrations of dopamine 20 - 40 μM have been reported to bind not only to the transporter but to exchange with [^{3}H]5-HT (Balkovetz et al., 1989; Wöfel and Graefe, 1992; Corey et al., 1994).

For neurotransmitter influx to occur, all Type I plasma membrane transporters, by definition, exhibit absolute requirement for Na^+ in the external medium. Na^+ concentration gradient (out > in; physiologically created by Na^+/K^+ ATPase) has been demonstrated as the driving force for 5-HT uptake. Na^+ may not be replaced by other cations and if the gradient is experimentally created, independently of ATPase activity, 5-HT uptake is insensitive to stimulation or inhibition of Na^+ ATPase, indicating that 5-HT and Na^+ fluxes are directly coupled by the transporter (Rudnick, 1977; Balkovetz et al., 1981; Kanner and Bendahan, 1985, O'Reilly and Reith, 1988). External Na^+ increases V_{max} and decreases K_m for 5-HT (Cool et al., 1990). In peripheral 5-HTTs, Na^+-5-HT stoichiometry is 1:1 since the uptake rate varies as an hyperbolic function of external Na^+ ion concentration (Talveinheimo et al., 1983; Cool et al., 1990). In brain membranes, the increase of 5-HT uptake with Na^+ showed a Hill coefficient of 2 suggesting a requirement of two Na^+ ions for a transport cycle (O'Reilly and Reith, 1988). More recently, Na^+ stoichiometry has been studied in stably expressed r5-HTT and an hyperbolic function consistent with a 1:1 stoichiometry was found (Gu et al., 1994). If the sodium gradient is kept, external Cl^- increases V_{max} and decreases K_m for 5-HT, fitting a Cl^-:5-HT stoichiometry of 1:1 (Nelson and Rudnick, 1982; Cool et al., 1990, Gu et al., 1994). Intracellular K^+ has also been shown to stimulate 5-HT uptake in platelets, placenta brush-border vesicles, brain vesicles (Nelson and Rudnick, 1979; Reith et al., 1989; Cool et al., 1990). Only in the case of the neuronal transporter, H^+ could not substitute for K^+ in enhancing uptake (Reith et al., 1989). The overall stoichiometry of a 5-HT uptake cycle as proposed by Rudnick and Clark is 5-HT/Na^+/Cl^-/K^+, 1:1:1:1 (1993). In their mechanistic model, the authors assume that the transporter may behave like an ion channel with a gate at each face of the membrane, but with only one gate that may open at any point in time. In a first step the transporter binds Na^+, Cl^- and 5-HT on the extracellular face of the membrane. To account for cotransport of the three species it would undergo conformational change and open the intracellular gate only once all three of them are bound to the permeation channel. K^+ countertransport consists of an inverse conformational change, reorienting the transporter

to its "active uptake state" once the K^+ ion is released to the extracellular space. Given that 5-HT is transported in its cationic form (5-HT^+; Keyes and Rudnick, 1982; Rudnick et al., 1989), the 1:1:1:1 stoichiometry should give place to an electroneutral process. In agreement with this concept, in most studies uptake of 5-HT by mammalian transporters was not affected by membrane potential (see Rudnick and Clark, 1993). However, studies of the r5-HTT present in basophilic leukaemia cells (Kanner and Bendahan, 1985), stably expressed h5-HTT (Laezza et al., 1994), r5-HTT (Mager et al., 1994) and drosophila 5-HTT (Corey et al., 1994) indicate that 5-HT uptake may be indeed electrogenic since it depends on membrane potential and/or generates a transport-associated current. These observations would imply not only that voltage gradients across the membrane may regulate uptake but also that electrogenic transporters may mediate nonvesicular 5-HT release.

Anatomical and cellular localization of the 5-HTT.- In the periphery, the 5-HTT is expressed in enteric 5-HT neurons (Wade et al., 1996) and non neuronal cells such as mast cells (Gripenberg et al., 1976), crypt epithelial cells and very discretely in enterochromaffin cells (Wade et al., 1996). It has also been found in platelets (Rudnick, 1977; Qian et al., 1995) lung membranes (Qian et al., 1995), and maternal brush-border of syncytiotrophoblast (Cool et al., 1990; Ramamoorthy et al., 1993a, 1993b).

In the brain the 5-HTT has been radiolabelled with [^{3}H]imipramine (Langer et al., 1980; Dawson and Wamsley, 1983; Hrdina et al., 1985), and more selective 5-HT uptake inhibitors such as [^{3}H]cyanoimipramine (Wolfe et al., 1987; Kovachich et al., 1988; Soucy et al., 1994), [^{3}H]paroxetine (Habert et al., 1985; De Souza and Kuyatt, 1987; Marcusson et al., 1988) and [^{3}H]citalopram (D'Amato et al., 1987). Though in general the autoradiographic binding pattern of [^{3}H]imipramine was found to be similar to that of [^{3}H]paroxetine (Hrdina et al., 1990) and that of [^{3}H]citalopram (Duncan et al., 1992), there are important regional differences in density of imipramine vs SSRI labelled sites, the former showing a much higher density of binding in forebrain areas such as the cortex and hippocampus. The reason for this discrepancy has been attributed to the fact that [^{3}H]imipramine binds to two classes of sites, high and low affinity, but only the high affinity ones seem related to 5-HT uptake (Moret and Briley, 1986; Marcusson et al., 1986; Hrdina et al., 1987, 1988, 1989; see review in D'Amato et al., 1987). For this reason, tritiated SSRIs are ligands of choice to label the brain 5-HT carrier *in vitro*. Recently, *in vitro*

[^{3}H]cyanoimipramine and [^{3}H]citalopram autoradiograms have been compared to the innervation pattern of 5-HT neurons as marked by [^{3}H]5-HT uptake in rat brain. A similar linear relationship was found for the labelling density of each of these ligands and density of 5-HT innervation, further indicating the high sensitivity of both [^{3}H]cyanoimipramine and [^{3}H]citalopram to label the 5-HTT (Descarries et al., 1995). [^{3}H]Citalopram binding has also been compared to that of [^{3}H]paroxetine binding in postmortem human brain tissue and it was concluded that both of these drugs are highly selective ligands but, because of its higher affinity for the carrier, [^{3}H]paroxetine was suggested as the radioligand of choice for *in vitro* studies (Arranz and Marcusson, 1994).

Though [^{3}H]paroxetine may be the ligand of choice for *in vitro* labelling of the 5-HTT, its *in vivo* binding distribution resembles that of *in vitro* imipramine (Biegon and Mathis, 1993), and successful conversion of paroxetine into PET or SPECT imaging agents has not been accomplished despite its very high potency for uptake inhibition (see Scheffel et al., 1992). Other drugs with high affinity for the uptake site such as cyanoimipramine, sertraline, citalopram or fluoxetine have been labelled with ^{11}C, but also displayed relatively low specific to non-specific binding ratios *in vivo* (Hashimoto et al., 1987; Lasne et al., 1989; Scheffel et al., 1990; Hume et al., 1991). McN-5652-Z (trans-1,2,4,5,6,10ß-hexahydro-6-[4-(methylthio)phenyl]pyrrolo[2,1-a]isoquinoline) is another potent 5-HT inhibitor, that has been ^{11}C-tagged and assessed as a PET radiotracer in mouse brain (Shank et al., 1988). This study suggested that McN-5652-Z may label 5-HT uptake sites *in vivo* with high target to non-target ratio, holding as a promising radiotracer for human PET studies (Suehiro et al., 1993). *In vivo* imaging of the 5-HT carrier has also been assessed in rat and non-human primate brain with the cocaine analog [(^{123}I]ß-CIT ([^{123}I]methyl 3ß-(-4-iodophenyl)tropane-2ß-carboxylate; Scheffel et al., 1992; Laruelle et al., 1993). Though this cocaine analog binds to the DA transporter as well as the 5-HTT, both sites may be discriminated because of kinetic differences in the way the ligand is taken up or washed out from rich 5-HT and DA innervation areas (Laruelle et al., 1993). Indeed, in a recent PET study in healthy human volunteers, the 5-HTT was distinctly recognized in medial frontal cortex, brainstem, hypothalamic area and visual occipital cortex 1 h after injection and DA transporters were recognized in the basal ganglia 20 h later (Kuikka et al., 1995). In this same study, 5-HT uptake sites were found to be reduced in frontal cortex of a patient with depression and increased in occipital cortex of a patient

with panic disorder.

An alternative way in which cellular localization of 5-HTTs in the CNS has been accomplished is using site-specific antibodies (Lawrence et al., 1995a; 1995b; Ovalle et al., 1995; Qian et al., 1995). Immunocytochemistry using antibodies directed against sites on the second and third outer loops of the 5-HT carrier revealed both neuronal and glial staining in areas containing 5-HT somata and terminals (dorsal raphe and hippocampus) of the rat brain (Lawrence et al., 1995b). In contrast, Qian et al., (1995), using an antibody developed against the intracellular N-terminus, found no evidence of glial staining. In this case, 5-HTT-immunoreactive somata and a dense network of 5-HTT immunoreactive processes were observed in the DRN as well as immunoreactivity corresponding to terminals in CA_{2-3} region of the hippocampus (Qian et al., 1995). It may then be possible, either that glial 5-HTT expression in adult rat brain is not very abundant or that the epitope on the N-terminus is not expressed (or masked) in the glial 5-HTT. Thus, 5-HTT expression in adult brain astrocytes remains a matter of debate. Using colocalization techniques for glial fibrillary acidic protein and radioactivity for [^{3}H]5-HT, 5-HT uptake activity has been found in primary astrocyte culture (Katz and Kimelberg, 1985; Kimelberg and Katz, 1985) and in 50% (frontal cortex) - 80% (periventricular region) of adult rat brain astrocytes (Anderson et al., 1992). Furthermore, Artigas et al., (1995) found that following intracortical perfusion of 5-HT, 5,7-DHT pretreated rats displayed similar *in vivo* SSRI-sensitive 5-HT uptake as control rats. Our group on the other hand, using an electrophysiological and *in vitro* uptake paradigm found no effect of paroxetine in 5,7-DHT treated rats (Piñeyro et al., 1994). These latter results are in agreement with *in situ* hybridization studies that have failed to detect any hybridization signal for mRNA in glial cells (Fujita et al., 1993). Like 5-HTT immunoreactivity, 5-HTT mRNA is present in neurons of caudal linear nucleus, DRN, MRN and caudal 5-HT nuclei matching however the distribution of cell bodies but not that of terminals (Fujita et al., 1993; Austin et al., 1994). Only in one study in which reverse transcriptase polymerase chain reaction was used for amplification a detectable level of 5-HTT mRNA expression was found in frontal cortex, hippocampus and neostriatum apart from the abundant expression observed in the midbrain raphe complex (Lesch et al., 1993c).

Pharmacological properties of the 5-HTT.- The 5-HTT is the pharmacological target for various therapeutic and abused substances. Compounds that block 5-HT reuptake

such as tricyclic antidepressants and SSRIs are among the first group while stimulants like amphetamine and its derivatives, which block 5-HT uptake and promote release are part of the latter category. Cocaine, though it binds and blocks the 5-HT carrier, is believed to exert most of its behavioral effects by blocking DA rather than 5-HT uptake (Woolverton and Klaven 1992; Barker and Blakely, 1995).

5-HT and NE transporters share their sensitivity for tricyclic antidepressants, tertiary amines like imipramine and chlomipramine being more potent at the 5-HTT and secondary amine tricyclics (*e.g.:* desipramine, nortriptyline) at the NE transporter (NET; see Hyttel, 1982; Thomas et al., 1987; Langer and Schoemaker, 1988; Bolden-Watson and Richelson, 1993). [^{3}H]Imipramine binding sites were among the first antidepressant binding sites to be described both on 5-HT neurons and platelets (Raisman et al., 1979; Langer et al., 1980a; 1980b). Based on early observations in which the binding of this radioligand was inhibited in a complex manner by 5-HT and SSRIs but competitively by imipramine itself (Langer and Raisman, 1983; Sette et al., 1983), it was initially proposed that imipramine-like antidepressants (imipramine, amitriptyline or chlomipramine) would act by allosterically regulating the function of the transporter, without directly binding to the substrate recognition site (Langer and Raisman, 1983). Conversely, the observation that imipramine, as well as other tricyclic as well as SSRIs inhibit the binding of the selective 5-HTT ligand [^{3}H]paroxetine in a competitive manner (Habert et al., 1985; Graham et al., 1989; Marcusson et al., 1989; Marcusson and Eriksson, 1988), later suggested that there is a single or at least overlapping binding site for tricyclic and non-tricyclic 5-HT uptake inhibitors on the 5-HTT. The idea of an overlapping binding site for the substrate, tricyclic and non-tricyclic uptake inhibitors is further supported by the following observations: i) in membranes from rat and human brain, 5-HT produces competitive displacement not only of [^{3}H]imipramine but also of [^{3}H]paroxetine which in turn may be displaced, fitting a single site binding model by citalopram, norzimelidine, paroxetine, fluoxetine, indalpine, chlomipramine and desipramine (Habert et al., 1985; Marcusson et al., 1988; Marcusson et al., 1989); ii) [^{3}H]5-HT uptake by d5-HTT is inhibited in a monophasic manner by paroxetine > fluoxetine > citalopram > cocaine ≥ 5-HT > desipramine ≥ imipramine (Demchyshyn et al., 1994); iii) Na^+ ions are needed for paroxetine and imipramine binding, as well as for 5-HT binding and translocation (Wood, 1987; Mann and Hrdina, 1992; iv) preincubation with imipramine protects against the reduction in total [^{3}H]paroxetine binding

caused by the sulphydryl group alkylating agent NEM (Graham et al., 1989). It is possible then that the early observations by Langer's group could be explained by taking into account the existence of two components for [^{3}H]imipramine specific binding: high affinity, Na^+-dependent and low affinity, Na^+-independent binding as defined using desipramine to determine non-specific binding (Hrdina, 1984; 1988; 1987). Indeed, the high affinity, Na^+-dependent component of [^{3}H]imipramine binding is completely displaced by 5-HT and non-tricyclic reuptake blockers in brain and platelets (Marcusson et al., 1986; Hrdina, 1988; Humphreys et al., 1988).

Even if the above-mentioned findings support the existence of a common recognition site for 5-HT, tricyclics and non-tricyclic antidepressants (as opposed to the regulatory subunit proposed in early studies), there is also compelling evidence indicating that although overlapping, these sites are distinct, sharing some but not all interacting chemical groups: i) chemical modification of the platelet 5-HTT with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a reagent that links carbonyl moieties to vicinal amino groups, significantly reduces the total number of [^{3}H]imipramine binding sites, an effect that is prevented by preincubation with imipramine and 5-HT but not fluoxetine and citalopram (Biessen et al., 1988); ii) oxidation of the 5-HTT in human platelets by the thiol reagent phenylarsine oxide (PAO) reduces [^{3}H]imipramine binding by 90%, an effect that is prevented by the preincubation with the tricyclic drugs imipramine, cyanoimipramine, chlomipramine, amitriptyline but not by non-tricyclic reuptake blockers: citalopram, fluoxetine, femoxetine and zimelidine (Biessen et al., 1988; on the other hand, in rat cortical membranes preincubation with imipramine did protect [^{3}H]paroxetine binding sites from NEM inactivation, Graham et al. 1989); iii) the sulphydryl reducing agent dithiotheithrol increases the affinity of the human platelet 5-HTT for [^{3}H]imipramine but not for [^{3}H]paroxetine (Tarrant and Williams, 1995); iv) antisera directed against the second extracellular loop of the 5-HTT produce a dose-dependent inhibition of [^{3}H]5-HT uptake but have no effect on [^{3}H]citalopram binding (Lawrence et al., 1995b) and v) incubation of [^{3}H]citalopram, [^{3}H]imipramine or [^{3}H]paroxetine in the presence of high μM concentrations of 5-HT, citalopram or paroxetine may induce very different types of changes in the dissociation kinetics of each radioligand, *eg:* 200 μM citalopram attenuated the dissociation of [^{3}H]citalopram four times more than that of [^{3}H]paroxetine while paroxetine has an opposite effect increasing the dissociation rate of [^{3}H]imipramine

(Wennogle and Meyerson, 1985; Humphreys et al., 1988; Plenge et al., 1990; 1991). This last set of observations is consistent with the existence of low affinity sites that may modulate the binding status of the high affinity site. Further support for allosterism is given by the fact that paroxetine (though in the low nM range) may decrease the affinity of the 5-HTT for [^{3}H]cocaine (Akunne et al., 1992). Cocaine, in turn, binds to a site that may be distinguished from the substrate/antidepressant site because binding of the latter but not that of 5-HT or antidepressants is insensible to Cl^- and inhibited by H^+ (Wall et al., 1993). Conversely, the ability of 5-HT to competitively displace cocaine analogues such as [^{3}H]CFT and ß-[^{125}I]CIT argues that cocaine and substrate sites, if not the same, are closely related (Rudnick and Wall, 1991; Wall et al., 1993). Other drugs of abuse such as neurotoxic amphetamine derivatives (*eg:* 3,4-dioxymethylene-methamphetamine, para-chloro-amphetamine) also induce competitive displacement of [^{3}H]imipramine and inhibition of [^{3}H]5-HT transport (Rudnick and Wall, 1992a; 1992b; 1992c). Site directed mutagenesis and chimera construction should help to determine which amino acid residues on the transporter interact with all or most of the drugs and which are unique to each ligand. Such studies have suggested: i) that phenylalanine 586 located on the twelfth transmembrane domain could be responsible for high affinity recognition of imipramine (present in human but not drosophila 5-HTT); ii) that (+)amphetamine interacts with multiple residues in this same transmembrane domain and iii) that contact sites for citalopram have been localized to the second transmembrane domain (Barker et al., 1995).

Tianeptine, a class by itself?.- Tianeptine is a tricyclic agent (dibenzothiazepine nucleus) with a long (aminoheptanoic acid) lateral chain (Labrid et al., 1988), which unlike other drugs from this class, has been characterized by its ability to enhance 5-HT uptake. In France it is indicated for the treatment of "neurotic and reactional depressive conditions" and it has been claimed to be a unique type of antidepressant, that unlike all others produces its effect by enhancing 5-HT uptake.

In rat cortical and hippocampal synaptosomes (Mennini et al., 1987, Fattacini et al., 1990), as well as in rat and human platelets (Kato and Weitsch, 1988; Chambda et al., 1991), the increase in 5-HT uptake induced by tianeptine is secondary to a 20% - 30% increase in V_{MAX}. Tianeptine does not displace [^{3}H]paroxetine, [^{3}H]imipramine nor [^{3}H]-d-fenfluramine and does not produce *in vitro* effects on 5-HT uptake (Kato and Weitsch,

1988). The increase in V_{MAX} which is observed *ex vivo* at least one h after acute administration of tianeptine (Mennini et al. 1987) should then be an indirect effect. This interpretation is supported by the observation that, if tianeptine is given one h before sacrifice at a similar dose as the one used in *ex vivo* experiments (10 mg/kg, i.p.) in which V_{MAX} is increased, it does not modify [^{3}H]imipramine binding to rat cortical membranes (Romero et al., 1992). Also given acutely (10 mg/kg) or chronically (10 or 20 mg/kg/day x 14 days), tianeptine has no significant effect on 5-HT uptake in mesencephalic synaptosomes, a region where 5-HTTs are most abundant (Mennini et al., 1987). In keeping with this observation, neither acute nor sustained tianeptine administration modify 5-HT neuron firing frequency (Dresse and Scuvée Moreau, 1988). Furthermore, electrophysiological data from our laboratory also indicate that the increase in the firing activity of hippocampal pyramidal neurons following acute administration of tianeptine does not depend on the integrity of 5-HT terminals and the presence of 5-HTTs since the effect of the drug is not modified by 5,7-DHT lesion (Piñeyro et al., 1995a). Moreover, analysis of the effect of sustained tianeptine administration also indicates that its effect on 5-HT uptake is not always reproducible: i) sustained tianeptine administration increases 5-HT uptake in rat platelets and brain synaptosomes (10-20 mg/kg/day x 14 days; Mennini et al., 1987; Kato and Weitsch, 1988) but not in rat brain slices (20 mg/kg/day x 14 days; Piñeyro et al., 1995) or human platelets (37, 5 mg/kg/day x 10 or 28 days; Chambda et al., 1991); ii) in brain the 5-HT uptake enhancing effect of tianeptine occurs following a 72 h but not a 24 h washout (Mennini et al., 1987; Mocaër et al., 1988); in platelets, on the other hand, a 24 h washout allows to demonstrate a 30% increase in V_{MAX} (Kato and Weitsch, 1988); iii) results from certain binding studies indicate that doses of 10-20 mg/kg/day x 14 days (which enhance cortical and hippocampal 5-HT uptake) produce no change in hippocampal [^{3}H]imipramine or [^{3}H]paroxetine binding parameters (Mennini et al., 1987; Mennini and Garattini, 1991; Frankfurt et al., 1993), while other studies have shown not only a decrease in B_{MAX} for [^{3}H]paroxetine binding sites in cortex, hippocampus and DRN (Watanabe et al., 1993; Kuroda et al., 1994) but a decrease in 5-HTT mRNA in DRN (Kuroda et al., 1994); prenatal exposure to tianeptine (20 mg/kg/day x 14 days) has also been shown to reduce B_{MAX} for [^{3}H]imipramine in rat cortex (Romero et al., 1992).

Except for biochemical studies that have assessed the actual effect of tianeptine

on 5-HT uptake, considerable evidence supporting the idea that this drug enhances 5-HT reuptake are indirect. Following one h after its acute administration (10 mg/kg, i.p.), tianeptine was shown to increase brain tissue concentration of 5-HIAA without modifying tissue levels of 5-HT (Fattacini et al., 1990). At a similar dose, it has also been shown to induce an increase in extracellular 5-HIAA in hippocampus, hypothalamus and medullary dorsal horn (De Simoni et al., 1992; Puig et al., 1993). Interestingly a dose of 20 mg/kg (i.p.) had an opposite effect on extracellular 5-HIAA in rat hippocampus (Mennini and Garattini, 1991) but increased plasma 5-HIAA levels (Ortiz et al., 1991). The observed increases in 5-HIAA have been interpreted as an increase in intracellular 5-HT turnover secondary to enhanced 5-HT uptake. The biphasic effect still remains unexplained. Another common approach that has been used to unveil the site of action of tianeptine is its interaction with drugs that are known to modify 5-HT reuptake activity (Fattacini et al., 1990; Datla and Curzon, 1993; Ortiz et al., 1991; De Simoni et al., 1992). In keeping with its 5-HT uptake enhancing capacity, tianeptine, given 30 min after different SSRIs or together with 5-HTP, respectively reduced or prevented the increase in plasma or extracellular cortical 5-HT caused by the aforementioned treatments. In both cases, also in agreement with an increase in 5-HT uptake and intracellular deamination, tianeptine administration potentiated the observed increase in 5-HIAA respectively induced by SSRIs in plasma or 5-HTP in the extracellular cortical fluid (Ortiz et al., 1991; Dalta and Curzon, 1993). However, in rat hippocampus as many other brain regions, SSRI administration reduced extracellular availability of 5-HIAA, and tianeptine had no effect on this reduction (De Simoni et al., 1992). Furthermore, in this same study a metabolite of the 5-HT releasing drug fenfluramine, produced the same increase in 5-HIAA independent of whether rats had been pretreated or not with tianeptine (De Simoni et al., 1992). Acute tianeptine administration was also without effect on the 5-HT depletion caused by d-fenfluramine in cortex and striatum (Fattaccini et al., 1990) and on the increase caused by paroxetine in the time it takes CA_3 pyramidal neurons to recover their firing activity following microiontophoretic application of 5-HT (Piñeyro et al., 1995a). On the other hand, the previous lack of interaction between tianeptine and drugs that modify the 5-HTT activity should be confronted with other findings indicating that such an interaction may exist: i) tianeptine did no longer induce an increase in tissue 5-HIAA when fenfluramine was previously administered (Fattacini et al., 1990), ii) in rat hippocampus the

administration of sertraline after tianeptine reverted the enhancing effect of the latter on 5-HIAA production (De Simoni et al., 1992) and iii) following its sustained administration, tianeptine antagonizes the increase in the time of recovery of firing of CA_3 pyramidal from microiontophoretic applications of 5-HT produced by the SSRI paroxetine (Piñeyro et al., 1995). The effect of tianeptine on 5-HT release has also been assessed and these experiments indicate that neither its acute nor sustained administration has an effect on basal 5-HT release (Mennini et al., 1987, Whitton et al., 1991a) but that it reduces (acutely and chronically given) K^+-induced 5-HT release from rat brain *in vitro* (IC_{50} 2 μM in cortex and 0.4 μM in hypothalamus) and *in vivo* (Mocaër et al., 1988; Whitton et al., 1991a). Most interestingly, as reported by Mocaër et al., (1988) the *in vitro* effect of tianeptine is partially blocked by methiothepin (1 μM), once again suggesting that a straight foward interpretation of a decreased 5-HT output due to increased 5-HT uptake may not be the only explanation possible for this effect. Further supporting this view, Bolaños-Jiménez et al., (1993) have recently reported that tianeptine dose-dependently reduced the effect of the 5-HT_{1B} agonist CGS 12066B on 5-HT release. This group has also shown that without modifying basal outflow, tianeptine (100 μM) may inhibit [^{3}H]ACh release from hippocampal rat synaptosomes (Bolaños-Jiménez et al., 1993). Interestingly, and in keeping with the high concentrations used in the previous study, only doses of 30 mg/kg, i.p. were found to inhibit *in vivo* ACh release from rat hippocampus (Bertorelli et al., 1992).

From the previous analysis, it can be concluded that there is no simple explanation for the effects of tianeptine on the 5-HT system. One of the most consistent observations is that acute as well as prolonged administration of tianeptine may reduce 5-HT neurotransmission when the latter is enhanced by 5-HTP, K^+-evoked release or SSRIs Moreover, in the study by Mennini et al., (1987) in which cortical and hippocampal synaptosomes were showed for the first time to increase V_{MAX}, the lowest 5-HT concentration used was 40 nM (at least 15 times higher than normal extracellular brain concentration of 5-HT) and in that by Fattaccini et al., (1990) [^{3}H]5-HT concentration was at least 5-fold higher than basal extracellular 5-HT. These neurochemical observations are supported by behavioral studies in which tianeptine has been shown to reduce some of the symptoms of 5-HTP-induced 5-HT syndrome (De Simoni et al., 1992; Datla and Gurzon, 1993). Moreover, tianeptine has been shown to attenuate behaviours that have been attributed to stress-induced increase in 5-HT activity: it attenuates stress-induced

open field behavioral deficits, without altering basal locomotion parameters (Broqua et al., 1991; Whitton et al., 1991b; Fontanges et al., 1993), it also abolishes stress-induced decrease in hypothalamic CRF (Delbende et al., 1994), it suppresses isolation rearing-induced decrease in glucocorticoid Type I hippocampal receptors without affecting basal levels of the latter nor Type II receptors (McEwen, 1991; McEwen et al., 1992) and it prevents stress and corticosterone-induced reduction in CA_3 pyramidal neuron apical tree (Watanabe et al., 1993). Tianeptine has not been found to alter basal ACTH or corticosteroid plasma levels (Delbende et al., 1993; Watanabe et al., 1993; Delbende et la., 1994) nor to modify the efficacy of 5-HT neurotransmission in basal conditions (Piñeyro et al., 1995b). On the other hand it reduces the recovery time from microiontophoretic applications of high currents of concentrated 5-HT (Piñeyro et al., 1995b).

The next question that naturally arises is whether the "protective" effect of tianeptine against stress-induced changes is linked to its uptake-enhancing capacity? A recent study by Mennini et al., 1993 directly addressed this question indicating that tianeptine (10 mg/kg, i.p. 1 h before stress) antagonizes the decrease in 5-HT uptake caused by acute noise stress. However, tianeptine has also been shown to induce specific changes in other brain monoamines involved in the response to stress: i) acute tianeptine administration decreases the firing activity of NA neurons in locus coeruleus without altering the activity of DRN 5-HT neurons (Dresse and Scuvée-Moreau, 1988); ii) without producing marked changes in the 5-HT system short-term administration of tianeptine (10 mg/kg/day x 4 days) increases NE content and decreases NE turnover in specific nuclei related with mood and condition, *i.e.:* preoptic area, DRN and sensory cortex (Frankfurt et al., 1995); iii) its sustained administration, like that of desipramine antagonizes the stress-induced increase in tyrosine-hydroxylase mRNA in the NA nucleus (see McEwen, 1991); iv) acute and prolonged tianeptine treatment may increase extracellular DA concentrations in striatum and nucleus accumbens in a 5-HT independent manner (Invernizzi et al., 1992), as well as increase DA turnover in the prefrontal cortex (Louilot et al., 1990). Hence, these results further indicate that an interaction with the 5-HT system may not be the only mechanism by which tianeptine attains its stress-protecting effects.

Finally, clinical proof of the antidepressant efficacy of tianeptine has been recently reviewed by Wilde and Benfield (1995). It was concluded that according to the available

information the antidepressant efficacy of this new drug, administered in the short term, appears similar to that of amitriptyline, imipramine and fluoxetine and in patients with coexisting anxiety and depression, tianeptine could be superior to maprotiline. However, it should be noticed that i) most of the studies lacked a placebo-controlled group; ii) that in amitriptyline and maprotlyline studies as well as in some of those in which tianeptine was compared to imipramine optimal dosages of the standard drugs were not used, iii) in multicenter double blind studies in which the efficacy of long-term tianeptine treatment was assessed, efficacy was evaluated in those patients who completed the treatment even if 65% of dropouts under tianeptine treatment abandoned the study due to ineffective treatment and iv) comparative trials of tianeptine and SSRIs other than fluoxetine are needed to further define its role in the treatment of depression. Based on basic and clinical facts it is thus our opinion that it may be premature to claim that "tianeptine is a new antidepressant class with a unique 5-HT uptake enhancing profile". Furthermore, if indeed 5-HT uptake is stimulated by the drug in conditions in which 5-HT neurotransmission is enhanced, then it would be unlikely that this is the mechanism involved in its antidepressant action since depression is a condition in which the 5-HT system is frequently if not consistently deficient (see Maes and Meltzer, 1995).

Regulation of 5-HT uptake activity by antidepressant drugs.- The results from radioligand and functional studies following prolonged antidepressant treatments have often been found controversial (Table 7) and the reasons for this controversy has been attributed to three main limitations: i) the use of [^{3}H]imipramine as a radioligand which due its binding to heterogeneous sites may confound interpretation of results (it is worth noting that when the effect of long-term antidepressant administration was assessed on high and low-affinity [^{3}H]imipramine sites, in the great majority of cases treatment-induced decreases in affinity for imipramine were observed at both sites; Hrdina, 1987) ii) examination of effects of long-term antidepressants being limited in most cases to cortical or hippocampus homogenates, which unlike autoradiographic analysis, prevents modest changes in discrete brain areas to be assessed and iii) the use low doses and mainly administration routes (i.p. and s.c. injections; oral administration) that cause important fluctuations (peak versus trough) in plasmatic concentration of antidepressant drugs may also account for a high proportion of negative results. Nevertheless, if these limitations are taken into account some general conclusions may be drawn. First, when selective ligands

TABLE 7

Effect of different antidepressant treatments on funtional and binding properties of the 5-HT transporter

Treatment	Dose/Route	ligand	Effect observed	References
Electroconvulsive shock	once	[^{3}H]paroxetine	Increase in number of binding sites in cortical homogenates, not hippocampus	Hayakawa et al., 1995
	1/d x 14 ds	"	Increase in number of binding sites in cortical homogenates, not hippocampus	Hayakawa et al., 1995
	1 every 2 ds x 10 ds	"	No change in frontal cortex homogenates	Cheetham et al., 1993
	1/d x 10 ds	"	No change in cortex homogenates	Gleiter and Nutt, 1988
	1/d x 10 ds	[^{3}H]imipramine/ functional	No change in Vmax or Bmax in cortical synaptosomes or homogenates	Craig et al., 1986
	1/d x 7 ds	[^{3}H]imipramine	Increase in Bmax in cortical homogenates	Barkai, 1986
Imipramine	20 mg/kg/d x 14 ds (i.p.)	[^{3}H]paroxetine	No change in hippocampal or cortical homogenates	Hayakawa et al., 1995
	5 mg/kg/d x 21 ds (i.p.)	"	No change frontal cortex homogenates	Dewar et al., 1993
	20 mg/kg/d x 21 ds (i.p.)	[^{3}H]imipramine	Increase in Vmax and decrease in Bmax in synaptosomes and homogenates	Barbaccia et al., 1983a, 1983
	3 mg/rat/d x 5 weeks (p.o.)	"	No change in cortical homogenates	Plenge and Mellerup, 1988
	20 mg/kg/d x 21 ds (i.p.)	"	No change in Vmax or Bmax in cortical synaptosomes or homogenates	Marcusson et al., 1988
	20 mg/kg/d x 10 ds (i.p.)	"	Decrease in Bmax in hippocampal homogenates No change in cortical homogenates	Kinnier et al., 1980
	5 mg/kg/d x 21 ds (s.c. minipumps)	mRNA hybridization	Decrease in midbrain raphe complex	Lesch et al., 1993
	20 mg/kg/d x 16 or 32 ds (i.p.)	mRNA hybridization	No change midbrain homogenates	Spurlock et al., 1994
	10 mg/kg/d x 21 ds (i.p.)	mRNA hybridization	Increase in optical density in DRN autoradiograph	Lopez et al., 1994
Desipramine	10 mg/kg/d x 14 ds (p.o.)	[^{3}H]paroxetine	No change frontal cortex homogenates	Cheetham et al., 1993
	20 mg/kg/d x 21 ds (i.p.)	[^{3}H]imipramine	Decrease in Bmax incortical homogenates	Barbaccia et al., 1983a, 1983
	10 mg/kg/d x 10 ds (i.p.)	"	No change in midbrain raphe area or limbic terminal projection areas	Biegon, 1986
	13.6 mg/kg/d x 22ds (p.o., in drinking water)	"	Decrease in Bmax in cortical homogenates	Butler, 1987
	5 mg/kg/d x 21 ds (s.c., via osmotic minipump)	"	No change in midbrain raphe complex	Raisman et al., 1980
	10 mg/kg/d x 21 ds (i.p.)	functional	No change in cortical or amygdala synaptosomes	Lesch et al., 1993
Trimipramine	5 mg/kg/d x 21 ds (i.p.)	[^{3}H]paroxetine	No change in frontal cortex homogenates	Dewar et al., 1993
Protriptyline	20 mg/kg/d x 21 ds (i.p.)	[^{3}H]cyanoimipramine	No change in cortex or hippocampus autoradiograms	Kovachich et al., 1992
Amitriptyline	10 mg/kg/d x 14 ds (p.o.)	[^{3}H]paroxetine	No change in frontal cortex homogenates	Cheetham et al., 1993
Nortryptiline	20 mg/kg/d x 21 ds (gastric intubation)	[^{3}H]imipramine/ functional	No change in Vmax, decrease in affinity for imipramine in frontal cortex homogenates	Hrdina, 1987
Chlomipramine	20 mg/kg/d x 19 ds (i.p.)	[^{3}H]paroxetine	No change in Kd or Bmax in cortex and hippocampus homogenates	Graham et al., 1987
	5 mg/kg/d. x 19 ds (s.c. via osmotic minipump)	[^{3}H]paroxetine	No change in Kd or Bmax in cortex and hippocampus homogenates	
	5 mg/kg/d. x 21 ds (s.c. via osmotic minipump)	mRNA hybridization	No significant change midbrain homogenates	Lesch et al., 1993
Clorgyline	1mg/kg/d x 18 ds (i.p.)	[^{3}H]paroxetine	No change Kd or Bmax in cortical and hippocampus homogenates	Graham et al., 1987
	1 mg/kg/d x 21 ds (s.c. via osmotic minipump)	mRNA hybridization	No change in midbrain raphe complex	Lesch et al., 1993
	4 mg/kg/d x 21 ds (i.p.)	mRNA hybridization	Increase in optical density in DRN autoradiographs	Lopez et al., 1994
Pargyline	100 µm/kg/d x 21 ds (i.p.)	[^{3}H]imipramine	No change in Kd and Bmax in cortical homogenates	Zsilla et al., 1983
Phenelzine	5 mg/kg/d x 21 ds (i.p.)	[^{3}H]cyanoimipramine	Increase in binding in perirhinal cortex autoradiographs	Kovachich et al., 1992
Tranylcypromine	10 mg/kg/d x 14 ds (p.o.)	[^{3}H]paroxetine	No change in frontal cortex homogenates	Cheetham et al., 1993
Deprenyl	0.5 mg/kg/d x 18 ds (i.p.)	[^{3}H]paroxetine	No change in Kd or Bmax in cortical and hippocampal homogenates	Graham et al., 1987
	1 µM/kg/d x 21 ds (s.c. injected daily)	[^{3}H]imipramine	Increase in Bmax in cortical and hippocampal homogenates	Zsilla et al., 1983
	0.25 mg/kg/d x 21 ds (i.p.)	[^{3}H]cyanoimipramine	Increase in binding in dentate gyrus and amygdala autoradiograph	Kovachich et al., 1992

TABLE 7 (cont)

Effect of different antidepressant treatments on funtional and binding properties of the 5-HT transporter

Treatment	Dose/Route	ligand	Effect observed	References
Citalopram	10 mg/kg/d x 14 ds (p.o.)	[^{3}H]paroxetine	No change in frontal cortex homogenates	Cheetham et al., 1993
	20 mg/kg/d x 21 ds (i.p.)	[^{3}H]cyanoimipramine	No change in autoradiographs	Kovachich et al., 1992
	20 mg/kg/d x 19 ds (i.p.)	-	No change in frontal cortex homogenates	Graham et al., 1987
	10 mg/kg/d x 16 o 32 ds (i.p.)	mRNA hybridization	No change in mesencephalic homogenates	Spurlock et al., 1994
	40 mg/kg/d 13 ds (p.o., given with diet)	functional	No change in whole rat brain synaptosomes	Hyttel et al., 1984
Fluoxetine	2 mg/kg/d x 21 ds (i.p.)	[^{3}H]paroxetine	No change in frontal cortex synaptosomes	Dewar et al., 1993
	10 mg/kg/d x 21 ds (i.p.)	[^{3}H]paroxetine	Increase in binding in corical and hippocampal autoradiographs	Hrdina and Vu, 1993
	10 mg/kg/d x 21 ds (i.p.)	[^{3}H]imipramine/ functional	Decrease in Vmax and affinity binding in frontal cortex synaptosomes and homogenates	Hrdina, 1987
	2.5 mg/kg/d x 21 ds s.c. via osmotic minipump)	mRNA hybridization	Decrease in midbrain raphe complex homogenates	Lesch et al., 1993
Fluovoxamine	50 mg/kg/d x 16 or 32 ds (i.p.)	-	No change in midbrain homogenates	Spurlock et al., 1994
Paroxetine	10 mg/kg/d x 21 ds (s.c., via osmotic minipump)	[^{3}H]paroxetine/ functional	Decrease in Bmax in cortical and hippocampal homogenated. Decrease in 5-HT uptake in hippocampal slices	Piñeyro et al., 1994
Sertraline	10 mg/kg/d x 21 ds (i.p.)	[^{3}H]cyanoimipramine	Decrease in binding in amygdala and perirhinal cortex autoradiograph	Kovachich et al., 1992
	10mg/kg/day x 21 ds (i.p.)	functional	Decrease in Vmax in cortex and amygdala synaptosomes	Butler, 1987
Zimelidine	10 mg/kg/d x 14 ds (p.o.)	[^{3}H]paroxetine	No change in frontal cortex homogenates	Cheetham et al., 1993
	20 mg/kg/d x 21 days (i.p.)	[^{3}H]imipramine	No change in Vmax or Bmax in cortical synaptosomes and homogenates	Marcusson et al., 1988

TABLE 7 (cont)

Effect of different antidepressant treatments on funtional and binding properties of the 5-HT transporter

Treatment	Dose/Route	ligand	Effect observed	References
Citalopram	10 mg/kg/d x 14 ds (p.o.)	[^{3}H]paroxetine	No change in frontal cortex homogenates	Cheetham et al., 1993
	20 mg/kg/d x 21 ds (i.p.)	[^{3}H]cyanoimipramine	No change in autoradiographs	Kovachich et al., 1992
	20 mg/kg/d x 19 ds (i.p.)	-	No change in frontal cortex homogenates	Graham et al., 1987
	10 mg/kg/d x 16 o 32 ds (i.p.)	mRNA hybridization	No change in mesencephalic homogenates	Spurlock et al., 1994
	40 mg/kg/d 13 ds (p.o., given with diet)	functional	No change in whole rat brain synaptosomes	Hyttel et al., 1984
Fluoxetine	2 mg/kg/d x 21 ds (i.p.)	[^{3}H]paroxetine	No change in frontal cortex synaptosomes	Dewar et al., 1993
	10 mg/kg/d x 21 ds (i.p.)	[^{3}H]paroxetine	Increase in binding in corical and hippocampal autoradiographs	Hrdina and Vu, 1993
	10 mg/kg/d x 21 ds (i.p.)	[^{3}H]imipramine/ functional	Decrease in Vmax and affinity binding in frontal cortex synaptosomes and homogenates	Hrdina, 1987
	2.5 mg/kg/d x 21 ds s.c. via osmotic minipump)	mRNA hybridization	Decrease in midbrain raphe complex homogenates	Lesch et al., 1993
Fluovoxamine	50 mg/kg/d x 16 or 32 ds (i.p.)	-	No change in midbrain homogenates	Spurlock et al., 1994
Paroxetine	10 mg/kg/d x 21 ds (s.c., via osmotic minipump)	[^{3}H]paroxetine/ functional	Decrease in Bmax in cortical and hippocampal homogenated. Decrease in 5-HT uptake in hippocampal slices	Piñeyro et al., 1994
Sertraline	10 mg/kg/d x 21 ds (i.p.)	[^{3}H]cyanoimipramine	Decrease in binding in amygdala and perirhinal cortex autoradiograph	Kovachich et al., 1992
	10mg/kg/day x 21 ds (i.p.)	functional	Decrease in Vmax in cortex and amygdala synaptosomes	Butler, 1987
Zimelidine	10 mg/kg/d x 14 ds (p.o.)	[^{3}H]paroxetine	No change in frontal cortex homogenates	Cheetham et al., 1993
	20 mg/kg/d x 21 days (i.p.)	[^{3}H]imipramine	No change in Vmax or Bmax in cortical synaptosomes and homogenates	Marcusson et al., 1988

are used to mark the 5-HTT, repeated electroconvulsive shocks (ECS) and long-term MAOI administration were the only types of treatment in which positive results have been consistent in showing an increase in the number of 5-HTT (Hayakawa et al., 1995; Kovachich et al., 1992). The use of [^{3}H]imipramine as a radioligand has also shown increases in 5-HTT sites following ECS or deprenyl administration (Zsilla et al., 1983; Barkai et al., 1986). The studies which yielded negative results were either performed in homogenates, smaller number of ECS were given, or oral/i.p. treatments were administered (Zsilla et al., 1983; Graham et al., 1987; Gleiter and Nutt, 1988; Cheetham et al., 1993). Hybridization studies similar to binding studies, show either no change or increased mRNA hybridization in the midbrain raphe complex following prolonged clorgyline administration (Lesch et al., 1993c; Lopez et al., 1994). The dose used for the negative hybridization study (Lesch et al., 1993c) was 4 times smaller than the one used in the positive one (López et al., 1994), in spite the fact that in the study by López et al. treatment was given i.p. and in that by Lesch et al., steady drug plasma levels were achieved by using osmotic minipumps. In the case of SSRIs functional studies show a decrease in V_{MAX} in cortical and amygdala synaptosomes of rats that had received prolonged fluoxetine or sertraline treatment as well a decrease in maximal [^{3}H]5-HT uptake in cortical and hippocampal slices obtained from rats that had received paroxetine for 21 days (Butler et al., 1987; Hrdina, 1987b). Rats that had received citalopram administered in their diets no functional changes were observed when assessed in whole rat brain synaptosomes (Hyttel et al., 1984). Positive observations from binding studies indicate that sustained administration of sertraline and paroxetine decreases the number of 5-HTT in amygdala, perirhinal cortex, hippocampus and rat frontal cortex (Kovachich et al., 1992; Piñeyro et al., 1994) and that fluoxetine induced an increase in [^{3}H]paroxetine binding in these two latter areas (Hrdina and Vu, 1993). However, at the same dose as in the last study and using the same route of administration fluoxetine was seen to decrease V_{MAX} in cortical synaptosomes (Hrdina, 1987). These results indicate that no simple explanation may explain all observations following prolonged antidepressant treatment. Since synaptosomes in which V_{MAX} was decreased were prepared from frontal cortex and increased numbers of in 5-HTT sites was observed in frontoparietal, striatal as well as hippocampal cortices, a possible explanation for the divergent observations following fluoxetine treatment could be accounted for by regional differences in the adaptative

properties of the 5-HTT. Hybridization studies indicate a decrease in 5-HTT mRNA in midbrain raphe nuclei following prolonged fluoxetine administration via osmotic minipumps (Lesch et al., 1993c). Whether this decrease in transcriptional activity is secondary to a decrease in 5-HTT protein turnover (in agreement with at least a *transitory* increase in the number of uptake sites) or is the cause for a reduction in 5-HTT number cannot be deduced from these results. Once again, studies in which prolonged SSRI administration induced no changes in 5-HTT activity or binding sites were performed in homogenates, and/or drugs were administered i.p (Graham et al., 1987; Kovachich et al., 1992; Dewar et al., 1993; Spurlock et al., 1994). The fact that in spite that both sertraline and citalopram were administered i.p., but only the first produced a reduction in [^{3}H]cyanoimipramine binding (Kovachich et al., 1992) and cortical 5-HT V_{MAX} (Butler et al., 1987) is probably due to the fact that of these two SSRIs, only sertraline has an active metabolite while citalopram is rapidly inactivated (see discussion in results section; article I). Finally, results from prolonged tricyclic administration may not be systematized: imipramine and desipramine have been shown to decrease [^{3}H]imipramine but not [^{3}H]paroxetine binding while chlomipramine, not only a tricyclic but also a potent and selective 5-HT uptake blocker, did not produce any significant change in 5-HTT sites or its mRNA (Table 7).

Based on the previous analysis, the observations in Table 7 could be summarized by saying that an increase in 5-HT uptake may be expected following repeated ECS or sustained MAOI administration while a decrease in this function is more likely to occur following prolonged SSRI treatment. Hence, it seems that the antidepressant effect is not correlated with a specific adaptative response of the 5-HT carrier. Given that 5-HT uptake activity is regulated by numerous physiological processes, understanding them and finding the similarities they might have with a given pharmacological treatment may help understand how antidepressant drugs or ECS regulate 5-HTT activity. ACTH and ACTH fragments upregulate 5-HTT expression during 5-HT neuron differentiation (Azmitia and Kloet, 1987; Eaton and Whittemore, 1995). A transcription increase also occurs in raphe neurons of aged rats, an effect that may compensate for 5-HT leakage from degenerating terminals and/or increased release induced by age-related decline in terminal autoreceptor regulation (Meister et al., 1995). Similarly, 5-HT neuron sprouting in raphe nuclei following 5,7-DHT lesion, has been correlated with an increase in [^{3}H]paroxetine B_{MAX} values in

brainstem (Pranzatelli and Martens, 1992). Up-regulation of the 5-HTT secondary to transcriptional activation may occur via an increase in cAMP (Cool et al., 1991; King et al., 1992; Ramamoorthy et al., 1993) which has been recently proposed to induce the activation of the h5-HTT gene promoter via immediate early genes products like transcription factors of the c-fos/c-jun family (Heils et al., 1995). cAMP-independent mechanisms activated by interleukin-1ß (Ramamoorthy et al., 1995a) or the PKC inhibitor staurosporine (Ramammoorthy et al., 1995b) may also activate 5-HTT mRNA production. The signalling pathways involved in these responses are still unknown. Alternatively, 5-HT uptake activity may be rapidly enhanced without altering transporter density, by a mechanism involving transporter phosphorylation/dephosphorylation. Nitric oxide-cGMP pathway activation has been shown to have this effect (Miller and Hoffman, 1994). Phosphorylation/ dephosphorylation of the transporter via other signalling pathways such as PKC and calmodulin, induce an opposite effect, reducing 5-HT uptake (Myers et al., 1989; Anderson and Horne, 1992; Jayanthi et al., 1994). It is unlikely however that these rapid regulatory responses may account for the decrease in 5-HT uptake observed following prolonged SSRI administration since the former take place within an hour or less following treatment and in the case of paroxetine tolerance to the drug was not expressed within a 48 h period but rather following various days of treatment (Piñeyro et al., 1994). Furthermore, a recent study that indicates that reduction in 5-HTT mRNA does not always result in a decrease in the number of 5-HTT in 5-HT neurons (Yu et al., 1995), suggests that a decrease in transcription might not always serve as an explanation for a long-latency down-regulation in the 5-HTT. On the other hand, a dysregulated expression of the 5-HTT gene (Heils et al., 1995) has been suggested as a possible explanation for one of the most consistent findings in biological psychiatry: the disease-associated decrease in brain and platelet 5-HT uptake and inhibitor binding observed in patients with affective disorders (see Lesch and Bengel, 1995).

EXPERIMENTAL PART

ARTICLES I, II AND III

The goal of this part of the project was to describe alternative ways in which antidepressant drugs might modify classically described autoregulatory properties of 5-HT neurons, apart from the clasically-described autoreceptor desensitization pathway (see Introduction). To attain this first goal our strategy was to investigate whether apart from inducing auto- and/or heteroreceptor desensitization, prolonged antidepressant drug administration would produce adaptive changes of the 5-HT transporter. The drugs used were tianeptine, a tricyclic drug which has been recently proposed to possess antidepressant properties, and the SSRI paroxetine. Paroxetine and tianeptine were chosen because they modify the activity of the 5-HT transporter either by decreasing[30] or by increasing 5-HT uptake, respectively.

ARTICLE I

The 5-HT reuptake process and the 5-HT transporter had been widely studied *in vitro* using both [^{3}H]5-HT uptake and radioligand binding techniques[32]. In contrast, the characterization of 5-HT reuptake activity *in vivo* had not been so extensive since previous studies had been mostly concerned with physiological, behavioral or neurochemical consequences of 5-HT reuptake inhibition. Moreover, reports on the occurrence of a down-regulation of the 5-HT transporter following long-term antidepressant treatments were controversial. Our first study was thus undertaken to address the following issues which remained unsolved: i) to devise a reliable method for

measuring electrophysiologically *in vivo* 5-HT reuptake activity; ii) to functionally assess the possibility of a desensitization of the 5-HT reuptake carrier following long-term 5-HT reuptake blockade; iii) to correlate functional modifications with changes in the [^{3}H]paroxetine binding parameters following long-term 5-HT reuptake blockade with paroxetine.

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Desensitization of the Neuronal 5-HT Carrier following Its Long-Term Blockade

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***In vivo* extracellular unitary recordings, *in vitro* ^{3}H-5-hydroxytryptamine (5-HT) uptake, and ^{3}H-paroxetine binding assays were used to assess the effect of acute and long-term administration of the 5-HT reuptake inhibitor paroxetine on the neuronal 5-HT transporter in the rat dorsal hippocampus. Recovery time of the firing activity of CA_3 hippocampus pyramidal neurons following microiontophoretic application of 5-HT was used as an index of *in vivo* reuptake activity. In a first series of experiments, the acute intravenous administration of paroxetine and 5-HT denervation with the neurotoxin 5,7-dihydroxytryptamine produced a marked prolongation of the suppressant effect of 5-HT, indicating that reuptake into 5-HT terminals plays a significant role in terminating the action of microiontophoretically applied 5-HT. In a second series of experiments, rats were treated with paroxetine (10 mg/kg/d, s.c.) for 2 or 21 d. In both treatment groups, there was a marked prolongation of the effect of microiontophoretically applied 5-HT; however, in rats treated for 2 d, the prolongation was significantly greater than that observed in rats treated for 21 d. After the 21 d treatment with paroxetine and a 48 hr washout, the prolongation of the effect of microiontophoretically applied 5-HT by acute intravenous paroxetine was significantly reduced, suggesting a decrease in the number of 5-HT carriers. In keeping with this interpretation, following the same treatment regimen, there was a 50% and 60% reduction of the *in vitro* ^{3}H-5-HT uptake in hippocampal and dorsal raphe slices, respectively, and a reduced effectiveness of paroxetine in blocking ^{3}H-5-HT uptake in both regions. The determination of the binding parameters of ^{3}H-paroxetine revealed that, in rats treated for 21 d with paroxetine (10 mg/kg/d, s.c.), following a 48 hr washout K_d values were unchanged but B_{max} values were reduced by 70% and 60% in hippocampal and cortical membranes, respectively.**

[Key words: 5-HT uptake, 5-HT transporter, 5-HT carrier, ^{3}H-paroxetine binding, downregulation, rat hippocampus]

The 5-hydroxytryptamine (5-HT) reuptake process and 5-HT transporter have been widely studied *in vitro* using both ^{3}H-5-HT uptake and radioligand binding techniques (Marcusson and Ross, 1990). In contrast, the characterization of 5-HT reuptake activity *in vivo* has not been so extensive, as studies have been mostly concerned with physiological, behavioral, or neurochemical consequences of reuptake inhibition (Fuller and Wong, 1990; Johnson, 1991). Using an *in vivo* electrophysiological paradigm, Wang et al. (1979) reported that 5-HT reuptake plays a significant role in terminating the action of microiontophoretically applied 5-HT onto lateral geniculate and amygdaloid neurons. However, the 5-HT reuptake process did not appear to play an important role in terminating the suppressant effect of microiontophoretically applied 5-HT onto CA_3 pyramidal neurons (de Montigny et al., 1980).

Reports on the occurrence of a downregulation of the 5-HT transporter following long-term antidepressant treatment are controversial. Radioligand binding studies indicate that the neuronal 5-HT transporter is associated with ^{3}H-imipramine (Langer and Raisman, 1983) and ^{3}H-paroxetine binding sites (Habert et al., 1985; Mellerup and Plenge, 1986) and that repeated administration of several classes of antidepressant drugs downregulate ^{3}H-imipramine (Plenge and Mellerup, 1982; Brunello et al., 1987), but not ^{3}H-paroxetine, binding sites (Graham et al., 1987; Cheetham et al., 1991; Foy et al., 1991). This controversy may stem in part from the observation that while ^{3}H-paroxetine binds to a single population of sites selectively located on 5-HT neurites (Marcusson et al., 1988; Hrdina et al., 1990), ^{3}H-imipramine has been shown to label a heterogeneous population of sites in brain tissue (Reith et al., 1983; Marcusson et al., 1985). Recently, Kovachich et al. (1992), using ^{3}H-cyanoimipramine to label the 5-HT transporter, have concluded that different types of antidepressants do not exert consistent effects on the density of 5-HT reuptake sites.

The present study was thus undertaken to address the following issues that remained unsolved: (1) to devise a reliable method for measuring electrophysiologically *in vivo* 5-HT reuptake activity; (2) to assess functionally the possibility of a desensitization of the 5-HT carrier following long-term reuptake blockade; and (3) to correlate functional modifications with changes in the ^{3}H-paroxetine binding parameters following long-term reuptake blockade with paroxetine.

Materials and Methods

Treatments. Male Sprague–Dawley rats (175–200 gm) were implanted subcutaneously with an osmotic minipump (Alza, Palo Alto, CA) that delivered 10 or 20 mg/kg/d of paroxetine (SmithKline Beecham, Harlow, England) for 2 or 21 d. The drug was dissolved in a 50% ethanol: water solution, and control rats were implanted with a minipump containing the vehicle. Unless otherwise specified, the experiments were

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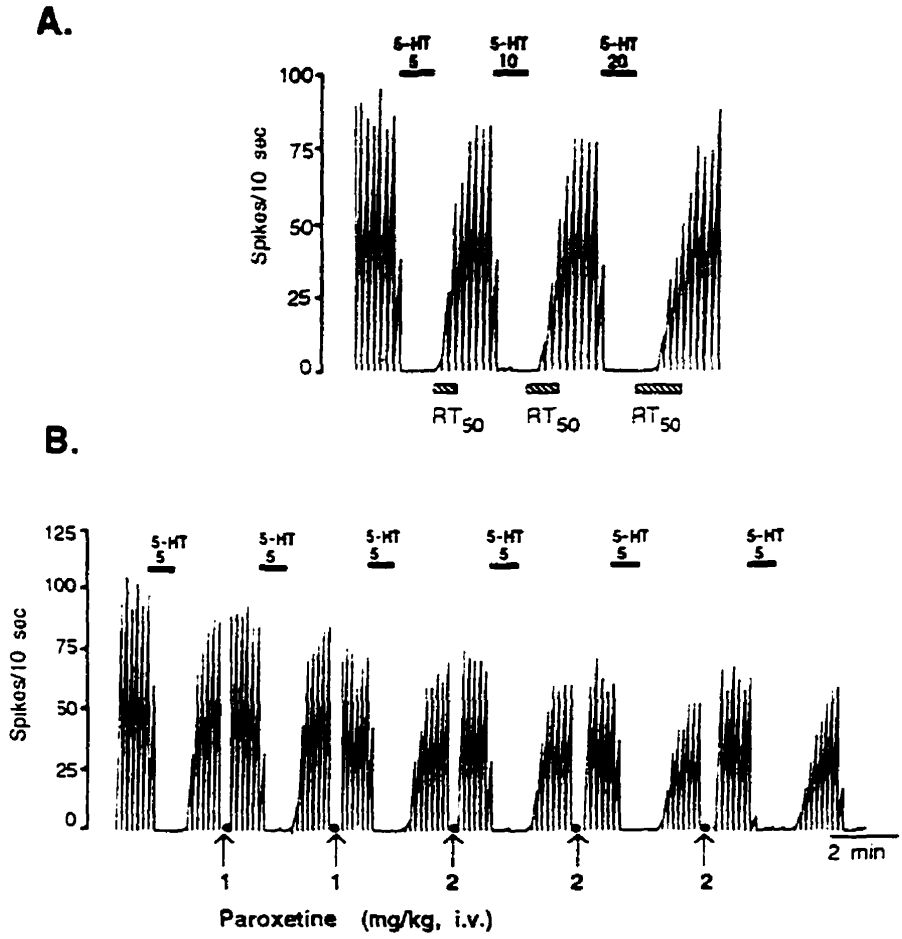

Figure 1. Integrated firing rate histograms showing the response of a representative dorsal hippocampus CA_1 pyramidal neuron to increasing microiontophoretic currents of 5-HT (*A*), and the effect of successive injections of paroxetine at 10 min intervals on the response to microiontophoretic application of 5-HT (*B*). The *solid bars above each trace* indicate the duration of application for which the ejection currents are given in nA. The *hatched bars below the histogram in A* indicate RT_{50}. This value is the time in seconds required by a neuron to recover by 50% its initial firing frequency calculated from the termination of the microiontophoretic application of drugs (*left edge of the hatched bar*). The *dots at the bottom of the histogram in B* represent periods of 10 min. Time scale applies to both traces.

carried out with the osmotic minipump in place. Two weeks before the experiments, another series of rats received an intracerebroventricular injection of 5,7-dihydroxytryptamine (5,7-DHT; 200 μg of free base in 20 μl of 0.9% NaCl and 0.1% ascorbic acid). This treatment has been shown to reduce 5-HT content in CA_1 region by at least 90% (Gerson and Baldessarini, 1975). Desipramine (25 mg/kg, i.p.) was administered 1 hr before 5,7-DHT to protect noradrenaline neurons (Bjorklund et al., 1975). Control rats were injected with saline. All experiments were carried out under chloral hydrate anesthesia (400 mg/kg, i.p.).

Electrophysiological experiments. Extracellular unitary recordings were obtained from pyramidal neurons in the CA_1 region of dorsal hippocampus. The microelectrode was descended 4 mm lateral and 4 mm anterior to lambda. Pyramidal neurons were identified by their high-amplitude (0.5–1.2 mV), long-duration (0.6–1 msec) complex spike discharges alternating with simple spike activity (Kandel and Spencer, 1961). A leak or a small current of ACh (0–5 nA) was used to activate the neurons within their physiological range (8–14 Hz; Ranck, 1975). Microiontophoretic applications were performed with five-barreled glass micropipettes that were pulled in a conventional manner and their tips broken back to 10–15 μm under microscopic control. The central barrel, filled with 2 M NaCl solution, was used for extracellular unitary recording. Three of the side barrels contained three of the following solutions: 5-HT creatinine sulfate (5 mM in 200 mM NaCl, pH 4; Sigma, St. Louis, MO); norepinephrine (NE) (50 mM in 200 mM NaCl, pH 4; Sigma); gepirone (25 mM in 200 mM NaCl, pH 4; Bristol-Myers Squibb, Wallingford, CT); or ACh (20 mM in 200 mM NaCl, pH 4; Sigma). The fourth side barrel, containing a 2 M NaCl solution, was used for automatic current balancing. Ejection periods were always of 50 sec.

The responsiveness of CA_1 pyramidal neurons to microiontophoretic application of drugs was assessed using $I \cdot T_{50}$ and RT_{50} values calculated by on-line computer with a 0.1 sec resolution. The $I \cdot T_{50}$ value represents the charge in nanocoulombs (1 nC = 1 nA × 1 sec) required to obtain a 50% decrease of the firing rate of the neuron recorded and has been shown to provide an index of the neuronal responsiveness to the microiontophoretically applied drug (de Montigny and Aghajanian, 1977; de Montigny et al., 1980). The presynaptic component of neuronal responsiveness to microiontophoretic application of drugs was evaluated using the RT_{50} method. RT_{50} is defined as the time in seconds required by the neuron to recover 50% of its initial firing frequency from the termination of microiontophoretic application. The RT_{50} value has been shown to provide a reliable index of the *in vivo* activity of the NE reuptake process in the rat hippocampus (de Montigny et al., 1980; Gravel and de Montigny, 1987) and of the 5-HT carrier in the rat amygdala and lateral geniculate body (Wang et al., 1979).

Determination of in vitro *^{3}H-5-HT uptake.* For determination of *in vitro* ^{3}H-5-HT uptake, animals were decapitated, their brains rapidly removed, and dissected on an ice-cold plate. Slices of 0.4 mm thickness from hippocampus or raphe region were prepared using a McIlwain chopper. They were incubated for 3 min at 37°C in a Krebs solution with various concentrations (0–1000 nM) of paroxetine, and bubbled with a mixture of 95% O_2 and 5% CO_2. The composition of the Krebs solution was 118 mM NaCl, 4.7 mM $CaCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 0.004 mM Na_2EDTA, and 0.11 mM ascorbic acid. After the incubation period, ^{3}H-5-HT (specific activity, 22.7 Ci/mmol; New England Nuclear Research Products, Mississauga, Ontario, Canada) was added at a final concentration of 5 nM, 20 nM, or 100 nM. Following a 3 min incubation period, uptake was terminated by transferring the slices to 5 ml of ice-cold buffer, and they were then solubilized in 0.5 ml of Soluene 350 (Packard Instruments, Downers Grove, IL). Radioactivity in the slices and the incubation medium was determined by liquid scintillation spectroscopy. Parallel experiments were carried out at 0°C as control for passive diffusion. All experiments were performed in duplicate and the amount of tritium actively captured by the tissue (C_t) was calculated according to the formula $C_t = C_T - C_n$, where

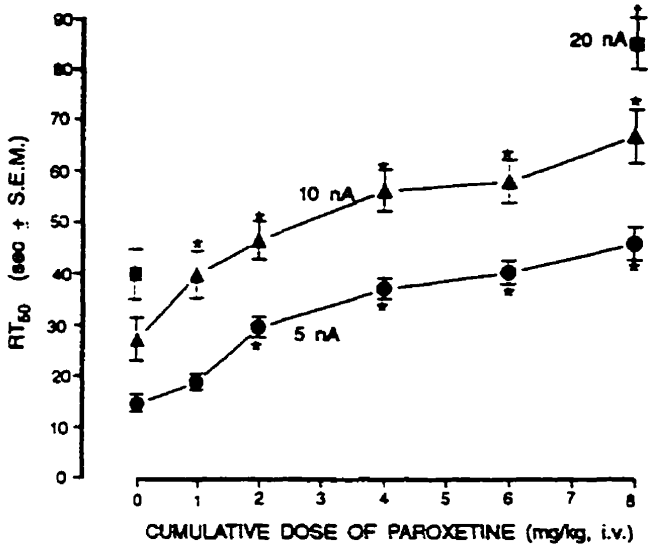

Figure 2. Effect of successive intravenous injections of paroxetine on the recovery time, expressed as RT_{50} values (means ± SEM), from microiontophoretic applications of 5-HT with 5 nA (●), 10 nA (▲), and 20 nA (■) ($N = 9$ for all currents used). *, $p < 0.001$, compared to preinjection value using the Student's paired t test.

C_T and C_P are the tissue:medium ratios of ^{3}H-5-HT at 37°C and 0°C, respectively. Inhibition of uptake was calculated by means of the formula % of inhibition = $[1 - C_{AC}/C_{AP}] \times 100$, where C_{AC} and C_{AP} are the amounts of actively captured ^{3}H-5-HT in a medium with or without paroxetine, respectively.

IC_{50} values for the uptake of ^{3}H-5-HT were determined by computer analysis (GRAPHPAD, Graphpad Software, San Diego, CA) from concentration–effect curves based on four concentrations of paroxetine.

^{3}H-paroxetine binding assays. Binding assays using ^{3}H-paroxetine (25 Ci/mmol; New England Nuclear Research Products) were performed according to a previously described protocol (Marcusson et al., 1988). Forty-eight hours after the removal of the osmotic minipump rats were decapitated, brains dissected, and cortex and hippocampus immediately frozen and kept at −70°C until the binding assays were carried out. Membranes were prepared by homogenizing brain tissue in 15 ml of ice-cold buffer (50 mM Tris HCl, 120 mM NaCl, 5 mM KCl; pH 7.4) and centrifuging at 48,000 × g for 10 min at 4°C. The resulting pellet was suspended in 15 ml of buffer and centrifuged. The final pellet was suspended to a final tissue concentration of 40–60 μg protein/ml (approximately 0.75 mg wet weight/ml) in the binding assay. The homogenates were incubated with ^{3}H-paroxetine (0.03–2 nM) at 22°C in a final volume of 1600 μl for 60 min. Incubations were terminated by addition of 4 ml ice-cold buffer and filtration through Whatman GF/C filters using a 24-channel cell harvester. Filters were then washed with four 4 ml rinses in cold buffer. Radioactivity trapped by the filters was determined by liquid scintillation spectroscopy (Beckman Counter LS 6000 SE, Beckman Instruments, Fullerton, CA). Nonspecific binding was estimated in the presence of 100 μM 5-HT. Binding was analyzed using the curve-fitting program LIGAND (G. A. McPherson, Elsevier-Biosoft, Cambridge, UK).

Statistical analysis. Results were expressed throughout as means ± SEM. When two means were compared, statistical significance of their difference was assessed using the two-tailed paired or nonpaired Student's t tests as indicated. For multiple comparisons, one-way or two-way ANOVA was used for independent and paired samples, respectively.

Results

Effect of acute intravenous administration of paroxetine on the recovery time from microiontophoretic applications of 5-HT

The duration of suppression following 5-HT applications was assessed in naive rats using increasing currents for applying

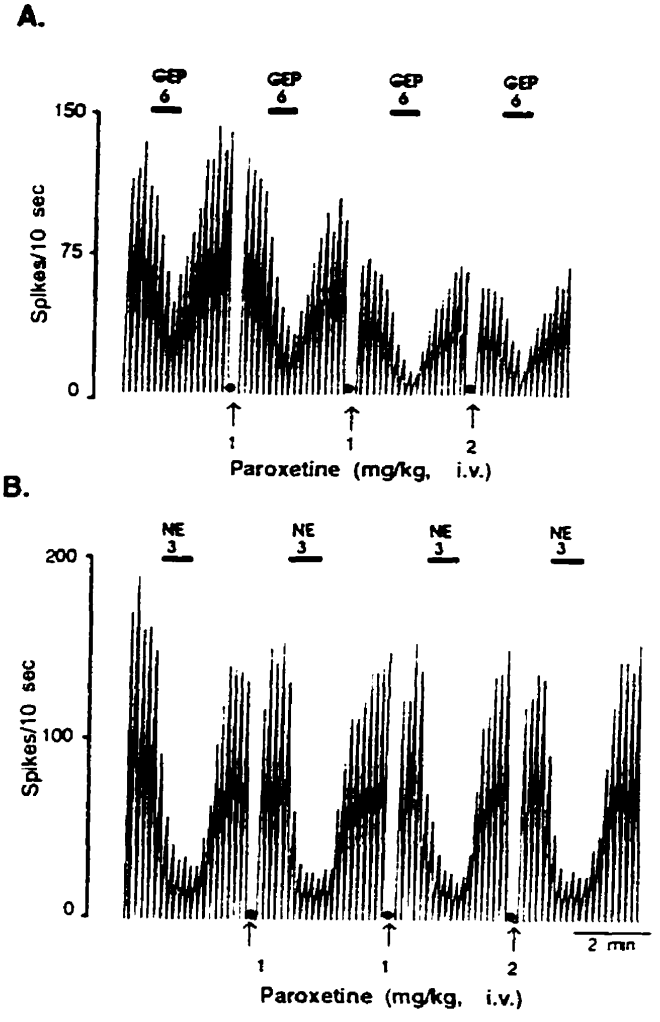

Figure 3. Integrated firing rate histograms of dorsal hippocampus CA$_1$ pyramidal neurons showing the effect of successive injections of paroxetine at 10 min intervals on the response of a dorsal hippocampus pyramidal neuron to microiontophoretic application of gepirone (*A*) and NE (*B*). *Dots at the bottom of each histogram* represent periods of 10 min. Time scale applies to both tracings.

5-HT. The recovery time (RT_{50} value) was proportional to the current used: 5, 10, and 20 nA applications yielded RT_{50} values of 16 ± 2, 27 ± 4, and 41 ± 5 sec, respectively ($n = 9$; Fig. 1*A*). Following intravenous administration of successive 1–2 mg/kg doses of paroxetine (cumulative doses of 2, 4, 6, and 8 mg/kg), the effect of microiontophoretically applied 5-HT was dose-dependently prolonged (Figs. 1*B*, 2). At the highest dose of paroxetine used (8 mg/kg, i.v.), the RT_{50} values for 5 nA, 10 nA, and 20 nA applications were increased by 206%, 145%, and 110%, respectively.

In order to confirm the selectivity of paroxetine for the 5-HT carrier in this paradigm, the effect of paroxetine was assessed on the recovery from microiontophoretic applications of the 5-HT$_{1A}$ agonist gepirone and NE, neither of which are substrates for the 5-HT transporter. In contrast to the prolongation of the effect observed for 5-HT, the RT_{50} values for these two compounds were not affected by successive doses of paroxetine (Fig. 3, Table 1). While in the dose range of 1–4 mg/kg, intravenous

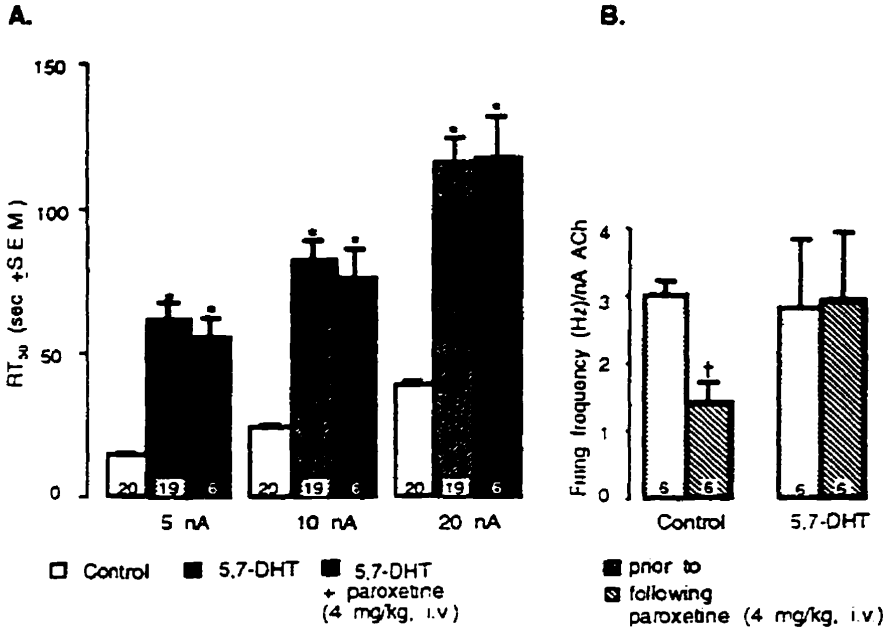

Figure 4. *A*, Recovery time, expressed as RT_{50} values (means ± SEM), of dorsal hippocampus CA_1 pyramidal neurons from microiontophoretic applications of 5-HT in control rats and in rats treated with 5,7-DHT, before and after intravenous administration of 4 mg/kg of paroxetine. *, $p < 0.001$ compared to control values using two-tailed Student's t test. *B*, Effect of intravenous paroxetine on pyramidal neuron firing frequency in control and 5,7-DHT–lesioned rats. †, $p < 0.01$ using two-tailed paired Student's t test. The number of neurons tested is given at the bottom of each column.

administration of paroxetine dose-dependently increased RT_{50} values of 5-HT applications ($r = 0.8$, $p < 0.001$). RT_{50} values for NE and gepirone were not correlated with the dose of paroxetine administered ($r = 0.01$, $p = 0.8$).

Effect of 5-HT denervation on the recovery from microiontophoretic applications of 5-HT

The prolongation of the recovery time from microiontophoretic applications of 5-HT, following acute 5-HT reuptake blockade, in the absence of a modification of its initial effectiveness [$I \cdot T_{50}$ values: 23 ± 3 nC prior to, and 25 ± 4 nC following, paroxetine (4 mg/kg, i.v.; $n = 14$)], suggested a pure presynaptic effect of paroxetine. This interpretation relied, however, on the assumption that the recovery of the firing rate of CA_1 pyramidal neurons from microiontophoretic applications of 5-HT is exerted mainly by the 5-HT transporters located on 5-HT terminals. To verify this hypothesis, the recovery from 5-HT applications was assessed in 5,7-DHT–lesioned rats. As expected, 5-HT denervation markedly prolonged the effect of 5-HT: the RT_{50} values were increased by 324%, 262%, and 197% for 5 nA, 10 nA, and 20 nA applications, respectively (Fig. 4*A*). Interestingly, as shown in Figure 4*A*, the injection of paroxetine (4 mg/kg, i.v.) in 5,7-DHT–lesioned rats did not produce a further increase in RT_{50}, indicating that pretreatment with 5,7-DHT had produced a complete denervation and that the effect of paroxetine observed in intact rats (Figs. 1, 2) was entirely attributable to the blockade of the 5-HT transporters located on 5-HT terminals.

In intact rats, intravenous administration of paroxetine per se reduced pyramidal neuron firing frequency. Consequently, the microiontophoretic current of the ACh had to be increased from 4 ± 0.7 to 9 ± 0.8 nA ($n = 6$) to restore the firing rate to its preinjection level. Brunel and de Montigny (1987) have previously demonstrated that ACh does not alter the response of pyramidal neurons to 5-HT. In 5,7-DHT–lesioned rats, paroxetine had no effect on the firing frequency of pyramidal neurons ($n = 6$; Fig. 4*B*).

It is also noteworthy that 5,7-DHT lesion and administration of paroxetine (10 mg/kg/d, s.c.) for 2 d yielded nearly identical RT_{50} values. When the RT_{50} values from 5-HT applications were

Table 1. Effect of acute intravenous paroxetine on the recovery from microiontophoretic applications of gepirone, norepinephrine and serotonin

Cumulative dose of paroxetine (mg/kg, i.v.)	RT_{50} (s ± SEM) Gepirone ($n = 3$)	NE ($n = 3$)	5-HT ($n = 3$)
0	23 ± 10	35 ± 12	10 ± 2
1	18 ± 4	35 ± 15	14 ± 5
2	18 ± 5	37 ± 12	26 ± 4*
4	20 ± 11	35 ± 9	39 ± 4*

* $P < 0.01$ using the two-tailed Student's t test.

Table 2. Effect of short-term paroxetine administration and 5,7-DHT lesion on the recovery from microiontophoretic applications of 5-HT

Current (nA)	RT_{50} (s ± SEM) Control ($n = 19$)	5,7-DHT ($n = 19$)	Paroxetine *low* dose* ($n = 15$)	Paroxetine *high* dose** ($n = 17$)
5	14.5 ± 1	61.5 ± 6***	62.3 ± 6***	66.2 ± 3***
10	24.3 ± 1	82.6 ± 7***	88.8 ± 7***	85.8 ± 3***
20	39.0 ± 2	116.0 ± 8***	122.0 ± 9***	109.4 ± 4***

In paroxetine treated rats the experiments were performed with the osmotic pump in place.

* 10 mg/kg/day, s.c. × 2 days.

** 20 mg/kg/day, s.c. × 2 days.

*** $p < 0.001$ using two-tailed Student's t test.

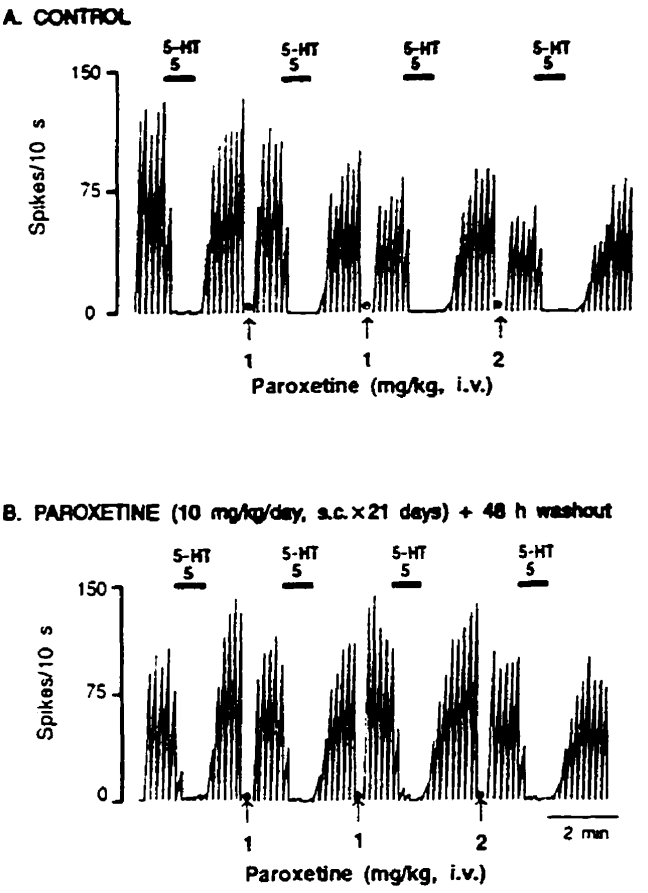

Figure 5. Integrated firing rate histograms showing the response of dorsal hippocampus CA_1 pyramidal neurons to microiontophoretic applications of 5-HT in a control rat (*A*) and a long-term paroxetine-treated rat (*B*), following successive intravenous injections of paroxetine at 10 min intervals. Rats were implanted subcutaneously with an osmotic minipump which delivered either vehicle or paroxetine. After 21 d the minipump was removed and a 48 hr washout period was allowed. Time scale applies to both traces.

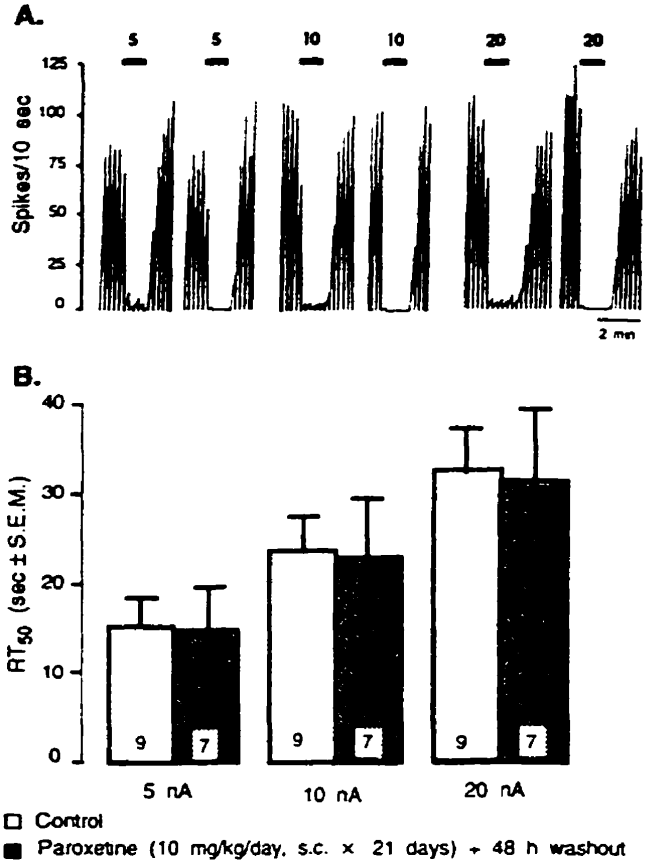

Figure 6. *A*, Integrated firing rate histograms illustrating the response of dorsal hippocampus CA_1 pyramidal neurons in a control and a long-term paroxetine-treated rat 48 hr after the removal of the osmotic pump. Each current is applied alternatively in the control rat and the paroxetine-treated rat. *B*, Recovery time expressed as RT_{50} values (mean ± SEM) of dorsal hippocampus pyramidal neurons from microiontophoretically applied 5-HT in control rats and in rats treated with paroxetine for 21 d. Note that RT_{50} values for the 5 nA current are significantly smaller than for the 20 nA current and this difference is maintained after long-term paroxetine administration ($p < 0.001$). Number of neurons tested is given at the bottom of each column.

assessed in rats that were still carrying the osmotic minipump during the experiment, this parameter was increased by 320%, 270%, and 212% for 5, 10, and 20 nA currents, respectively (as compared to 324%, 262%, and 197% for the same currents in 5,7-DHT-lesioned rats). This suggests that, at a dose of paroxetine of 10 mg/kg/d, all the 5-HT transporters were blocked, thus simulating denervation. Furthermore, when the dose of paroxetine was doubled (20 mg/kg/d × 2 d), RT_{50} values did not further increase (Table 2), thereby confirming that the 10 mg/kg/d dose of paroxetine maximally inhibits 5-HT reuptake.

Table 3. Effect of long-term paroxetine treatment on the ED_{50} of acute intravenous paroxetine for increasing the recovery from 5-HT applications[a]

Current (nA)	Dose of paroxetine (mg/kg, i.v.) Control ($n = 9$)	Paroxetine[**] ($n = 7$)	p[***]
5	1.7 ± 0.2	3.2 ± 0.6	<0.03
10	2.4 ± 0.2	4.1 ± 0.4	<0.01
20	2.4 ± 0 3	4.0 ± 0.7	<0.05

[a] ED_{50} was calculated as the dose of paroxetine necessary to increase RT_{50} by 50%.
[**] 10 mg/kg/day, s.c. × 21 days.
[***] Using the two-tailed Student's *t* test.

Effect of long-term administration of paroxetine on the recovery time from microiontophoretic applications of 5-HT

To test the effect of long-term reuptake blockade on the 5-HT transporter, the effectiveness of successive intravenous doses (1–2 mg/kg) of paroxetine to prolong RT_{50} values was assessed in control and paroxetine-treated rats (10 mg/kg/d, s.c. × 21 d) following a 48 hr washout period to ensure complete drug elimination. As exemplified in Figure 5, the effectiveness of acute intravenous paroxetine was reduced in the paroxetine-treated group. Before the injection of paroxetine, the RT_{50} values were similar in treated and in control rats (Fig. 6), indicating, first, that the reuptake process was no longer blocked after the washout period, and second, that at the concentrations attained with the different microiontophoretic currents of 5-HT used, the ability of 5-HT terminals to take up 5-HT was unchanged after

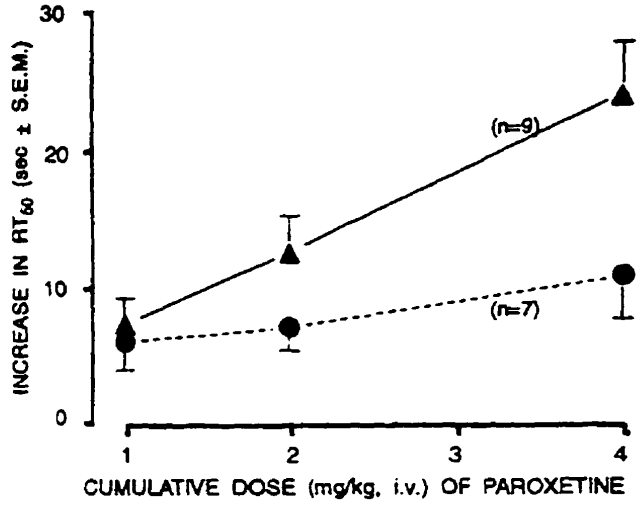

Figure 7. Effect of successive injections of paroxetine on the recovery time from 5 nA microiontophoretic applications of 5-HT in control rats (▲) and in rats treated with paroxetine (10/mg/kg/d, s.c. × 21 d) following 48 hr washout (●). Values are expressed as increase in RT_{50} (mean ± SEM). $p < 0.05$, comparing the two curves by ANOVA.

long-term blockade by paroxetine. The first dose of paroxetine (1 mg/kg, i.v.) increased the RT_{50} values to a similar extent in control and long-term paroxetine-treated rats. However, subsequent cumulative doses of 2 and 4 mg/kg produced smaller effects in long-term paroxetine-treated rats than in controls (Figs. 5, 7), indicating a decreased efficacy of intravenous paroxetine in blocking 5-HT reuptake following long-term paroxetine treatment. For all currents used, the dose of paroxetine required to increase RT_{50} values by 50% (ED_{50}) was doubled in long-term paroxetine-treated rats (Table 3).

To investigate further the induction of a "tolerance" by long-term paroxetine administration, the suppressant effect of 5-HT was determined in control rats and in rats treated with paroxetine (10 mg/kg/d, s.c.) for 2 or 21 d without removing the osmotic pump. For all currents used, recovery from microiontophoretic applications of 5-HT was prolonged in both treated groups, but to a significantly lesser extent in long-term– than in short-term–treated rats (Fig. 8). The RT_{50} values from 5, 10, and 20 nA applications of 5-HT were increased by 377%, 300%, and 198% in 2 d paroxetine-treated rats, and by 254%, 196%, and 149% in 21 d paroxetine-treated rats (Fig. 9).

Recovery from microiontophoretic applications of gepirone was unchanged by long-term administration of paroxetine, in keeping with the fact that it is not a substrate for the 5-HT transporter. RT_{50} values from 4 and 10 nA applications of gepirone were 28 ± 4 sec and 53 ± 8 sec in control rats, and 24 ± 4 sec and 50 ± 6 sec in rats treated with paroxetine for 21 d. The initial responsiveness ($I \cdot T_{50}$ values) to microiontophoretic applications of gepirone and of 5-HT was also unchanged following long-term paroxetine treatment, ruling out the possibility that the reduced effectiveness of acute paroxetine to prolong the effect of 5-HT could be ascribed to a decrease in neuronal responsiveness to the activation of postsynaptic $5\text{-}HT_{1A}$ receptors. For 5-HT, respective $I \cdot T_{50}$ values for 5 and 10 nA applications were 24 ± 3 nC and 39 ± 4 nC in control rats (n = 18), and 23 ± 2 nC and 42 ± 3 nC following the 21 d paroxetine treatment (n = 18).

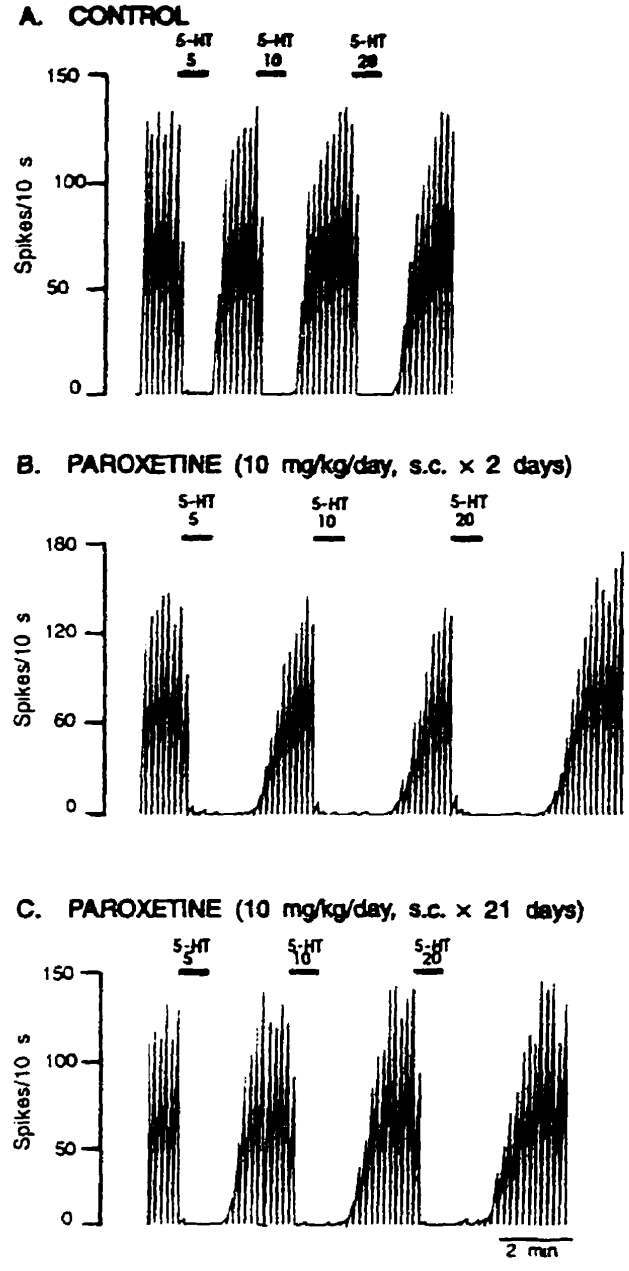

Figure 8. Integrated firing rate histograms showing the response of CA, dorsal hippocampus pyramidal neurons to microiontophoretically applied 5-HT in a control rat (*A*) and in rats treated with paroxetine (10 mg/kg/d, s.c.) for 2 d (*B*) or 21 d (*C*). In *B* and *C*, the experiments were carried out with the minipump in place.

Effect of long-term paroxetine administration on in vitro *^{3}H-5-HT uptake*

The following series of experiments were carried out to verify *in vitro* the induction of desensitization of the 5-HT transporter by long-term paroxetine administration. In control experiments, increasing the concentration of paroxetine in the incubation medium caused a concentration-dependent reduction in the amount of radioactivity captured by the slices. In slices from 5,7-DHT–lesioned rats, the uptake of ^{3}H-5-HT was reduced by

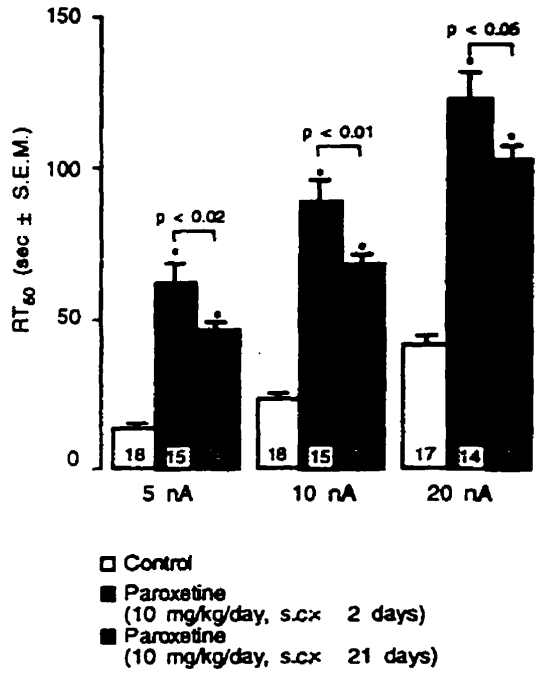

Figure 9. Recovery time, expressed as RT_{50} values (mean ± SEM), of dorsal hippocampus pyramidal neurons from microiontophoretically applied 5-HT in control rats and in rats treated with paroxetine (10 mg/kg/d, s.c.) for 2 or 21 d. The number of neurons tested is given at the bottom of each column. *, $p < 0.001$ compared to control values using two-tailed Student's t test. p values indicated on the figure also were obtained using the nonpaired Student's t test.

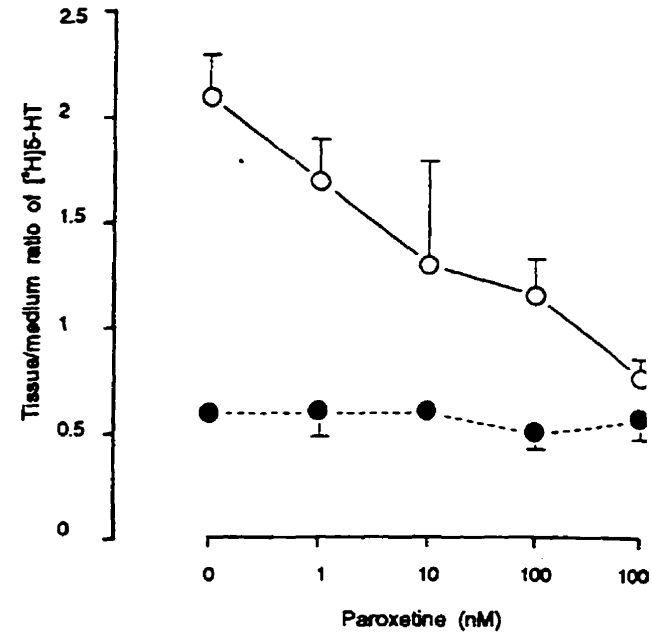

Figure 10. Effect of increasing concentrations of paroxetine on ^{3}H-5-HT uptake in hippocampal slices of control (○) and 5,7-DHT-lesioned rats (●). $n = 3$ in each group. $p < 0.001$ comparing both curves by ANOVA.

70% (Fig. 10). Moreover, the incubation with increasing paroxetine concentrations did not produce further inhibition (Fig. 10). As ^{3}H-5-HT uptake in control slices incubated with the highest paroxetine concentration (1000 nM) was not significantly different from that observed after 5,7-DHT lesion, it can be assumed that maximal blockade of 5-HT uptake sites was attained at this concentration.

In slices from control rats, paroxetine in the incubation medium was more effective in blocking reuptake using a 100 nM than a 5 nM concentration of ^{3}H-5-HT (Fig. 11*D,F*). From each of these paroxetine concentration–effect curves, the concentration of paroxetine inducing 50% of the maximal inhibition (attained with 1000 nM paroxetine) was determined. Paroxetine was six times more potent in blocking reuptake when the ^{3}H-5-HT concentration in the incubation medium was high (100 nM) than when it was low (5 nM): respective calculated IC_{50} values were 18 nM and 107 nM.

Following a 48 hr washout period, in slices incubated with 100 nM ^{3}H-5-HT, radioactivity taken up by the tissue was reduced by 50% in the long-term paroxetine-treated group. This effect was significant in slices incubated with 100 nM ^{3}H-5-HT and with paroxetine concentrations lower than 1000 nM (Fig. 11*A*).

Following long-term treatment with paroxetine, the *in vitro* effectiveness of paroxetine to block 5-HT uptake was reduced by 43%, thus confirming results from electrophysiological experiments reported above. However, this *in vitro* effect was only seen when incubation was carried out in the presence of 5 nM ^{3}H-5-HT (Fig. 11*F*). When slices were incubated with 20 nM ^{3}H-5-HT, long-term paroxetine treatment did not have any detectable effect (Fig. 11*B,E*). Moreover, it is noteworthy that after long-term paroxetine treatment the difference in IC_{50} for paroxetine at different ^{3}H-5-HT concentrations was abolished. Calculated IC_{50} values were 14 nM and 13 nM for ^{3}H-5-HT concentrations of 5 nM and 100 nM, respectively.

The effect of long-term reuptake blockade was also assessed in slices from the raphe region. It induced changes similar to those observed in the hippocampus: ^{3}H-5-HT uptake was reduced by 60% (Fig. 12*A*) and 10 nM, but not 100 nM, of paroxetine produced a much smaller inhibition of ^{3}H-5-HT uptake than in slices from control rats (Fig. 12*B*).

Effect of long-term paroxetine administration on ^{3}H-paroxetine binding parameters

Specific saturable binding of ^{3}H-paroxetine to rat cortical and hippocampal membranes was concentration dependent (28% specific binding at 2 nM ^{3}H-paroxetine). Scatchard transformation of the binding data produced linear plots in all cases (Fig. 13) and fitted better a one- rather than a two-site model. Mean K_d and B_{max} values in control and long-term paroxetine-treated rats for both regions are summarized in Table 4. After long-term paroxetine administration, the total number of binding sites was reduced by 60% in cortex and by 70% in hippocampus, thus confirming a downregulation of the 5-HT transporter. The fact that K_d values remained unchanged after the treatment ruled out the possibility that the changes observed could be due to a competitive inhibition of binding by residual paroxetine after the 48 hr washout period.

Discussion

Electrophysiological data obtained in the present study confirm and extend previous observations that RT_{50} values following

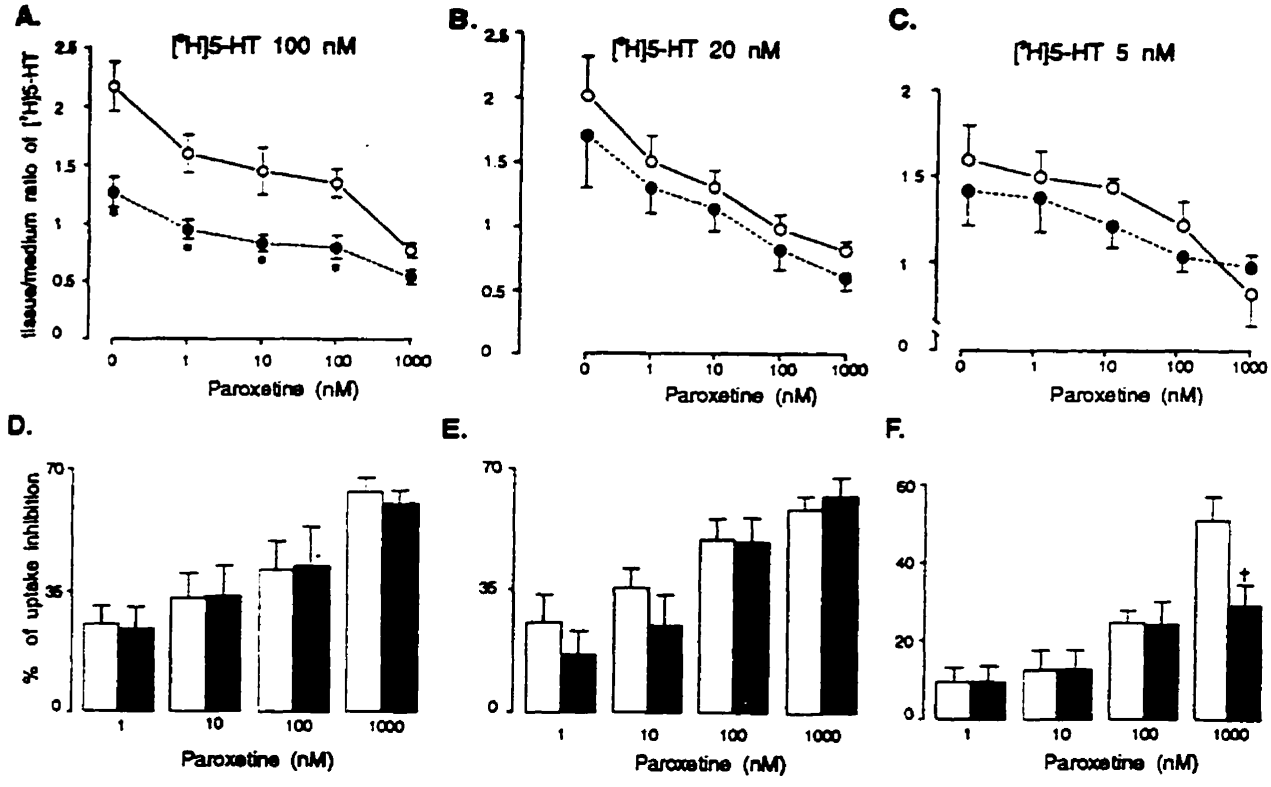

Figure 11. ³H-5-HT reuptake activity (*A–C*) and effectiveness of paroxetine to inhibit ³H-5-HT reuptake, expressed as percentage of uptake inhibition (*D–F*) in hippocampal slices from control ($n = 5$; ○) and long-term paroxetine-treated rats ($n = 5$; ●) (10 mg/kg/d, s.c. × 21 d + 48 h washout). *, $p < 0.02$ as compared to control values using nonpaired Student's *t* test; †, $p < 0.05$ as compared to control using nonpaired Student's *t* test.

microiontophoretic applications of 5-HT provide a reliable index of the *in vivo* activity of the 5-HT reuptake process, since it is prolonged three- to fourfold by acute, short-term, and long-term administration of paroxetine (Figs. 2, 9) as well as by lesioning 5-HT terminals (Fig. 4). The fact that a selective 5-HT reuptake inhibitor (SSRI) prolongs recovery from 5-HT applications, leaving unaffected the effects of gepirone and NE (Table 1), which are not substrates for the 5-HT transporter, strongly suggests that the recovery of the firing frequency of pyramidal neurons following microiontophoretic applications of 5-HT depends on the activity of the 5-HT carrier. In a previous report we had concluded that, in dorsal hippocampus, 5-HT reuptake did not play a significant role in terminating the action of microiontophoretically applied 5-HT (de Montigny et al., 1980). The apparent contradiction between the latter report and the present one is probably due to the fact that, herein, microiontophoretic applications were carried out using higher currents of a more concentrated solution of 5-HT to ensure that it would diffuse from the ejection site (near the cell body) to strata radiatum and oriens, where the majority of 5-HT terminals are located (Oleskevich and Descarries, 1990).

In keeping with previous observations that lesioning of 5-HT terminals abolishes ³H-paroxetine binding (de Souza and Kuyatt, 1987; Hrdina et al., 1990; Dewar et al., 1991), in our electrophysiological paradigm the 5,7-DHT pretreatment completely abolished the effect of acute paroxetine on the duration of suppression of firing activity by the microiontophoretic application of 5-HT (Fig. 4*A*). A similar effect was observed *in vitro* where paroxetine failed to decrease ³H-5-HT uptake in slices obtained from 5,7-DHT–lesioned rats (Fig. 10). Even if 5-HT transporters have been found in primary astrocyte cultures from neonatal rat brain (Kimelberg and Katz, 1985), the majority of

Table 4. Binding parameters of [³H]paroxetine in control and long-term paroxetine-treated rats

	Kd (nM)		Bmax (fmol/mg of prot.)	
Brain region	Control	Paroxetine[a]	Control	Paroxetine[a]
Hippocampus	0.3 ± 0.05	0.2 ± 0.08	114 ± 8	31 ± 9[**]
Cortex	0.3 ± 0.04	0.2 ± 0.06	138 ± 19	55 ± 14[***]

[a] 10 mg/kg/day, s.c. × 21 days + 48 h washout.
[**] $p < 0.001$, using two-tailed Student's *t* test.
[***] $p < 0.01$, using two-tailed Student's *t* test ($n = 4$).

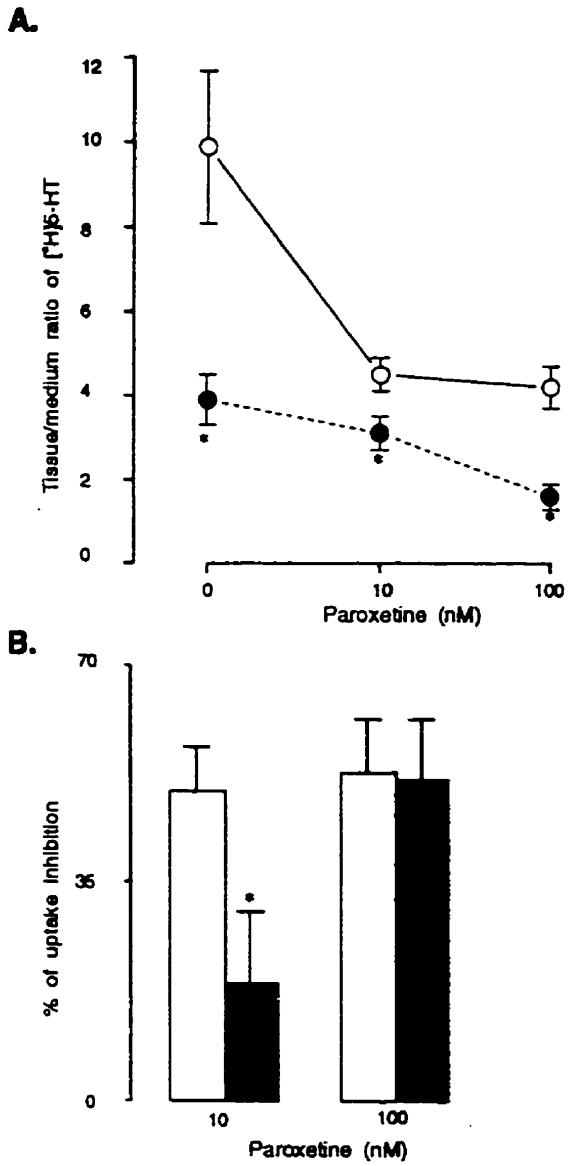

Figure 12. ^{3}H-5-HT reuptake (*A*) and effectiveness of paroxetine to inhibit 5-HT reuptake (*B*) in dorsal raphe slices from control (○) and long-term paroxetine-treated rats (●) (10 mg/kg/d, s.c. × 21 d + 48 hr washout; $N = 6$ in each group). *, $p < 0.05$ as compared to corresponding control values using nonpaired Student's t test. The concentration of ^{3}H-5-HT in the incubation medium was 100 nM.

autoradiographic studies of ^{3}H-5-HT reuptake in adult rat brain show that 5-HT uptake by astrocytes is minimal relative to that by 5-HT terminals (Katz and Kimelberg, 1985). Our results further support the notion that functionally relevant transporters are confined to 5-HT terminals.

The electrophysiological paradigm we devised was not only sensitive enough to detect the effect of paroxetine on exogenously applied 5-HT through changes in RT_{50}, but it could also detect the effect of 5-HT reuptake blockade on endogenously released 5-HT via the reduction of pyramidal neuron firing frequency (Figs. 1*B*, 4*B*, 5*A*). Since denervation abolished the consistent suppressant effect produced by paroxetine (Fig. 4*B*) on pyramidal neuron firing frequency, the latter observation clearly demonstrates the physiological role played by the 5-HT transporter in terminating the action of endogenously released 5-HT.

Long-term paroxetine treatment results in adaptive changes of the 5-HT transporter. These changes were detected not only in hippocampus (Figs. 7, 9, 11) but also in cerebral cortex (Fig. 13) and in the raphe region (Fig. 12), indicating that 5-HT transporters in multiple pre- and postsynaptic regions of the rat CNS share the ability to adapt to their sustained occupation.

Though previous studies using ^{3}H-imipramine have reported a decreased number of binding sites following long-term antidepressant treatment (Barbaccia et al., 1983; Arora and Meltzer, 1986; Brunello et al., 1987), these studies did not take into account the heterogeneity of ^{3}H-imipramine binding sites. In the present study, the use of paroxetine as the tritiated ligand allows us to conclude that the changes observed are directly related to the 5-HT transporter as it has been shown to selectively label this carrier. In contrast with our observations, Graham et al. (1987), Brunswick et al. (1991), and Cheetham et al. (1991), using ^{3}H-paroxetine, and Hrdina et al. (1990), taking into account high- and low-affinity ^{3}H-imipramine binding sites, did not detect any change in B_{max} following long-term antidepressant treatment. These divergent results could be explained by the fact that in the aforementioned studies reuptake blockers were administered by intraperitoneal injections. Since SSRIs have been shown to be rapidly metabolized in rodents (Buus Lassen, 1978; Fredricson, 1982), intraperitoneal injections, given usually every 12 or 24 hr, lead to large fluctuations in drug plasma levels, and consequently in the degree of reuptake blockade. For example, for the SSRI citalopram, which in rats has a half-life of 3 hr (Fredricson, 1982), its suppressant effect on the firing activity of 5-HT neurons is no longer detectable 15 hr after the injection of a high dose (Chaput et al., 1986). The observation of the lack of 5-HT reuptake blockade 48 hr after a 21 d treatment (Fig. 6) is thus fully consistent with the rapid metabolism of paroxetine in rodents (Buus Lassen, 1978). In the present study, to avoid fluctuations and produce stable plasma levels throughout the treatment period, paroxetine was administered by sustained subcutaneous infusion. Interestingly, Lesch et al. (1993) have recently reported the induction of downregulation of 5-HT transporter mRNA following long-term subcutaneous administration of fluoxetine, imipramine, and chlorimipramine. It therefore seems that stable plasma concentrations of the reuptake blocker administered are essential for the induction of an adaptive response at the level of the 5-HT transporter. Moreover, Kovachich et al. (1992) have reported that intraperitoneal administration of sertraline, but not that of citalopram, can reduce ^{3}H-cyanoimipramine binding in different limbic regions. The fact that desmethylsertraline is more potent and has a longer half-life than mono- and dimethylated citalopram metabolites (Boyer and Feighner, 1991) further supports the notion that a sustained occupation is required to induce a downregulation of the neuronal 5-HT transporter. Since Lesch et al. (1993), using 5-HT reuptake inhibitors less potent than paroxetine, still observed a downregulation of the mRNA encoding the 5-HT transporter, it is improbable that uptake blocking capacity of the different SSRI might account for the differences between this and previous studies (Graham et al., 1987; Hrdina et al., 1990; Brunswick et al., 1991; Cheetham et al., 1991).

In the present electrophysiological experiments, desensitization of the 5-HT transporter in long-term paroxetine-treated rats was evidenced by a reduction in the effectiveness of acute

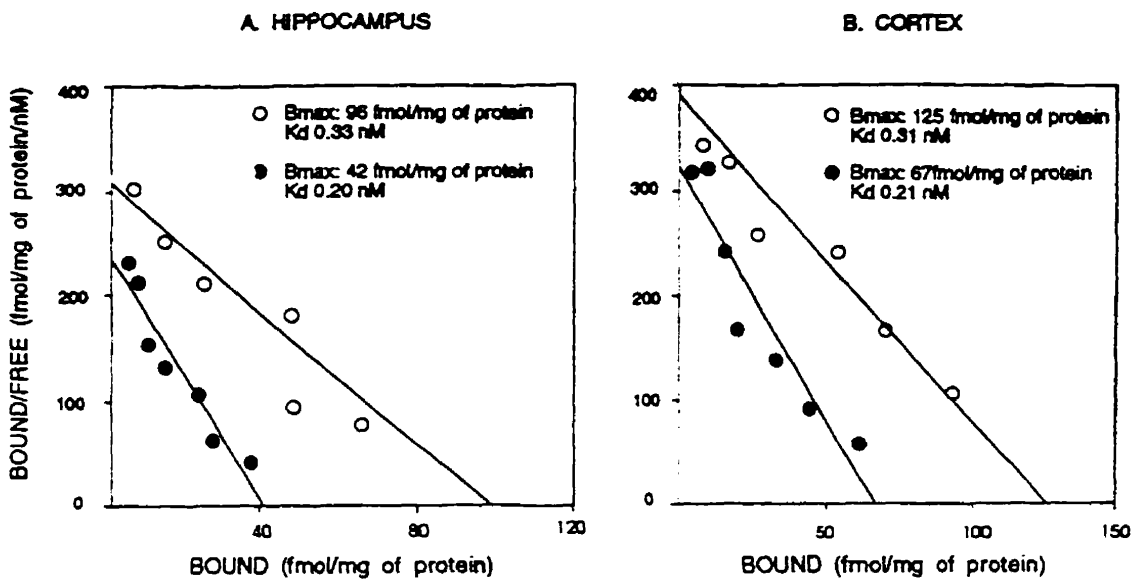

Figure 13. Representative Scatchard plots of specific ^{3}H-paroxetine binding to hippocampal (*A*) and cortical (*B*) membranes in a control rat (○) and in a rat treated with paroxetine (●) (10 mg/kg/d, s.c. × 21 d + 48 hr washout).

paroxetine to prolong the duration of suppression of firing by microiontophoretic applications of 5-HT (Fig. 7). Indeed, the ED_{50} of intravenous paroxetine to prolong RT_{50} was doubled in long-term paroxetine-treated rats (Table 3). Furthermore, the effect of subcutaneous administration of paroxetine on RT_{50} was significantly smaller in rats treated for 21 d than in those treated for 2 d (Fig. 9). Since the prolongation of RT_{50} values produced by 2 d subcutaneous administration of 10 mg/kg/d of paroxetine was similar in magnitude to that produced by a dose of 20 mg/kg/d, it can be assumed that, at the dose of 10 mg/kg/d, paroxetine blocked all available uptake sites. The fact that the effect of the 2 d treatment with 10 mg/kg/d of paroxetine was greater than that of the 21 d treatment suggests that the mechanism underlying this tolerance could be a reduction in the number of 5-HT transporters. This assumption was confirmed by radioligand binding studies that showed a 70% decrease in the number of ^{3}H-paroxetine binding sites in hippocampal membranes following long-term paroxetine administration (Fig. 13*A*).

The current model for the 5-HT transporter proposes that 5-HT, tricyclic, and nontricyclic uptake inhibitors all bind to the same or overlapping sites on the carrier (Marcusson et al., 1989; Marcusson and Ross, 1990). There is an apparent discrepancy between the reduced effectiveness of acute injection of paroxetine (Fig. 7), the reduced ^{3}H-5-HT uptake (Fig. 11), and the reduced number of ^{3}H-paroxetine binding sites (Fig. 12) 48 hr after the long-term paroxetine treatment, indicating a decrease in the number of 5-HT transporters, and the observation that, at this time point, the effect of microiontophoretically applied 5-HT was not prolonged (Fig. 6). This could be explained by the presence of spare 5-HT transporters. Hence, even if the long-term paroxetine treatment downregulates 5-HT transporter sites, the number of remaining sites would be sufficient to take up all of the microiontophoretically applied 5-HT, even when applied with the highest current (20 nA) used. This explanation is all the more likely since 5-HT is applied at the level of the soma, so that only minute amounts of the neurotransmitter would actually reach the 5-HT terminals, located mostly on remote dendritic trees. The existence of spare transporters could also explain the observation that, although paroxetine is a competitive antagonist for 5-HT reuptake, its IC_{50} was six times higher at 5 nM than at 100 nM ^{3}H-5-HT concentrations. At the low ^{3}H-5-HT concentration (5 nM), before interfering with ^{3}H-5-HT uptake, paroxetine would first need to occupy a large number of sites, thus explaining the high IC_{50} values observed in these conditions. At the high ^{3}H-5-HT concentration (100 nM), all (or most) of the transporters being operant, even low doses of paroxetine can interfere with reuptake. A reduction in the number of 5-HT transporters could also explain the fact that IC_{50} values for paroxetine at different ^{3}H-5-HT concentrations did not differ any longer following long-term paroxetine treatment since the number of spare sites would be minimal.

The proportion of occupied/free transporters could then explain the different ways in which desensitization was expressed in the *in vitro* reuptake experiments (Fig. 11). At 100 nM ^{3}H-5-HT (when all transporters are operant) a reduction in the number of sites was detected by reduction in ^{3}H-5-HT uptake capacity, whereas at the low concentration of ^{3}H-5-HT (5 nM), even in the presence of a reduced number of transporters, the remaining ones were in sufficient number to take up as much radioactivity as the control slices. Thus, in the latter condition, the reduction in the number of transporters was reflected by a decreased efficacy of the maximally effective paroxetine concentration (1000 nM).

The reduction in ^{3}H-paroxetine binding sites after long-term paroxetine administration confirms the downregulation of 5-HT transporters. The fact that K_d values were unchanged after treatment rules out the possibility of affinity changes being involved in the desensitization mechanism. This is in agreement with the observation that, even though paroxetine and 5-HT show different affinity for the 5-HT transporter, the present functional electrophysiological and uptake studies show that 5-HT reuptake activity and paroxetine effectiveness were reduced to a similar extent (45–60%) after long-term reuptake blockade.

Though K_d values found with ^{3}H-paroxetine in the present study were in the range of those previously reported by several groups, B_{max} values found in control rats were lower than those initially reported by Marcusson et al. (1988) for rat cortical membranes. However, values similar to those obtained in the present study were subsequently reported by Marcusson's group in the human cortex (Anderson et al., 1992) and by Foy et al. (1991) in rat diencephalon.

Blakely et al. (1991) and Haber and Goldman (1992) have cloned and expressed a 5-HT transporter from rat brain. Comparison of amino acid sequences demonstrates a 70% similarity between this protein and the NE transporter. In keeping with this observation, functional (Lacroix et al., 1991) and binding (Bauer and Tejani-Butt, 1992) assays of the NE transporter after long-term desipramine administration showed results similar to the ones observed for the 5-HT transporter in the present study. Therefore, monoaminergic transporters seem to possess common adaptive mechanisms elicited by their sustained occupation. Since long-term blockade of the 5-HT transporter induces a decrease in dorsal raphe mRNA codifying this protein (Lesch et al., 1993), the regulatory process most probably takes place at the transcriptional level.

In conclusion, the data presented in this study show (1) that the 5-HT reuptake process can play a significant role in terminating the action of microiontophoretically applied 5-HT in the rat hippocampus and that the RT_{50} method provides a reliable index of the *in vivo* activity of this process; and (2) that the 5-HT transporter, though not a receptor per se, shares with some of them the characteristic of adapting to its sustained occupation through a downregulation mechanism. Thus, it is possible that the enhancement of the efficacy of 5-HT synaptic transmission following long-term administration of SSRIs would not only be due to desensitization of somatodendritic and terminal autoreceptors (Chaput et al., 1986), but also to a decrease in 5-HT reuptake activity.

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ARTICLES II and III

The second study was devoted to study the effect of tianeptine on the 5-HT transporter. Whereas most tricyclic antidepressant drugs inhibit the neuronal reuptake of monoamines, tianeptine had been reported to increase this function[31,42]. However, since substantial evidence indicates that enhancement of 5-HT neurotransmission is an effect shared by several antidepressant treatments[15], the capacity of tianeptine to enhance 5-HT reuptake appeared to contrast with its purported antidepressant properties. On the other hand, the antidepressant properties of SSRIs, MAOIs or 5-HT_{1A} agonists are not linked to their acute pharmacological actions but rather to their ability to induce plastic changes in the neurons mediating 5-HT synaptic transmission[15]. Given then that tianeptine produces an acute increase in uptake, it was of interest to determine whether long-term treatment with tianeptine could induce changes of the 5-HT carrier that would lead to an altered 5-HT transmission. However, since the efficacy of 5-HT transmission may be modified by different mechanisms, we also assessed whether sustained tianeptine administration could increase 5-HT function either by altering the firing activity of 5-HT neurons, by desensitizing presynaptic inhibitory 5-HT autoreceptors, or by increasing the responsiveness of postsynaptic neurons to 5-HT.

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ORIGINAL ARTICLE

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Effect of acute and prolonged tianeptine administration on the 5-HT transporter: electrophysiological, biochemical and radioligand binding studies in the rat brain

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Abstract In the present study, in vivo extracellular unitary recordings, in vitro [^{3}H]5-HT uptake and [^{3}H]cyanoimipramine binding assays were used to assess the effect of acute and prolonged administration of the putative antidepressant tianeptine, on the 5-hydroxytryptamine (5-HT) transporter. Microiontophoretic application of tianeptine onto dorsal hippocampus CA_3 pyramidal neurons, as well as its intravenous administration (2 mg/kg), increased their firing frequency. Following intracerebroventricular administration of 5,7-dihydroxytryptamine, the activation induced by the microiontophoretic application of tianeptine remained unchanged, thus suggesting that the 5-HT carrier is not involved in this effect. Furthermore, in spite of its activating effect on CA_3 pyramidal neuron firing frequency, the intravenous administration of tianeptine did not alter the time of recovery of these neurons from microiontophoretic applications of 5-HT, an index of 5-HT uptake activity. In keeping with this observation, the acute administration of tianeptine did not change the effectiveness of the 5-HT reuptake blocker paroxetine (1 mg/kg, i.v.) in prolonging the suppressant effect of microiontophoretically-applied 5-HT. However, in rats that had received tianeptine for 14 days (20 mg/kg/day, s.c.), the recovery time from the suppressant effect of microiontophoretic applications of 5-HT was reduced by 40% and the effectiveness of paroxetine (1 mg/kg, i.v.) was decreased. These effects were no longer observed following a 48 h washout period. In a second series of experiments, the ability of tianeptine to interfere with the uptake blocking capacity of paroxetine was assessed in vitro, using hippocampal slices obtained from rats that had been treated with tianeptine for 14 days (20 mg/kg/day, s.c.; by minipump). The effectiveness of paroxetine to block [^{3}H]5-HT uptake was unchanged in slices obtained from rats still bearing the osmotic minipump at the time of the sacrifice, as well as from those which had undergone a 48 h washout period. To assess whether prolonged administration of tianeptine would induce adaptive changes on 5-HT uptake sites, [^{3}H]cyanoimipramine-binding parameters were measured following a 48 h washout period. Affinity values remained unchanged while density values were significantly increased in cortex (+22%) but not in hippocampus (+12%). It is concluded that, i) the activation of CA_3 pyramidal neurons observed following acute tianeptine administration cannot be attributed to its 5-HT uptake enhancing properties and ii) the prolonged administration of tianeptine induces adaptive changes on cortical but not on hippocampal 5-HT transporters.

Key words 5-HT uptake · 5-HT Transporter
[^{3}H]Cyanoimipramine binding · Up-regulation
Hippocampus

Introduction

Tianeptine is a new tricyclic agent, used in France for neurotic and reactional depressive conditions, and for anxio-depressive state with somatic complaints. In contrast with most tricyclic antidepressant drugs which have been shown to inhibit neuronal reuptake of monoamines, tianeptine has been reported to increase the reuptake of 5-HT (Mennini et al. 1987; Fattaccini et al. 1990). Using extracellular unitary recordings, Dresse and Scuvée-Moreau (1988) have shown that the acute administration of tianeptine increases the firing activity of CA_1 pyramidal neurons and that it reduces the recovery time of the firing activity of the latter neurons following microiontophoretic applications of 5-HT, but also of GABA. Since the effect of tianeptine on 5-HT-induced suppression of firing was opposite to that of the 5-HT reuptake blocker chlorimipramine, these authors suggested that the reduction in recovery time constituted electrophysiological evi-

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dence of the 5-HT reuptake enhancing capacity of tianeptine. In the present study, we assessed the role of the 5-HT transporter on the activating effect of acute tianeptine administration on the firing activity of pyramidal neurons of the rat hippocampus.

Substantial evidence indicates that enhancement of 5-HT neurotransmission is an effect shared by several antidepressant treatments (Blier and de Montigny 1994), therefore, the 5-HT reuptake enhancing capacity of tianeptine appears inconsistent with its purported antidepressant activity. However, an overall increase in the efficacy of 5-HT synaptic transmission does not depend on the acute pharmacological effects of antidepressant drugs. Rather, this enhancement occurs only after 5-HT neurons have undergone adaptive changes, e.g.: following long-term administration of selective 5-HT reuptake inhibitors (SSRIs) or monoamine oxidase inhibitors (MAOIs; Blier and de Montigny 1994). It would then be possible that adaptive changes induced by sustained tianeptine administration, rather than its acute 5-HT reuptake enhancing properties, might account for an increase in 5-HT function. Prolonged administration of SSRIs has been shown to decrease not only mRNA encoding the carrier protein (Lesch et al. 1993), but also the number of 5-HT transporters (Piñeyro et al. 1994). Since this reduction in the efficacy of 5-HT reuptake is consistent with enhanced 5-HT neurotransmission, we thus investigated whether prolonged tianeptine administration could induce adaptative changes of the 5-HT carrier.

Materials and methods

Treatments. Male Sprague Dawley rats (Charles River, St. Constant, Quebec, Canada) weighing 200–250 g were implanted subcutaneously with an osmotic minipump (Alza, Palo Alto, CA, USA) which delivered 20 mg/kg/day of tianeptine (Institut de Recherches Internationales, Servier, France) for 14 days. This dose has been frequently used in previous work on the effect of tianeptine on the 5-HT system (Mennini et al. 1987; Bertorelli et al. 1992; Ortiz et al. 1991; Mennini and Garattini 1991; Whitton et al. 1991; Datla and Curzon 1993). Experiments were performed with the minipump in place and after a 48 h washout period. Control rats were implanted with a pump containing saline. In experiments in which the acute effect of tianeptine was tested, a dose of 2 mg/kg (i.v.) was used. This dose is within the range of that used by Dresse and Scuvee-Moreau (1988) to increase the firing activity of hippocampal pyramidal neurons (0.2 mg/kg/min infused for 8–13 min).

A second group of rats was pretreated with desipramine (25 mg/kg, i.p.) to protect noradrenergic neurons from the neurotoxic action of 5,7-dihydroxytryptamine (5,7-DHT; Bjorklund et al. 1975) and 30 min thereafter, 200 µg (free base) of 5,7-DHT in 20 µl of 0.9% NaCl containing 0.1% ascorbic acid were injected into a lateral ventricle. This dose has been previously shown in our laboratory to reduce 5-HT content in CA_3 region by 90% (Chaput et al. 1990) and to completely abolish the inhibitory effect of paroxetine on 5-HT reuptake (Piñeyro et al. 1994). Control rats were injected with the vehicle.

All electrophysiological experiments were performed under chloral hydrate anesthesia (400 mg/kg, i.p.). To assess the effect of acute intravenous injection of tianeptine or paroxetine, naive rats were given the drug in a lateral vein of the tail.

Electrophysiological experiments. Five-barrelled glass micropipettes were pulled in a conventional manner and their tips broken to 10 µm under microscopic control. The central barrel was filled with 2 M NaCl solution and used for extracellular recording. Three of the side barrels contained one of the following solutions: 5-HT creatinine sulphate (5 mM in 200 mM NaCl, pH 4; Sigma, St. Louis, MO, USA); tianeptine (50 mM in 50 mM NaCl, pH 8; Servier, France); quisqualate (15 mM in 400 mM NaCl, pH 8; Sigma) or acetylcholine (ACh; 20 mM in 200 mM NaCl, pH 4; Sigma). The fourth side barrel, containing a 2 M NaCl solution was used for automatic current balancing. Ejection periods were kept constant at 50 s.

The microelectrodes were lowered 4 mm lateral and 4 mm anterior to lambda into the CA_1 region of the dorsal hippocampus (3.5–4.5 mm ventral). Pyramidal neurons were identified by their high amplitude (0.5–1.2 mV) and long duration (0.6–1.2 ms) action potentials (Kandel and Spencer 1961). Since most hippocampal pyramidal neurons are not spontaneously active under chloral hydrate anesthesia, a leak or small current of ACh or quisqualate (0–6 nA) was used to activate them within their physiological firing frequency (Ranck 1975). Quisqualate was used for experiments in which tianeptine was microiontophoretically applied and ACh was used throughout the rest of study.

The in vivo activity of the 5-HT reuptake process in the rat hippocampus was assessed using the RT_{50} method. RT_{50} is defined as the time in seconds required by a neuron to recover 50% of its initial firing frequency from the termination of microiontophoretic application of drugs. Since RT_{50} values from applications of highly concentrated solutions of 5-HT have been shown to be prolonged 3- to 4 fold by paroxetine administration or destruction of 5-HT terminals, it can be considered to provide a reliable index of the in vivo activity of the 5-HT carrier (see Piñeyro et al. 1994). For tianeptine, the response to its microiontophoretic application was measured by quantifying the number of spikes generated from the beginning of the application until complete recovery to the pre-injection baseline value. The effect of its intravenous administration on pyramidal neuron firing frequency was assessed by comparing the mean firing frequency 4 min before and 4 min after the administration of tianeptine.

Determination of in vitro [^{3}H]5-HT uptake. In vitro [^{3}H]5-HT uptake was determined in hippocampal slices obtained from rats with the minipump still delivering tianeptine and after a 48 h washout period, by using the method described by Langer et al. (1980). Briefly, animals were decapitated, their brains rapidly removed and dissected on an ice cold plate. Slices from hippocampus of 0.4 mm thickness were prepared using a McIlwain chopper. They were incubated for 3 min at 37 °C in a Krebs solution with various concentrations (0–1000 nM) of paroxetine, and bubbled with a mixture of 95% O_2 and 5% CO_2. The composition of the Krebs solution was 118 mM NaCl, 4.7 mM $CaCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 0.004 mM Na_2EDTA and 0.11 mM ascorbic acid. After the incubation period, [^{3}H]5-HT (specific activity 22.7 Ci/mmol; NEN Research Products, Mississauga, Ontario, Canada) was added to a final concentration of 100 nM. Following a 3-min incubation period, [^{3}H]5-HT uptake was terminated by transferring the slices to 5 ml of ice cold buffer and were then solubilized in 0.5 ml of Soluene 350 (Packard Instruments, Downers Grove, IL, USA). Radioactivity in the slices and in the incubation medium was determined by liquid scintillation spectroscopy. Parallel experiments were carried out at 0 °C as control for passive diffusion. All experiments were performed in duplicate and the amount of tritium actively captured by the tissue (C_A) was calculated according to the formula: $C_A = C_T - C_P$, where C_T and C_P are the tissue/medium ratios of [^{3}H]5-HT at 37 °C (total) and 0 °C (passive), respectively. Inhibition of uptake was calculated by means of the formula: % of inhibition $= [1 - C_{AC}/C_{APc}] \times 100$, where C_{APc} and C_{AC} are the amounts of actively captured [^{3}H]5-HT in a medium with or without paroxetine, respectively.

[^{3}H]Cyanoimipramine binding assays. [^{3}H]Cyanoimipramine binding to cortical and hippocampal membranes was determined by

modification of a previously described protocol (Dumbrille-Ross and Tang 1983). In brief, 48 h after the removal of the osmotic minipump, rats were decapitated, the brains removed, the cortex and hippocampus immediately frozen and kept at −70 °C until the binding assays were performed. Membranes were prepared by homogenizing brain tissue in 50 volumes of ice cold buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl; pH 7.4) and centrifugation at 48,000 g for 10 min at 4 °C. The resulting pellet was resuspended in 50 volumes of buffer and centrifuged again. This procedure was repeated once more and the final pellet was suspended to a tissue concentration of 800 µg/ml in the binding assay. The homogenates were incubated with [^{3}H]cyanoimipramine (0.1 – 4 nM; 85 Ci/ml, American Radiolabelled Chemicals, St. Louis, MO, USA) at 24 °C in a final volume of 280 µl for 90 min. Incubations were terminated by addition of 4 ml ice cold buffer and filtration through Whatman GF B filters using a 24-channel cell harvester. Filters were then washed with four 4-ml rinses of ice cold buffer. Radioactivity trapped by the filters was determined by liquid scintillation spectroscopy (Beckman Counter LS 6000 SE, Beckman Instruments, Fullerton, CA, USA). Non-specific binding was estimated in the presence of 10 µM fluoxetine. Binding was analyzed using the curve fitting programme LIGAND (G. A. McPherson, Elsevier Biosoft, Cambridge, UK).

Statistical analysis. Results are expressed as means ± SEM. When two means were compared, statistical significance of their difference was assessed using the two-tailed paired or non-paired Student's *t*-tests as indicated. The level of statistical significance was fixed at $p < 0.05$.

Results

Effect of acute tianeptine administration on dorsal hippocampus CA_3 pyramidal neuron firing frequency

Direct microiontophoretic application of tianeptine onto dorsal hippocampus CA_3 pyramidal neurons increased their firing rate (Fig. 1A). The number of spikes generated was proportional to the microiontophoretic current used (Fig. 2), with high currents producing not only greater maximal activation but also a longer lasting effect (Fig. 1A). In order to determine whether this activation was due to an interaction of tianeptine with the 5-HT transporter, located on 5-HT terminals (de Souza and Kuyatt 1987; Hrdina et al. 1990; Dewar et al. 1991), the drug was applied onto CA_3 pyramidal neurons in 5,7-DHT-lesioned rats. The activation observed was similar to that produced in control rats (Figs. 1B and 2). In naive rats, within 4 min of its intravenous administration, tianeptine (2 mg/kg) increased CA_3 pyramidal neuron firing frequency by 35% (Fig. 3A and B).

Interestingly, in rats that had received tianeptine for 14 days (20 mg/kg/day, s.c.) and still carried the osmotic minipump at the time of the experiment, the firing frequency of hippocampal pyramidal neurons was not different from that of controls. The observed frequency values were 8 ± 1 Hz (n = 170 neurons) and 9 ± 1 Hz (n = 170 neurons) for control and tianeptine-treated rats respectively. The ACh currents used for neuronal activation were similar (0 – 6 nA) in both groups.

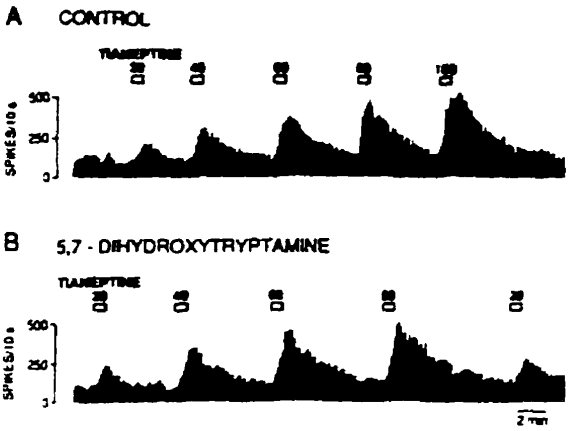

Fig. 1 Integrated firing rate histograms of dorsal hippocampus pyramidal neurons recorded in an intact rat (A) and in a rat pretreated with 5,7-dihydroxytryptamine (B), showing their responsiveness to microiontophoretic applications of tianeptine. The bars indicate the duration of microiontophoretic applications for which the currents are given in nA. Time scale applies to both traces

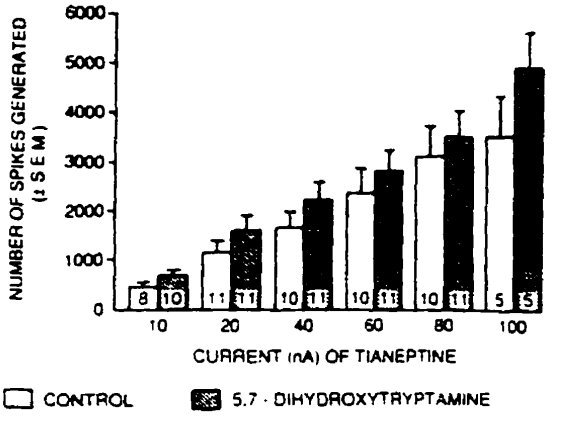

Fig. 2 Effect of tianeptine application onto CA_3 pyramidal neurons with different microiontophoretic currents in control and 5,7-dihydroxytryptamine treated rats. The number of neurons tested is given at the bottom of each column

Effect of tianeptine administration on 5-HT uptake in vivo

Acute administration of tianeptine

Acute administration of tianeptine (2 mg/kg, i.v.) did not alter the recovery time (RT_{50} values) from microiontophoretic applications of 5-HT (Fig. 3C) nor did it interfere with the effectiveness of the 5-HT reuptake blocker paroxetine to prolong recovery (RT_{50} values) from microiontophoretic applications of 5-HT. In control rats, the intravenous administration of paroxetine dose-dependently prolonged RT_{50} values. The administration of tianeptine (2 mg/kg, i.v.) 15 min prior to paroxetine did

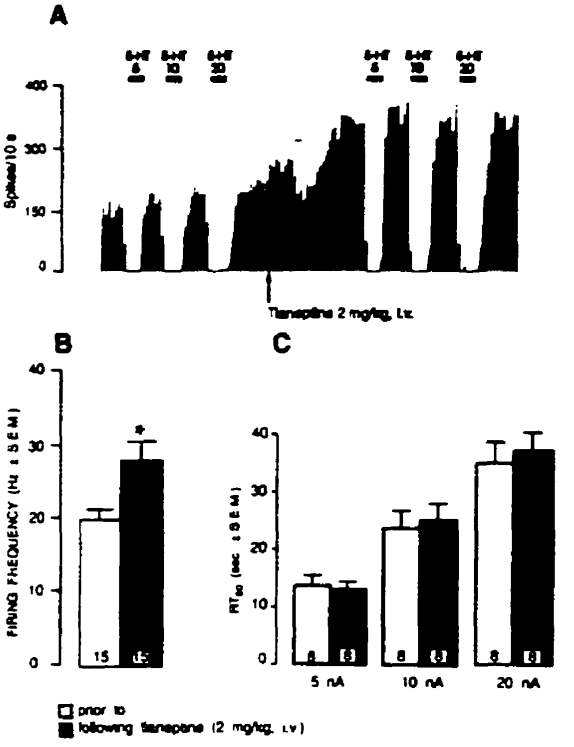

Fig. 3 A–C Integrated firing rate histogram showing the response of a dorsal hippocampus CA_1 pyramidal neuron to increasing microiontophoretic currents of 5-HT, and the effect of intravenous injections of tianeptine on the firing frequency and the response to microiontophoretic applications of 5-HT (**A**). Effect of intravenous tianeptine on pyramidal neuron firing frequency (**B**). Recovery time, expressed as RT_{50} values (mean ± SEM), of dorsal hippocampus pyramidal neurons from microiontophoretically applied 5-HT prior to and following the intravenous administration of tianeptine (**C**). The number of neurons tested is given at the bottom of each column. $^*p < 0.01$ using two-tailed paired Student's *t*-test

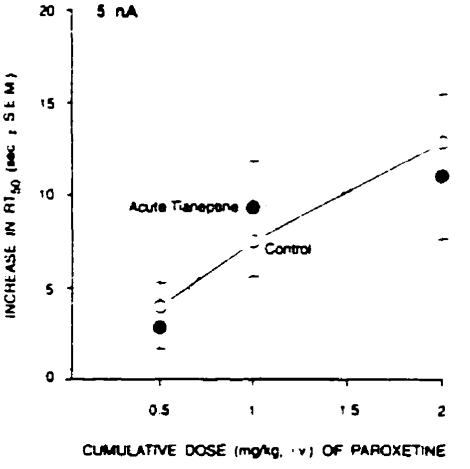

Fig. 4 Effect of successive injections of paroxetine on the recovery time from 5 nA microiontophoretic applications of 5-HT in 8 control rats (○) and in 8 rats treated with tianeptine (2 mg/kg, i.v.) (●)

not prevent the prolonging effect of the latter drug (Fig. 4). The intravenous administration of a cumulative dose of 2 mg/kg of paroxetine prolonged RT_{50} values from 5-HT applications of 5, 10 and 20 nA by 13 ± 3 s, 16 ± 3 s and 24 ± 4 s, respectively, in control rats ($n = 8$) and by 11 ± 3 s, 19 ± 3 s and 29 ± 8 s in rats ($n = 8$) that had received tianeptine 15 min beforehand. The administration of tianeptine (2 mg/kg, i.v.) 15 min after paroxetine (1 mg/kg, i.v.) did not reverse the prolonging effect of this SSRI on RT_{50} values. In six rats which had received 1 mg/kg paroxetine, the respective RT_{50} values for microiontophoretic currents of 5, 10 and 20 nA of 5-HT were 22 ± 3 s, 34 ± 4 s and 46 ± 4 s prior to and 22 ± 2 s, 34 ± 4 s and 43 ± 8 s following tianeptine administration (2 mg/kg, i.v.).

Prolonged administration of tianeptine

The recovery time (RT_{50} values) from 5-HT applications onto dorsal hippocampus pyramidal neurons was assessed in control and tianeptine-treated rats (20 mg/kg/day, s.c. × 14 days). RT_{50} values were reduced by 40% in rats that had received prolonged tianeptine treatment and were still carrying the minipump at the time of the experiment (Fig. 5). The effect of prolonged tianeptine administration was not observed when minipumps were removed 48 h prior to the experiment.

The effect of intravenous paroxetine was also assessed in rats that had received tianeptine (20 mg/kg/day, s.c.) for 14 days (Fig. 6). Following sustained tianeptine administration, the prolonging effect of 1 mg/kg of paroxetine on RT_{50} values was reduced (Figs. 6, 7). For microiontophoretic currents of 5 and 10 nA of 5-HT, the prolonging effect of 1 mg/kg of paroxetine on RT_{50} values was reduced by 64% and 73%, respectively. For the 20 nA current, the reduction was of 21% and it did not reach statistical significance. It was surprising to find that, in these same rats, the effect of 0.5 mg/kg of paroxetine on RT_{50} values remained unchanged.

To verify whether the reduced effectiveness of paroxetine was due to the presence of tianeptine and/or its metabolite(s), or to adaptive changes of the 5-HT transporter, the effect of paroxetine was assessed in tianeptine-treated rats (20 mg/kg/day, s.c. × 14 days) which had undergone a 48 h washout period to ensure complete drug elimination. After such a washout period, RT_{50} values and the effectiveness of paroxetine in prolonging the suppressant effect of 5-HT no longer differed from controls (data not shown).

Effect of prolonged tianeptine administration on in vitro [^{3}H]5-HT uptake

The following series of experiments was performed to verify ex vivo the enhancing effect of tianeptine on 5-HT reuptake activity. In experiments in which paroxetine was omitted from the incubation medium the total amount of

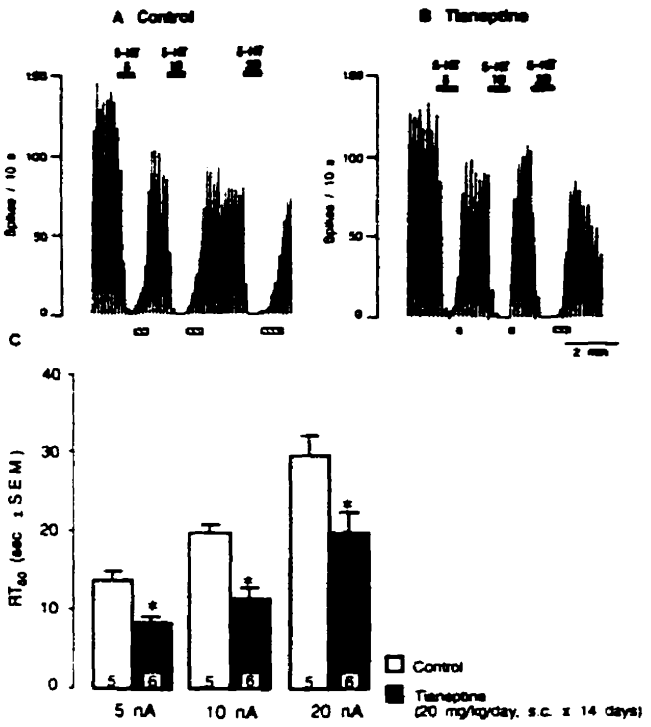

Fig. 5 Integrated firing rate histograms showing the response of dorsal hippocampus CA_3 pyramidal neurons to microiontophoretic applications of 5-HT in a control rat (**A**) and a rat treated with tianeptine (**B**). The *hatched bars* below the histograms indicate RT_{50}. Time scale applies to both traces. Decrease in RT_{50} values (mean ± SEM), of dorsal hippocampus pyramidal neurons from microiontophoretic applications of 5-HT, in control rats and in rats that received tianeptine for 14 days (**C**). The number of rats is given at the bottom of each column and in each rat RT_{50} was assessed in 4 different neurons. $^*p < 0.02$ using two-tailed non-paired Student's *t*-test

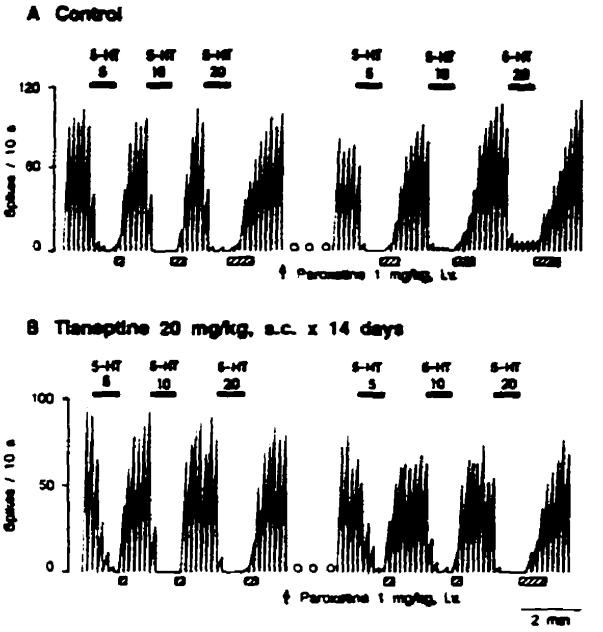

Fig. 6 Integrated firing rate histograms showing the response of dorsal hippocampus CA_3 pyramidal neurons to microiontophoretic applications of 5-HT in a control rat (**A**) and a rat treated with tianeptine (**B**), prior to and following the intravenous injection of paroxetine. Time scale applies to both traces. The hatched bars below the histograms indicate RT_{50}

radioactivity taken up by the tissue was similar in control rats and in rats treated with tianeptine (20 mg/kg/day, s.c.) for 14 days, whether the osmotic minipump was still in place at the time of the sacrifice or had been removed 48 h earlier (Fig. 8A). Inhibition of uptake caused by different concentrations of paroxetine in the incubation medium remained unchanged following prolonged tianeptine administration (Fig. 8B).

[^{3}H]Cyanoimipramine binding assays

To assess whether prolonged tianeptine administration induced adaptive changes of 5-HT uptake sites, [^{3}H]cyanoimipramine binding parameters were determined in cortical and hippocampal membranes. Assays were performed 48 h after removal of the osmotic minipump to ensure

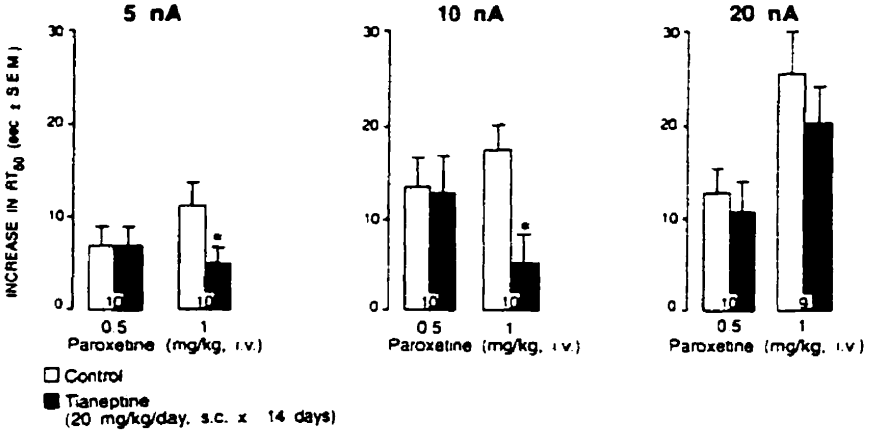

Fig. 7 Absolute increase in RT_{50} values of dorsal hippocampus pyramidal neurons from microiontophoretically-applied 5-HT produced by the intravenous injection of paroxetine in control rats and in rats treated with tianeptine for 14 days. The number of neurons tested is given at the bottom of each column. $^*p < 0.001$ compared to control values, using the two-tailed non-paired Student's *t*-test

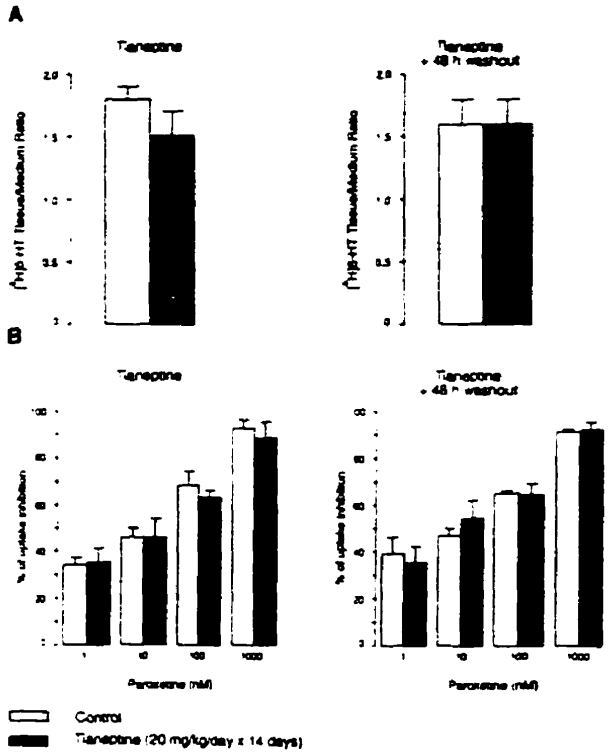

Fig. 8 [^{3}H]5-HT reuptake activity (**A**) and effectiveness of paroxetine to inhibit [^{3}H]5-HT uptake in hippocampal slices (**B**) from control (□) and tianeptine treated rats (■), $n = 4$ in each group

Table 1 Binding parameters of [^{3}H]cyanoimipramine in controls and long-term tianeptine-treated rats

	K_D (nM)		B_{max} (fmol mg of protein)	
	Control	Tianeptine[a]	Control	Tianeptine[a]
Hippocampus	0.5 ± 0.1	0.4 ± 0.1	322 ± 31	362 ± 24
Cortex	0.4 ± 0.1	0.4 ± 0.1	336 ± 27	410 ± 15*

[a] 20 mg kg day, s.c. × 14 days − 48 h washout ($n = 6$ rats)
* $p < 0.03$ using non-paired Student's t-test

that, if changes were observed, these would be due to the adaptative properties of the 5-HT carrier and not to the presence of residual drug. As shown in Table 1, K_D values were not altered by prolonged tianeptine administration. In frontal cortex, the total number of binding sites was significantly increased (22%) whereas, in the hippocampus, this increase was smaller (12%) and not statistically significant ($p = 0.32$).

Discussion

The results obtained in the present study indicate that: i) tianeptine has an activating effect on CA_3 pyramidal neurons; ii) its prolonged administration enhances 5-HT reuptake and interferes with the action of the SSRI paroxetine; iii) its prolonged administration increases the density of [^{3}H]cyanoimipramine binding sites in cortical, but not hippocampal, membranes.

Immediately following the microiontophoretic (Fig. 1) or intravenous (Fig. 3) administration of tianeptine, the firing activity of CA_3 pyramidal neurons was increased. This immediate response and the effectiveness of local microiontophoretic application of tianeptine suggest that activation is most probably due to a direct effect of the drug itself rather than to its metabolites. Indeed, since the latter are generated by cytochrome P-450 (Letteron et al. 1989) and since drugs administered via a tail vein bypass portal circulation, it makes it even more likely that the effect produced by intravenous administration of tianeptine was due to the drug and not to its metabolites. It is interesting to note that in vitro tianeptine did not directly enhance [^{3}H]5-HT uptake in brain slices (Kato and Weitsch 1988) or synaptosomes (Mennini et al. 1987). If tianeptine itself does not stimulate reuptake, it is thus likely that the direct activation of CA_3 pyramidal neurons by tianeptine, observed in the present study, is not due to enhanced 5-HT reuptake activity. This possibility is further supported by the present observation that intracerebroventricular administration of 200 µg of 5,7-DHT, a dose which has been shown to completely abolish the effect of paroxetine on 5-HT reuptake parameters (Piñeyro et al. 1994), did not modify the effect of microiontophoretically-applied tianeptine on the firing activity of hippocampus pyramidal neurons. Moreover, RT_{50} values, which constitute a reliable index of the in vivo activity of the 5-HT reuptake process (see Materials and methods), were not affected by the intravenous administration of tianeptine (Fig. 3C). The latter results differ from those of Dresse and Scuvée-Moreau (1988) who have found that intravenous infusion of tianeptine reduced the recovery time from 5-HT applications. These authors also reported a reduction in the recovery time from GABA applications for the same neurons. It appears then, that the accelerated recovery rate might be non-specific and secondary to the activating effect of acute tianeptine administration. The mechanism underlying CA_3 pyramidal neuron activation thus remains elusive. Since tianeptine has no affinity for monoamine or excitatory amino acid receptors (Mennini and Garattini 1991), direct activation of these receptors does not represent a likely possibility. Alternatively, tianeptine could induce the release or interfere with the metabolism of neurotransmitters that stimulate hippocampal pyramidal neuron firing activity, namely ACh or excitatory amino acids. An increased release of ACh seems an improbable explanation since Bertorelli et al. (1992) have shown that tianeptine reduces hippocampal release of this neurotransmitter. On the other hand, though the possibility that tianeptine might interfere with mechanisms eliminating ACh or quisqualate may not be excluded, it would be surprising that it affected both processes since, in the present experimental series, quisqualate was used to activate CA_3 pyramidal neurons

when tianeptine was applied microiontophoretically, while ACh was used for assessing the effect of intravenous tianeptine.

The effect of acute tianeptine administration on neuronal firing frequency has also been studied on medial septal neurons (Bassant et al. 1991). At variance with the present results and those of Dresse and Scuvée-Moreau (1988), both obtained in the dorsal hippocampus, the intravenous injection of tianeptine (0.2 – 1 mg/kg) had an inhibitory effect in the septum. These opposite effects might be due to different mechanisms being involved in the effect of tianeptine in different regions. Indeed, unlike hippocampal pyramidal neuron activation, which involves local factors, the inhibition of medial septum neurons is probably indirect since microiontophoretic application of tianeptine did not inhibit their spontaneous firing activity (Bassant et al. 1991).

In keeping with its lack of effect on the rate of recovery of firing from 5-HT applications (Figs. 3A, C), the acute intravenous administration of tianeptine did not modify the effect of paroxetine on RT_{50} values (Fig. 4). The lack of efficacy of tianeptine to reverse the effect of paroxetine observed in the present study is in agreement with a previous report by de Simoni et al. (1992). Indeed, in the latter study it was shown that the administration of tianeptine, 15 min after the SSRI sertraline, did not reverse the reduction induced by the latter drug in the voltammetric indoleamine peak recorded in hippocampus. Furthermore, using microdialysis, Whitton et al. (1991) have also shown that a single dose of tianeptine (10 mg/kg, i.p.) had no effect on extracellular 5-HT concentrations, when the SSRI citalopram was infused through the dialysis probe into the dorsal hippocampus of the rat.

Unlike its acute administration, prolonged treatment with tianeptine (20 mg/kg/day, s.c. × 14 days) produced a reduction in the recovery time (RT_{50}) from microiontophoretic applications of 5-HT (Fig. 5) and in the efficacy of paroxetine to prolong this parameter (Figs. 6, 7). Both these effects are consistent with an enhanced activity of the 5-HT carrier, but the mechanism whereby tianeptine produces this enhancement remains elusive. A tentative explanation could be an increase in the total number of 5-HT transporters as suggested by the observed increase in B_{max} for [^{3}H]cyanoimipramine binding sites in cortical membranes. However, the increase in B_{max} in hippocampus was small and non significant (Table 1) and the enhancing effect of tianeptine on the reuptake process vanished after a 48 h washout period. These two observations suggest that even if adaptative changes of the 5-HT transporter could account for the effects observed while the minipump was still in place, they do not seem to be long-lasting enough to be detectable following a 48 h washout period. Tianeptine has a short half-life (2.5 h) and 80% of its metabolites are eliminated in the first 24 h (Royer et al. 1988). Since tianeptine effects on 5-HT uptake disappeared after a 48 h washout period, it can be concluded that tianeptine or its metabolites must be present to enhance in vivo 5-HT uptake activity in rat hippocampus. In contrast, prolonged intraperitoneal tianeptine administration (10 mg/kg twice daily × 15 days) has been shown to enhance 5-HT uptake ex vivo, following a 72 h washout period (Mennini et al. 1987).

The observation that tianeptine reduced the effect of paroxetine to a greater extent for currents of 5 and 10 nA than for those of 20, could be explained by the fact that the relative contribution of diffusion from the recording site to the recovery of firing frequency, is more important when the amount of microiontophoretically-applied 5-HT is greater. It is then reasonable to presume that tianeptine, by enhancing 5-HT reuptake, will exert a smaller effect on higher ejection currents. In keeping with this interpretation, the prolonging effect of paroxetine on RT_{50} values of naive rats, was smaller for the 20 nA than for the 5 nA current (relative increases as compared to preinjection RT_{50} values were 64% and 101%, respectively). Tianeptine antagonized the effect of paroxetine in a non competitive way since it reduced the effectiveness of the dose of 1 mg/kg but not that of the 0.5 mg/kg. This interpretation is consistent with the fact that tianeptine does not bind to the 5-HT transporter (Mennini and Garattini 1991).

Ex vivo [^{3}H]5-HT uptake and the effectiveness of paroxetine to inhibit the latter were unaffected by prolonged tianeptine administration (Fig. 7). Since this same experimental procedure has been proven adequate to detect changes in reuptake activity (Blier et al. 1993; Piñeyro et al. 1994), it is then possible that the lack of effect observed in the present study could be due to the variability of the response to sustained tianeptine administration. As stated by Mennini et al. (1987), enhancement of 5-HT uptake by tianeptine "is not always reproducible".

[^{3}H]Cyanoimipramine binds with high affinity and specificity to the 5-HT transporter (Kovachich et al. 1988). Thus, the observed increase in cortical B_{max} values following sustained tianeptine administration indicates an increase in the number of 5-HT transporters in this region (Table 1). The increase in B_{max}, in the presence of unaltered K_D values, is consistent with the observation that, in cortical synaptosomes, tianeptine enhanced [^{3}H]5-HT uptake by increasing V_{max} without affecting K_m (Mennini et al. 1987). Changes in cortical B_{max} were observed after a 48 h washout period and therefore strongly suggest that tianeptine may induce adaptive changes in the 5-HT transporter. However, there seem to be regional differences in this adaptive response. In the hippocampus the increase observed in B_{max} was much smaller (+ 12%) and non significant.

The binding results obtained in the present study differ from those obtained by Mennini and Garattini (1991) who, using a similar dose of tianeptine administered for a similar time period, reported no change in [^{3}H]imipramine binding parameters. This could be due to the fact that, whereas [^{3}H]cyanoimipramine selectively binds to the 5-HT transporter (Kovachich et al. 1988), [^{3}H]imipramine binds to heterogeneous sites (Reith et al. 1983; Marcusson et al. 1985). However, results obtained with the more selective ligand [^{3}H]paroxetine have also been inconsistent. Watanabe et al. (1993) observed a significant

decrease in [^{3}H]paroxetine binding in hippocampus and cortex, when tianeptine was administered for 14 days at a total dose of 30 mg/kg/day. It is noteworthy that this same group did not reproduce such an effect when tianeptine was given at a dose of 10 mg/kg/day for 21 days (Frankfurt et al. 1993). It is thus possible that the different doses used in the present and the latter studies might account for divergent observations.

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ORIGINAL ARTICLE

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Effect of prolonged administration of tianeptine on 5-HT neurotransmission: an electrophysiological study in the rat hippocampus and dorsal raphe

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Abstract Extracellular unitary recordings of dorsal hippocampus CA_3 pyramidal neurons and of dorsal raphe 5-hydroxytryptamine (5-HT) neurons were used to assess the effect of tianeptine, a putative antidepressant, on the efficacy of 5-HT neurotransmission. Sustained tianeptine administration (20 mg/kg/day, s.c. × 14 days) did not modify the firing activity of 5-HT neurons in the dorsal raphe. Their responsiveness to the intravenous injection of LSD, an agonist of the somatodendritic 5-HT autoreceptor, and of 8-OH-DPAT, a selective 5-HT_{1A} agonist, was also unaffected by this treatment. The responsiveness of CA_3 pyramidal neurons to microiontophoretic application of 5-HT remained unchanged after sustained tianeptine administration, but it was markedly enhanced in rats treated with repeated electroconvulsive shocks. Finally, the duration of suppression of firing activity of CA_3 pyramidal neurons produced by electrical stimulation of the ascending 5-HT pathway, delivered at 1 Hz and 5 Hz, was not modified in rats treated with tianeptine. Methiothepin, an antagonist of the terminal autoreceptor enhanced the effectiveness of 5-HT pathway stimulation to the same extent in control and tianeptine-treated rats. The present results indicate that, administered at a dose known to stimulate 5-HT reuptake (20 mg/kg/day, s.c.; by minipump), and for a period of time (14 days) for which other antidepressant treatments have been shown to enhance 5-HT function, tianeptine does not modify the efficacy of 5-HT synaptic transmission in the rat hippocampus.

Key words Antidepressant treatments
5-HT neurotransmission · 5-HT reuptake
5-HT transporter · Hippocampus · Dorsal raphe

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Introduction

Preclinical and clinical evidence suggest an involvement of the 5-HT system in the mechanism of action of different types of antidepressant treatments in major depression (Blier et al. 1990; Price et al. 1990; Blier and de Montigny 1994). In particular, electrophysiological investigations have documented an enhancement of 5-HT neurotransmission following various long-term antidepressant treatments. The latter can be categorized into two classes: those that exert their effect by increasing the sensitivity of postsynaptic neurons to 5-HT, and those that increase the efficacy of 5-HT neurons themselves. The sensitivity of postsynaptic neurons to microiontophoretically-applied 5-HT is enhanced by tricyclic antidepressant drugs and electroconvulsive shocks (de Montigny and Aghajanian 1978; de Montigny 1984; Chaput et al. 1991). Whereas 5-HT reuptake blockers would exert their effect by way of a desensitization of somatodendritic and terminal 5-HT autoreceptors (Blier et al. 1988) and also possibly 5-HT transporters (Piñeyro et al. 1994), monoamine oxidase inhibitors would achieve the same result via the enhanced availability of releasable 5-HT and also possibly by desensitizing α_2-adrenoceptors located on 5-HT terminals (Blier and Bouchard 1994; Mongeau et al. 1994).

Tianeptine is a new tricyclic agent, used in France for neurotic and reactional depressive conditions, and for anxiodepressive state with somatic complaints. It has been demonstrated that, unlike most other tricyclic antidepressant drugs, tianeptine increases the uptake of [^{3}H]5-HT in the cerebral cortex and hippocampus without affecting noradrenergic and dopaminergic uptake systems (Mennini et al. 1987; Fattaccini et al. 1990). It has also been shown that tianeptine increases [^{3}H]5-HT uptake in human platelets, following both acute and chronic administration (Kato and Weitsch 1988; Mocaër et al. 1988).

In a previous study, we have shown that, unlike long-term paroxetine administration which down-regulates the number of 5-HT carriers (Piñeyro et al. 1994), changes in-

duced by prolonged tianeptine treatment on the 5-HT carrier are not consistent with an enhancement of 5-HT neurotransmission (Piñeyro et al. 1995). However, as described above, the efficacy of 5-HT transmission can be modified by other mechanisms. In the present study, we therefore assessed whether sustained tianeptine administration could increase 5-HT function either by altering the firing activity of 5-HT neurons, by desensitizing presynaptic inhibitory autoreceptors, or by increasing the responsiveness of postsynaptic neurons to 5-HT.

Materials and methods

Treatments. Male Sprague Dawley rats (Charles River, St. Constant, Quebec, Canada) weighing between 200 – 250 g were implanted subcutaneously with an osmotic minipump (Alza, Palo Alto, CA, USA) which delivered 20 mg/kg/day of tianeptine (Servier, France) for 14 days. Experiments were performed with the minipump in place. Control rats were implanted with a minipump containing saline. A third group of rats were administered a series of electroconvulsive shocks (ECS) every other day for two weeks. These rats were lightly anesthesized with halothane, needles were inserted subcutaneously in the temporal region, and the ECS (150 V, 10 ms pulses at 50 Hz for 1 s) produced a seizure lasting between 15 and 30 s.

Recordings of 5-HT neurons of the dorsal raphe nucleus. Electrophysiological experiments were carried out under chloral hydrate anesthesia (400 mg/kg, i.p.). Supplemental doses (100 mg/kg, i.p.) were given to prevent any nociceptive reaction to pinching of the tail or of a hind paw. The rats were mounted in a stereotaxic apparatus and a burr hole was drilled on midline 1 mm anterior to lambda. Single-barrelled glass micropipettes were used, with the tips broken back to 1 – 3 µm and filled with 2 M NaCl solution. Dorsal raphe 5-HT neurons were encountered on a distance of 1 mm starting at the ventral border of the Sylvius aqueduct, and were identified using the criteria of Aghajanian (1978): slow (0.8 – 1.2 ms), regular firing rate (0.5 – 2.5 Hz) and positive action potential of long duration. Each neuron encountered was recorded for at least 50 s. Additional electrode descents were carried out 200 µm anterior, 200 µm posterior and 200 µm lateral to the first descent. At the end of the experiment, various doses of the 5-HT autoreceptor agonist LSD or the 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) were injected in a lateral vein of the tail to assess the responsiveness of 5-HT neurons to these drugs.

Recording pyramidal cells of the dorsal hippocampus. Five-barrelled glass micropipettes were pulled in a conventional manner and their tips were broken back to approximately 10 µm under microscopic control. The central barrel, used for recording, was filled with a 2 M NaCl solution. Three side barrels, used for microiontophoresis, were filled with the following solutions: 5-HT creatinine sulphate (0.5 mM or 5 mM in 200 mM NaCl, pH 4; Sigma Chemical, St. Louis, MO, USA), 8-OH-DPAT (0.5 mM in 100 mM NaCl, pH 4; Lilly, Indianapolis) or acetylcholine chloride (ACh; 20 mM in 200 mM NaCl, pH 4; Sigma). The fourth barrel was used for automatic current balancing, and contained a 2 M NaCl solution. CA$_1$ pyramidal neurons were recorded extracellularly at the depth of 3.5 to 3.8 mm from cortical surface and identified by their characteristic large-amplitude (0.5 – 1.2 mV) and long-duration (0.8 – 1.2 ms) single action potentials alternating with complex spike discharges. A small ejection current of ACh (0 – 6 nA) was used to activate silent or slowly discharging neurons to a firing rate of 8 – 12 Hz. The responsiveness of CA$_1$ pyramidal neurons to microiontophoretic application of 5-HT and 8-OH-DPAT was assessed by determining the number of spikes suppressed from the beginning of the ejection period (which always lasted 50 s), to a recovery of 85% of the pre-ejection baseline for each current tested. The same micropipette was always used to assess postsynaptic receptor sensitivity in pairs of electroconvulsive shock and tianeptine-treated rats.

The response of pyramidal neurons following the activation of the ascending 5-HT pathway was also studied. A concentric bipolar electrode (NE-100, David Kopf) was positioned on midline at a backward angle of 10° in the ventromedial tegmentum (P = 5.8, V = 8, according to the atlas of Paxinos and Watson 1986). Square pulses of 0.5 ms in duration were delivered at the frequency of 1 and 5 Hz with intensities of 100, 200, 300 µA. The pulses generated by a stimulator (S88, Grass) were delivered through an isolation unit (SIU 478A, Grass). The unitary activity was analyzed on-line with an IBM-PC computer equipped with a Tecmar interface. Peristimulus time histograms of the firing activity of dorsal hippocampus neurons were generated to assess the suppression of firing produced by the stimulations. The duration of the suppression of firing was quantified as the absolute silence value (SIL). This value was calculated by dividing the total number of events suppressed by the mean frequency of firing of the neuron recorded. It thus corresponds to a duration of suppression of firing normalized for the firing activity of the neuron tested. That the suppressive effect of the stimulations on the firing activity of hippocampus pyramidal neurons was due to the release of endogenous 5-HT, is supported by several lines of evidence: the effect of the stimulations is virtually abolished by 5,7-dihydroxytryptamine lesion of 5-HT neurons (Blier and de Montigny 1983); it is blocked by the postsynaptic 5-HT$_{1A}$ receptor antagonist BMY 7378 (Chaput and de Montigny 1988); it is enhanced by blocking and decreased by activating the terminal 5-HT autoreceptor (Chaput and de Montigny 1988).

Two approaches were used to evaluate the sensitivity of the terminal 5-HT autoreceptor. In a first series of experiments, the effect of two frequencies of stimulation (1 and 5 Hz), were determined while recording from the same neuron. This approach is based on the assumption that the higher the frequency of stimulation, the greater should be the degree of activation of the terminal autoreceptor at the time of arrival of the next stimulation-triggered action potential. Therefore, each pulse of the higher frequency of stimulation is expected to produce a smaller effect on postsynaptic neurons. Indeed, using in vivo electrophysiological techniques (Chaput et al. 1986) and in vitro evoked release of [^{3}H]5-HT in rat hypothalamic slices (Blier et al. 1989) stimulations delivered at high frequencies (5 Hz) have been shown to release smaller amounts of 5-HT than low (1 Hz) frequency stimulations. In a second series of experiments, the effect of stimulating the ascending 5-HT pathway while recording from the same postsynaptic neuron was determined prior to, and following, the intravenous injection of methiothepin (1 mg/kg), an antagonist of the terminal 5-HT autoreceptor. In control rats, this dose of methiothepin enhances the suppression of firing of pyramidal neurons without altering the responsiveness of postsynaptic neurons to microiontophoretic application of 5-HT (Chaput et al. 1986).

Plasma and brain concentrations of tianeptine. After completing electrophysiological experiments and before sacrifice to remove their brains, 0.5 – 1 ml of blood were obtained from the left ventricle of 10 tianeptine-treated rats (20 mg/kg/day, s.c. for 14 days) and immediately centrifuged. Brains and plasma were kept at – 70 °C and sent for analysis at the Institut de Recherches Internationales, Servier, Cedex, France. Determinations of the concentration of tianeptine and its main metabolite, resulting from a β-oxidation of its aminoheptanoic lateral chain, were done by HPLC according to the method described by Nicot et al. (1986), and expressed as nanograms per milliliter of plasma or nanograms per gram of brain tissue.

Statistical analysis. Results were expressed as means ± SEM. When two means were compared, statistical significance of their difference was assessed using the two-tailed paired or non-paired Student's t-test as indicated. For multiple comparisons, one-way ANOVA for independent samples was used.

Results

Effect of prolonged tianeptine treatment on the firing activity of dorsal raphe 5-HT neurons

Figure 1 presents sequential integrated firing rate histograms of 5-HT neurons recorded during a single electrode descent in the nucleus raphe dorsalis of a control rat and of a rat treated with tianeptine (20 mg/kg/day s.c. × 14 days). The mean firing rate of dorsal raphe 5-HT neurons, as well as the mean number of neurons spontaneously active per electrode descent, were similar in control and tianeptine-treated rats (Table 1). No statistically significant difference between the two groups was found for either the mean firing rate or for the mean number of spontaneously active 5-HT neurons per descent.

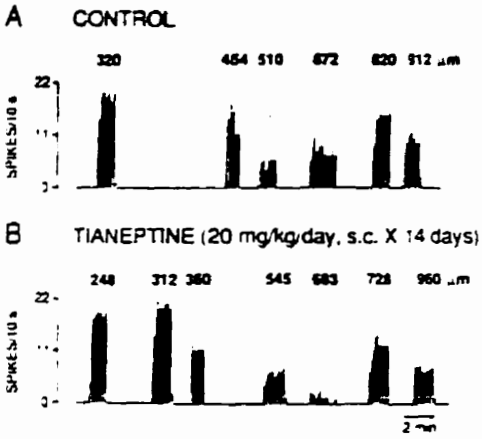

Fig. 1 Integrated firing rate histograms of 5-HT neurons recorded during a single electrode descent through the raphe dorsalis in a control rat (**A**) and in a rat treated with tianeptine (**B**). The depth of recording for each neuron is indicated in micrometers from the ventral border of the Sylvius aqueduct

Table 1 Firing activity of dorsal raphe 5-HT neurons[a]

	Firing rate (Hz)	Number of neurons/ electrode descent
Control	0.97 ± 0.05 (220)[b]	4.3 ± 0.4 (51)[c]
Tianeptine (20 mg/kg/day, s.c. × 14 days)	1.02 ± 0.06 (136)[b]	4.7 ± 0.7 (29)[c]

[a] Results expressed as mean ± SEM
[b] Number of neurons recorded
[c] Number of electrode descents performed

Effect of intravenous injection of 8-OH-DPAT and LSD on 5-HT neuron firing activity following prolonged tianeptine administration

In order to determine whether tianeptine modified the sensitivity of 5-HT_{1A} receptors controlling the firing ac-

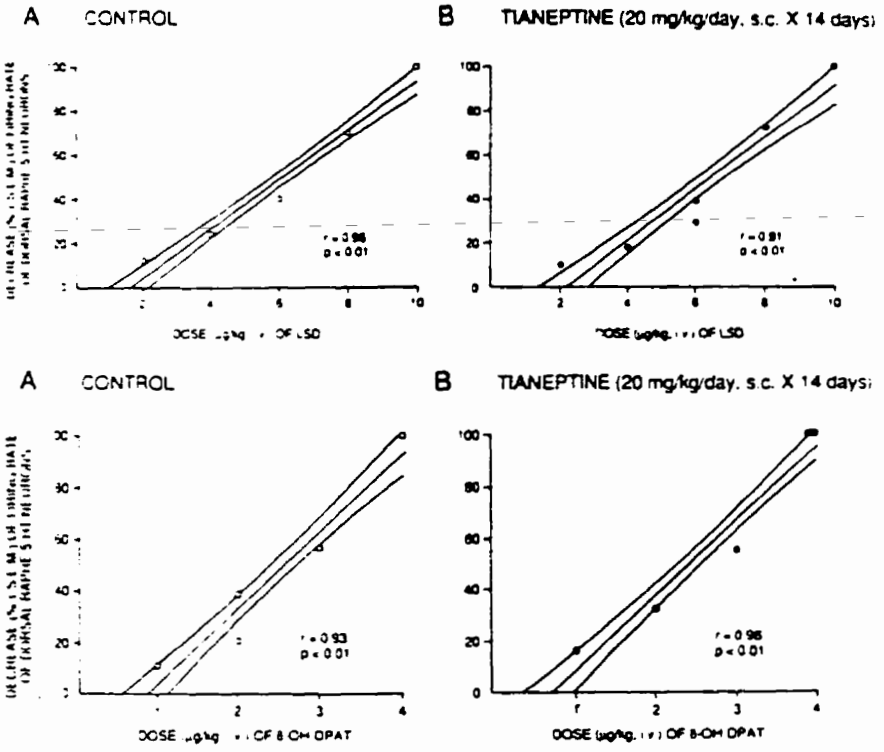

Fig. 2 Relationship between the degree of suppression of spontaneous firing activity of dorsal raphe 5-HT neurons and the dose of 8-OH-DPAT or LSD injected in control rats (**A**) and in tianeptine-treated rats (**B**). Each dot represents the response of one neuron to one dose of 8-OH-DPAT or LSD in one rat. The curved lines depict the standard error of the regression lines. There was no statistically significant difference between the ED_{50} doses obtained in control and tianeptine-treated rats, using the 95% confidence limit method

tivity of 5-HT neurons, the effects of intravenous 8-OH-DPAT and LSD on the firing activity of 5-HT neurons in the dorsal raphe nucleus were examined. In control rats, ED_{50} values for reducing the spontaneous firing activity of 5-HT neurons were 2.5 ± 0.3 μg/kg and 6.1 ± 0.5 μg/kg for 8-OH-DPAT and LSD, respectively. Following sustained tianeptine administration (20 mg/kg/day s.c. × 14 days), dose-response curves were similar to those obtained in control rats, the ED_{50} values for 8-OH-DPAT and LSD being 2.4 ± 0.3 μg/kg and 6.5 ± 0.6 μg/kg, respectively (Fig. 2).

Effect of prolonged tianeptine-treatment on the responsiveness of dorsal hippocampus CA_3 pyramidal neurons to microiontophoretic application of 5-HT and 8-OH-DPAT

Since the responsiveness of dorsal hippocampus pyramidal neurons to microiontophoretic application of 5-HT is

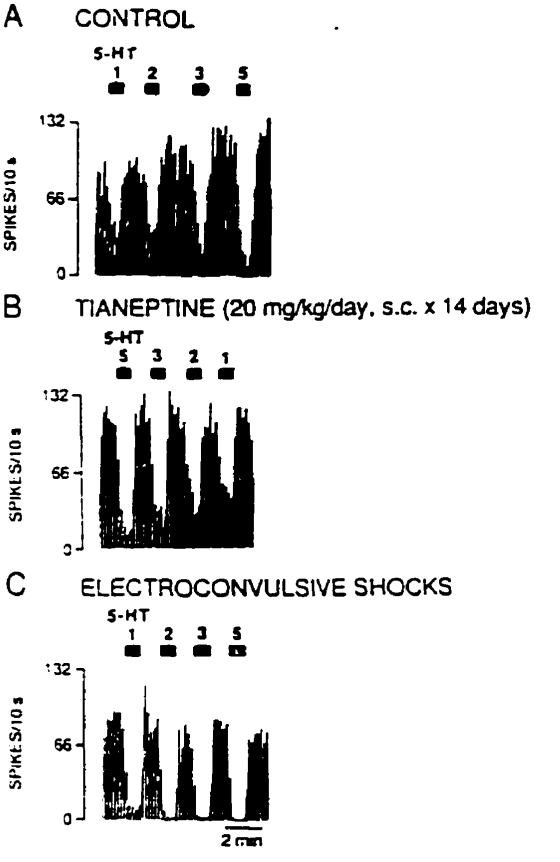

Fig. 3 Integrated firing rate histograms of CA_3 dorsal hippocampus pyramidal neurons showing their response to microiontophoretic application of 5-HT in a control rat (**A**), in a rat treated with tianeptine (**B**) and, in a rat treated with repeated electroconvulsive shocks (**C**). Recordings in **B** and **C** were obtained using the same micropippette. The bars indicate the duration of the application for which the ejection current is given in nA. Time scale applies to all traces

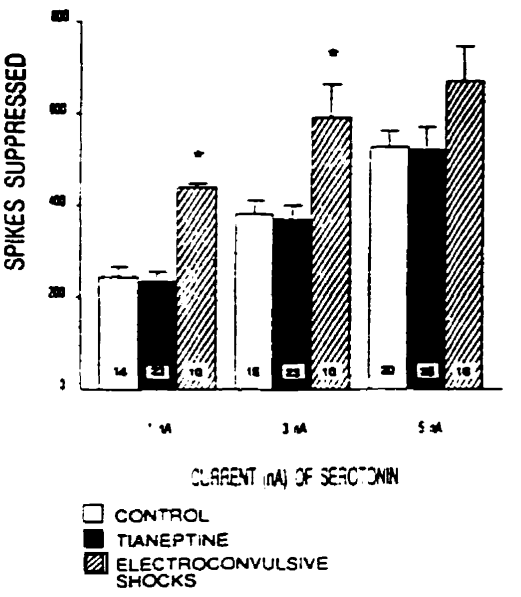

Fig. 4 Responsiveness, expressed as spikes suppressed (mean ± SEM), of dorsal hippocampus pyramidal neurons to microiontophoretically-applied 5-HT in control rats, rats pretreated with tianeptine (20 mg/kg/day, s.c. × 14 days) and in rats that had received six electroconvulsive shocks over a two week period. The number of neurons tested is indicated at the bottom of each column. $^*p < 0.001$ using paired Student's *t*-test

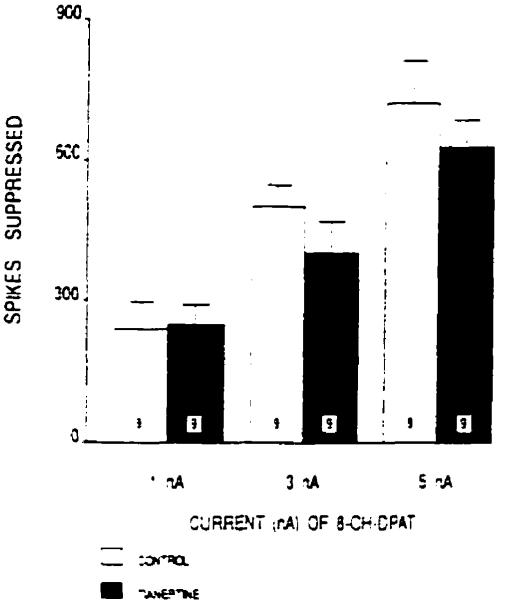

Fig. 5 Responsiveness, expressed as spikes suppressed (mean ± SEM) of dorsal hippocampus pyramidal neurons to microiontophoretically-applied 8-OH-DPAT in control rats, and rats pretreated with tianeptine (20 mg/kg/day, s.c. × 14 days). The number of neurons tested is indicated at the bottom of each column

markedly enhanced by electroconvulsive shocks (de Montigny 1984; Chaput et al. 1991), this treatment was included in the present study to ascertain that the experimental conditions were adequate to detect a sensitization. Whereas the responsiveness of dorsal hippocampus pyramidal neurons to 5-HT was significantly enhanced in rats that had received repeated electroconvulsive shocks, in rats treated with tianeptine for 14 days the responsiveness of CA_3 pyramidal neurons to 5-HT and 8-OH-DPAT was unchanged (Figs. 3–5).

Effect of prolonged tianeptine treatment on the efficacy of stimulation of the ascending 5-HT pathway in suppressing CA_3 pyramidal neuron firing activity

The efficacy of 5-HT pathway stimulation to suppress dorsal hippocampus pyramidal neuron firing activity was assessed by constructing peristimulus time histograms (Fig. 6). In control and tianeptine-treated rats, the duration of suppression of firing was proportional to the intensity of current used (Fig. 7), and no significant difference was observed in the degrees of suppression between the two groups.

The function of the terminal 5-HT autoreceptor was assessed using two approaches (see Materials and methods). First, the suppressant effect on pyramidal neuron firing activity of two different stimulation frequencies (1 and 5 Hz) was compared in control and tianeptine-treated rats. Similar to what was observed in controls, in tianeptine-treated rats, the effectiveness of the stimulation delivered at 5 Hz was less than that delivered at 1 Hz. Respective SIL values for stimulations delivered at 1 and 5 Hz were 53 ± 5 ms and 44 ± 5 ms in controls, and 56 ± 7 and 45 ± 5 ms in tianeptine-treated rats. Second, the degree of suppression of pyramidal neuron firing activity

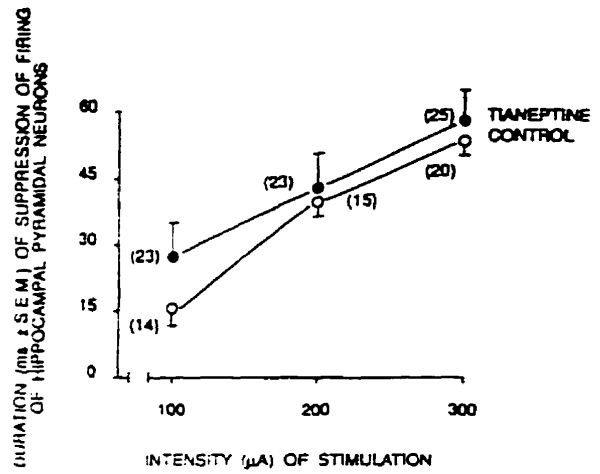

Fig. 7 Relationship between the duration of suppression of firing of CA_3 hippocampus pyramidal neurons and the intensity of the current used to stimulate the ascending 5-HT pathway in control rats (○) and in rats pretreated with tianeptine (20 mg/kg/day, s.c. × 14 days) (●). The number of neurons tested is given in parentheses

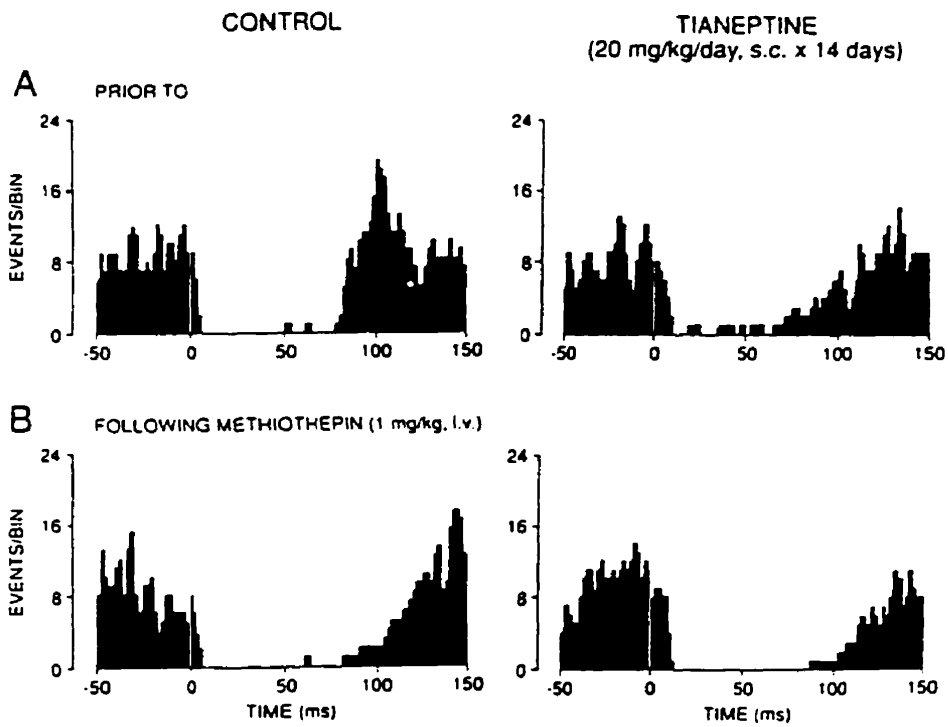

Fig. 6 Peristimulus time histograms showing the effect of the electrical stimulation of the ascending 5-HT pathway on the firing activity of two dorsal hippocampus pyramidal neurons, one recorded in a control rat and one in a rat treated with tianeptine, prior to (**A**) and following (**B**) the injection of methiothepin. Each peristimulus time histogram was constructed from 150 stimulations using 0.5 ms square pulses of 300 μA delivered at time 0. Bin width is 2 ms

secondary to 5-HT pathway stimulation was assessed prior to, and following, the administration of the terminal 5-HT autoreceptor antagonist, methiothepin (1 mg/kg, i.v.). The latter drug enhanced the effectiveness of stimulation to the same extent in control and tianeptine-treated rats (Fig. 6). Following methiothepin administration SIL values were increased by 26 ± 6 and 29 ± 9 ms in controls and tianeptine-treated rats, respectively.

Plasma and brain concentrations of tianeptine and its main metabolite

In 10 rats that had received tianeptine for 14 days (20 mg/kg/day, s.c.) and were still carrying the osmotic minipump at the time of sacrifice, the concentration of tianeptine in plasma was 22 ± 5 ng/ml and that of its main metabolite (obtained by β-oxidation of the lateral amino-heptanoic side chain), almost six fold higher, 119 ± 29 ng/ml. Brain levels were more variable, tianeptine and its β-oxidation product being undetectable in 3 and 2 of the 10 rats, respectively. The mean concentration of tianeptine in the brain of the seven remaining rats was 18 ± 7 ng/g of tissue and that of its metabolite was 11 ± 2 ng/g of tissue.

Discussion

The sustained administration of tianeptine did not modify the spontaneous firing activity of dorsal raphe 5-HT neurons (Table 1, Fig. 1) nor did it alter the sensitivity of somatodendritic 5-HT autoreceptors to intravenously injected LSD (Fig. 2A and B). Evidence supporting the fact that, apart from somatodendritic 5-HT$_A$ autoreceptors, postsynaptic 5-HT$_A$ receptors might be implicated in controlling the firing activity of 5-HT neurons is now considerable (Blier and de Montigny 1987; Ceci et al. 1994; Jolas et al. 1994; Romero et al. 1994). It has therefore been suggested that intravenous 8-OH-DPAT may not exert its effect on 5-HT neuron firing directly via the somatodendritic 5-HT$_A$ autoreceptor, but rather by activating postsynaptic 5-HT$_A$ receptors involved in a putative negative feedback loop controlling 5-HT neuron firing. Since ED_{50} for 8-OH-DPAT was not changed by prolonged tianeptine administration, it thus appears that this treatment does not modify the postsynaptic 5-HT$_A$ receptors involved in this postulated feedback loop controlling 5-HT neuron firing activity.

The sensitivity of postsynaptic 5-HT$_A$ receptors of dorsal hippocampal pyramidal neurons to microiontophoretically-applied 5-HT or 8-OH-DPAT was also unchanged following prolonged treatment with the tricyclic drug tianeptine. This lack of effect stands in contrast with previous observations showing that long-term tricyclic antidepressant drug administration produces an increase in the responsiveness of these neurons to microiontophoretically-applied 5-HT (de Montigny and Aghajanian 1978; Chaput et al. 1991). The structure of tianeptine is not identical to other classical tricyclic antidepressant drugs; tianeptine possesses a long amino-heptanoic chain and a terminal carboxylic acid group whereas most tricyclic antidepressant drugs have a short side chain that ends with a terminal amino group (Labrid et al. 1988). The lack of change in postsynaptic neuron responsiveness to 5-HT and 8-OH-DPAT in tianeptine-treated rats also stands in contrast with the enhanced response to both 5-HT and 8-OH-DPAT observed in the present study following repeated electroconvulsive shock treatments.

The effectiveness of the terminal 5-HT autoreceptor antagonist methiothepin in increasing the efficacy of the stimulation of the 5-HT pathway was also unchanged following prolonged treatment with tianeptine. These results indicate that the function of the terminal 5-HT autoreceptor was not altered by this treatment. In contrast, selective 5-HT reuptake inhibitors have been shown to attenuate the function of this release-modulating 5-HT autoreceptor (see Blier and de Montigny 1994). In keeping with this conclusion, prolonged treatment with tianeptine did not induce any change of the differential effectiveness of 1 and 5 Hz stimulations.

The results described above show that prolonged tianeptine administration at a dose of 20 mg/kg/day did not produce any changes in the overall efficacy of 5-HT neurotransmission. Interestingly, plasma tianeptine concentrations obtained in the present study were similar to those observed by Chambda et al. (1991) in depressed patients 12 h after the last administration of a long-term tianeptine treatment (37.5 mg/day, p.o. < 28 days). These observations thus suggest that, at the doses used in clinical trials, tianeptine would not produce its effect by enhancing 5-HT neurotransmitter function. Indeed, the present study shows that, unlike all other antidepressant drugs studied thus far using an electrophysiological approach, tianeptine does not modify 5-HT neurotransmission.

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ARTICLES IV, V, VI and VII

The goal of this part of the project was to investigate new autoregulatory processes of 5-HT neurotransmission, as well as the way in which they might be modified following prolonged antidepressant administration (see Introduction). As previously described, 5-HT neurons are under potent autoregulatory control and antidepressant treatments only enhance 5-HT synaptic transmission once this mechanism becomes desensitized allowing for autoregulatory negative feedback to be overcome. Specifically, at the somatodendritic level, local regulatory mechanisms influence the efficacy of 5-HT neurotransmission in forebrain projection areas. Indeed, it has been extensively documented that the activation of somatodendritic 5-HT_{1A} autoreceptors by endogeneous 5-HT reduces its own release in forebrain projection areas. In spite of the fact that the activation of this potent feedback mechanism depends on the degree of stimulation of somatodendritic 5-HT_{1A} autoreceptors, determined by the concentration of 5-HT in their biophase, the regulation of extracellular levels of 5-HT in the midbrain raphe nuclei is not well known. Unlike somatodenritic 5-HT_{1A} autoreceptors, terminal 5-$HT_{1B/1D}$ autoreceptors control 5-HT release without altering 5-HT neuron firing activity. In fact, there was already some evidence suggesting that this firing-independent control of 5-HT release could take place at the cell body level. For example, several autoradiographic and binding studies had documented the presence of 5-HT binding sites, other than 5-HT_{1A}, in the rat raphe nuclei, but the role of these other receptors in modulating 5-HT release at the somatodendritic level has not been characterized. The lack of effect of 1-[3-(trifluoromethyl)-phenylpiperazine (TFMPP) and *m*-chlorophenylpiperazine (*m*CPP) on the firing activity of 5-HT neurons led Sprouse and Aghajanian to conclude that 5-HT_{1B} receptors were not present on the cell body of 5-HT neurons in the rat brain. However,

more recent observations indicated that in anesthesized rats, the 5-HT_1 agonist RU 24969 could modulate extracellular availability of 5-HT in the dorsal raphe nucleus without altering 5-HT neuron firing frequency. Hence, the purpose of our next series of studies was to explore whether the extracellular availability of 5-HT at the cell body level could be regulated by a firing-independent manner and if so, to determine the pharmacological profile of the 5-HT receptor involved, its response to prolonged antidepressant administration, and finally determine whether it is located on 5-HT neurons themselves.

SYNAPSE 22:54–62 (1996)

Autoregulatory Properties of Dorsal Raphe 5-HT Neurons: Possible Role of Electrotonic Coupling and 5-HT_{1D} Receptors in the Rat Brain

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KEY WORDS 5-HT_{1D} receptors, Dorsal raphe, Release, Somatodendritic, Serotonin, Gap junctions, Electrotonic coupling

ABSTRACT In the present study, the hypothesis that somatodendritic availability of 5-hydroxytryptamine (5-HT) could be regulated independently of the firing activity of dorsal raphe 5-HT neurons was tested. The 5-HT pathway was electrically stimulated at the level of the ventromedial tegmentum and the ensuing action potentials, recorded in the dorsal raphe, met all criteria for antidromic invasion of 5-HT neurons. The latency of antidromic spikes was current-dependent and the changes in latency were of quantal nature. This observation suggests an electrotonic coupling between 5-HT neurons. Stimulation of the ventromedial tegmentum also induced a decrease in the probability of firing of 5-HT neurons. This reduction in 5-HT neuron firing activity is a 5-HT-mediated response, due to an increased bioavailability of the neurotransmitter in the biophase of somatodendritic 5-HT_{1A} autoreceptors. The intravenous administration of the 5-HT_1 agonists TFMPP and RU 24969 reduced the duration of suppression of firing induced by the 5-HT-pathway stimulation, without altering the spontaneous firing rate of 5-HT neurons. The effect of TFMPP and RU 24969 on duration of suppression was blocked by (±)mianserin, a drug with high affinity for the rat 5-HT_{1D}, but not 5-HT_{1B}, receptors. On the other hand, (−)propranolol, a mixed 5-HT antagonist also blocked the effect of TFMPP. However, the selective 5-HT_{1A} antagonist (+)WAY 100135 did not alter the effect of TFMPP. These results, in keeping with previous anatomical studies, suggest the existence of electrotonic coupling of 5-HT neurons and indicate that 5-HT release in the rat dorsal raphe nucleus may be controlled independently of firing-regulating 5-HT_{1A} autoreceptors. They also suggest that 5-HT_{1D} receptors may play a role in this regulatory function of 5-HT neurons.

INTRODUCTION

Serotonin neurons are under potent autoregulatory control. Since their firing frequency is negatively regulated by somatodendritic 5-HT_{1A} autoreceptors (Aghajanian, 1978; de Montigny et al., 1984), the activation of these receptors inhibits 5-HT neuron activity causing a reduction of 5-HT release in projection areas (Bel and Artigas, 1992; Celada and Artigas, 1993). The latter observation is an indication of the extent to which local regulatory mechanisms at the somatodendritic level may influence the efficacy of 5-HT neurotransmission in projection areas. There is a large body of evidence supporting the fact that 5-HT release in projection areas is regulated locally by terminal autoreceptors and that their degree of activation depends on the concentration of 5-HT in their biophase (see Starke et al., 1989). On the other hand, the mechanisms regulating the concentration of 5-HT in the biophase of somatodendritic receptors are less well known. Whether 5-HT release in raphe nuclei is of somatodendritic (Héry et al., 1982) or axon collateral origin still remains a matter of debate. Regardless of its origin, Bosker et al. (1994) have recently reported that 5-HT release in the median raphe nucleus is impulse flow dependent, and thus regulated by 5-HT_{1A} autoreceptors. The purpose of this study was thus to explore, whether somatodendritic availability of 5-HT could be regulated in an alternative, firing-independent manner.

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Serotonin concentration in the biophase of 5-HT_{1A} autoreceptors was assessed indirectly by determining the duration of suppression of firing of 5-HT neurons following the electrical stimulation of the 5-HT pathway. Wang and Aghajanian (1977) have previously demonstrated that the consistent suppression of firing of 5-HT neurons, observed following the stimulation of the 5-HT pathway, is a 5-HT-mediated response since it is blocked by the tryptophan hydroxylase inhibitor parachlorophenylalanin and restored by 5-hydroxytryptophan (Wang and Aghajanian, 1978).

Terminal 5-HT autoreceptors control 5-HT release in projection areas without altering 5-HT neuron firing activity. They have been described as being of the 5-HT_{1B} subtype in the rat (Engel et al., 1986; Maura et al., 1986) and of the 5-HT_{1D} subtype in other species including humans (Galzin et al., 1992; Hoyer and Middlemiss, 1989). Recently, a gene encoding 5-HT_{1D} receptors in the rat has been cloned and mRNA for this receptor has been found in rodent brain (Neumaier et al., 1993). It was thus deemed of interest to determine whether one of these receptor subtypes, known to control 5-HT release in terminal regions, would also be involved in controlling extracellular availability of 5-HT at the somatodendritic level, in a firing independent manner.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 250–300 g were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted in a stereotaxic apparatus. Single-barrelled glass micropipettes were preloaded with fibreglass filaments to facilitate filling, pulled in a conventional manner, their tip broken back to about 1 μm under microscopic control, and filled with a 2 M NaCl solution to obtain an impedance of 3–8 MΩ. The microelectrode was positioned 1 mm anterior to lambda and descended into the nucleus raphe dorsalis, which was reached immediately below the ventral border of the Sylvius aqueduct, usually 4.5–5 mm from the brain surface; 5-HT neurons were encountered over a distance of about 1 mm from this point and identified using the criteria of Aghajanian (1978).

To activate the 5-HT pathway, a bipolar concentric electrode (NE-100, David Kopf Instruments, Tujunga, CA) was positioned on the midline at an anterior angle of 10° in the ventromedial tegmentum (VMT), 3 mm anterior to lambda, V = 8.3 mm. Square pulses of 0.5 ms duration were delivered at 1 or 50 Hz with variable intensities (100–3,500 μA). Pulses were generated by an S88 stimulator (Grass) and were delivered through a direct-coupled isolation unit (SIU 478A, Grass). Peristimulus time histograms of the firing activity of 5-HT neurons were generated by a computer equipped with a Techmar interface. The duration of suppression of 5-HT neuron firing activity produced by the stimulation was quantified as absolute silence values (SIL, ms). This value was obtained by dividing the total number of missing events after the stimulation by the mean prestimulation firing frequency. Thus, the absolute silence value corresponds to the duration of suppression of firing normalized for the firing activity of the neuron recorded (Chaput et al., 1986). The following lines of evidence indicate that VMT-induced cessation of firing 5-HT neurons is indeed due to the release of 5-HT: i) it is abolished by destroying 5-HT axons (Wang and Aghajanian, 1977), and ii) it is prevented by the tryptophan hydroxylase inhibitor pCPA and subsequently restored following the injection of 5-hydroxytryptophan, the immediate precursor of 5-HT (Wang and Aghajanian, 1978).

Statistical analysis

All results are expressed as means ± S.E.M. For the pharmacological studies, data were always generated in pairs by measuring, in the same rat and for the same neuron, the effect of 5-HT pathway stimulation prior to and following intravenous administration of drugs. Results were thus analyzed for statistical significance using the paired Student's *t* test.

Drugs

1-[3-(Trifluoromethyl)phenyl]-piperazine (TFMPP; Research Biochemicals Natick, MA), 5-methoxy-3(1,2,3,6-tetrahydropyridinyl)-1H-indole (RU 24969; Roussel, Romainville, France), (−)propranolol (Imperial Chemical Industries, Cheshire, UK), (±)mianserin (Research Biochemical, Natick, MA), (−)WAY 100135 (Wyeth Research, Berkshire, UK), and spiperone (Research Biochemicals, Natick, MA) were used. When given systemically neither TFMPP (Sprouse and Aghajanian, 1987) nor RU 24969 (Crespi et al., 1990) produced an activation of 5-HT_{1A} autoreceptors since they failed to modify 5-HT neuron firing frequency, a 5-HT_{1A}-mediated response. In spite of this lack of effect on 5-HT neuron firing activity, the systemic administration of RU 24969 (Blier et al., 1990) and TFMPP (Piñeyro et al., 1994a) induced a significant decrease in the voltammetric 5-hydroxyindole signal obtained from the dorsal raphe nucleus of anesthetized rats (Blier et al., 1990; Piñeyro et al., 1994a). Both agonists were thus chosen to characterize the firing-independent regulation of 5-HT concentration in the dorsal raphe nucleus.

RESULTS

Seventy-three 5-HT neurons with characteristic slow (0.5–2.5 Hz), regular, long duration (0.8–1.2 ms) action potentials were recorded from the dorsal raphe nucleus. Stimulation of the 5-HT pathway produced antidromic spikes which were identified according to the following criteria: i) collision with spontaneous spikes which occurred immediately before the stimulation, ii) ability to follow high stimulation frequency (up to 50 Hz), and iii) constant latency at constant stimulus intensity.

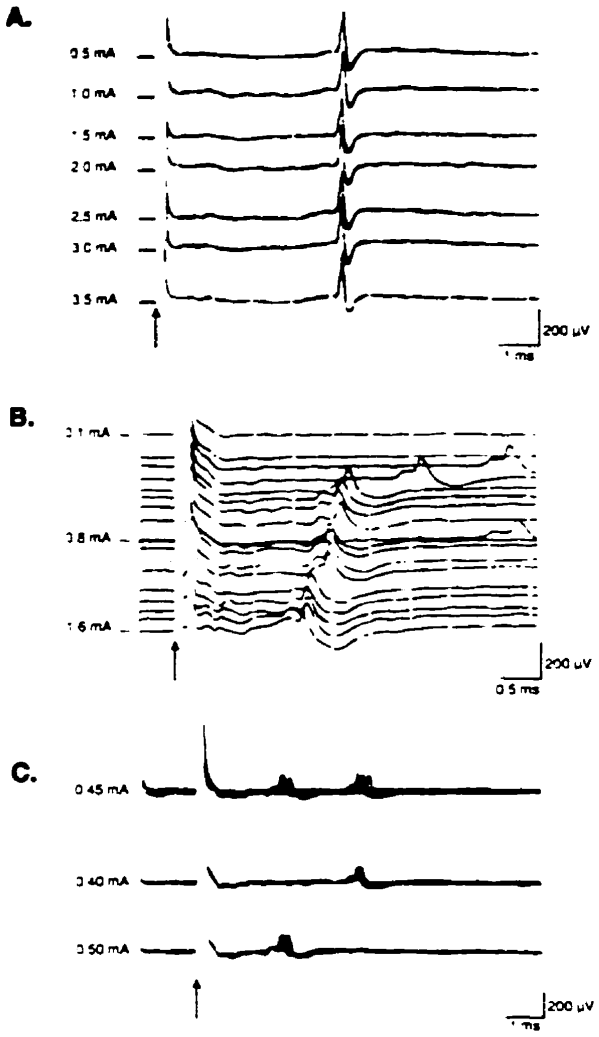

Fig. 1. Extracellular recordings obtained from dorsal raphe 5-HT neurons showing antidromically evoked responses at different current intensities. Upward deflection is positive. A neuron in which latency was constant (4.3 ms) at different stimulation intensities is shown in (A). (B) and (C) show quantal variations of antidromic spike latencies. Arrows indicate the time of delivery of the electrical stimulations. Stimulation frequency was kept constant at 1 Hz.

The effect of different stimulus intensities was tested in 22 neurons. In seven of them, currents ranging from 200 µA to 3,500 µA induced no change in evoked-spike latency. An example of such neurons is given in Figure 1A. In the remaining 15 neurons, latency of activation was current-dependent, ranging from 1 to 12 ms at maximal and threshold intensities, respectively. Collision was always present for short- as well as long-latency responses. Observed variations in latency were always quantal in nature, a typical example being shown in Figure 1B. For this neuron, 100 µA and 150 µA currents elicited no response while 200 µA, 300 µA, and 400 µA stimuli induced spikes at 3.9 ms, 2.9 ms, and 1.9 ms, respectively. The quantal nature of this phenomenon is further illustrated in Figure 1C which shows that the response of a 5-HT neuron to a 450 µA stimulus, half of the responses were of short latency (2 ms) and the other half of a long one (4 ms). In this neuron, if current intensity was reduced to 400 µA or increased to 500 µA, all responses were fixed at 4 ms or 2 ms, respectively. Current intensities beyond 500 µA did not produce further "quantal steps"; instead decrease in latency was linear (see Fig. 1B), probably due to the fact that higher currents triggered the spikes nearer to the recorded soma.

In the remaining 51 neurons, stimulus intensity was kept constant at 300 µA and peristimulus time histograms were constructed from 150–200 stimuli delivered at 1 Hz. The activation of the 5-HT pathway suppressed the firing activity of 5-HT neurons with a mean SIL value of 181 ± 8 ms. The observed latency for the suppressant effect was of 13 ± 1 ms. Neurons in which inhibition occurred within a short latency (< 10 ms; n = 34), presented significantly higher SIL values than those with longer latency (10–20 ms; n = 17). The respective SIL values were 211 ± 24 and 100 ± 16 ms ($P < 0.01$).

The effects of intravenous administration of 0.5 mg/kg of TFMPP or RU 24969 on the duration of suppression of dorsal raphe 5-HT neurons, elicited by the 5-HT pathway stimulation, are shown in Figure 2. Though these drugs reduced SIL values by 56% and 35%, respectively (Fig. 3A), neither of them modified significantly the mean spontaneous firing activity of dorsal raphe 5-HT neurons (see Figs. 2, 3B). TFMPP and RU 24969 are 5-HT_1 agonists, but do not distinguish among the different 5-HT_1 receptor subtypes (Doods et al., 1985; Schoeffter and Hoyer, 1989). Therefore, to characterize the type of 5-HT_1 receptor activated by TFMPP and RU 24969 in this paradigm, the effect of these drugs on SIL values was assessed in rats pretreated with different 5-HT_1 antagonists. Administered by themselves, neither (−)-propranolol (2 mg/kg, i.v.) nor (+)WAY 100135 (0.5 mg/kg, i.v.) significantly affected SIL values (Fig. 4). Only spiperone (1 mg/kg, i.v.) reduced the duration of suppression of firing induced by the antidromic activation of the 5-HT pathway by 39% (Fig. 4D). However, while (+)WAY 100135, a selective 5-HT_{1A} antagonist (Fletcher et al., 1993), did not prevent TFMPP from reducing silence values (Figs. 5A, 6A), (−)propranolol, a mixed 5-HT_1 antagonist (Hoyer, 1991), blocked the effect of TFMPP (Figs. 5B, 6B). Moreover, the injection of (−)propranolol (2 mg/kg, i.v.) also restored duration of suppression to preinjection values in three TFMPP (0.5 mg/kg, i.v.) pre-treated rats (control SIL: 136 ± 30 ms; SIL following TFMPP: 58 ± 30 ms; SIL following (−)propranolol: 147 ± 20 ms). Given the ineffectiveness of (+)WAY 100135 to block the TFMPP-induced response, it was deemed possible that 5-HT_1 receptors,

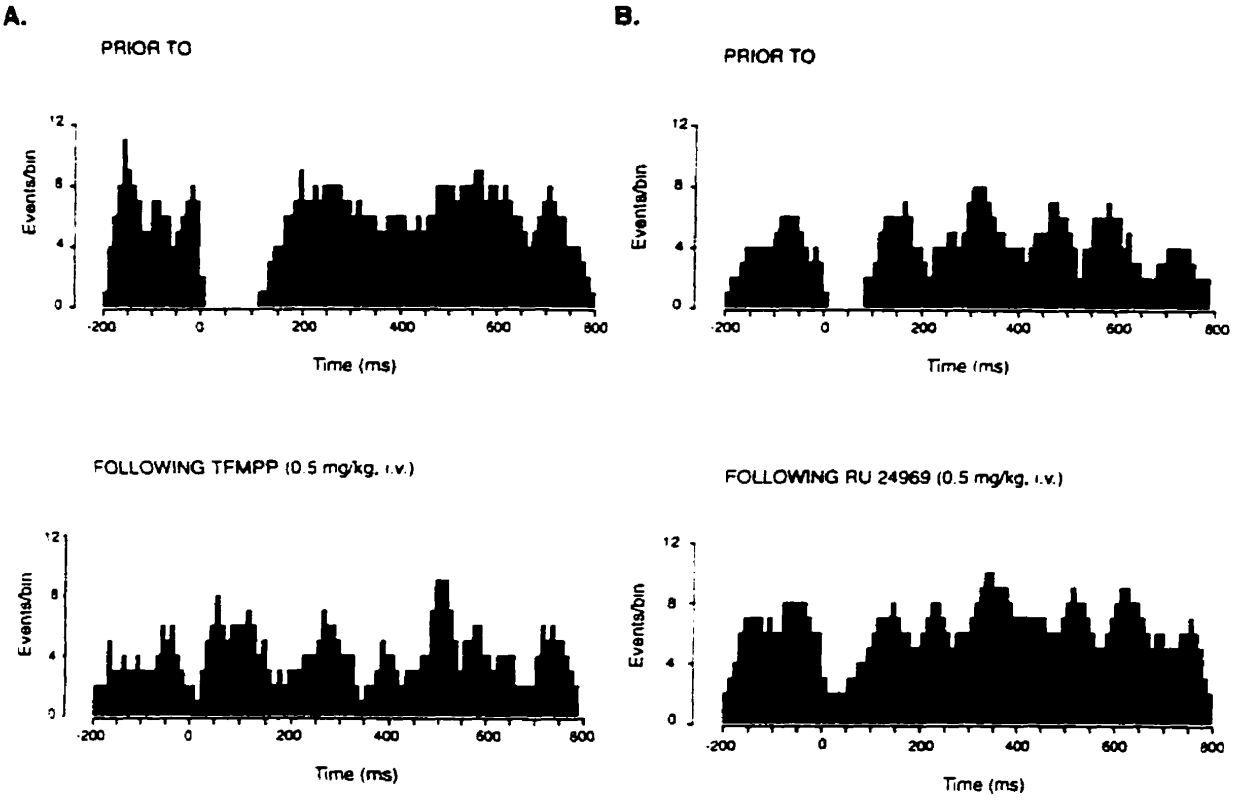

Fig. 2. Peristimulus time histograms obtained from a dorsal raphe 5-HT neuron prior to and following the intravenous administration of TFMPP (A) and RU 24969 (B). Each histogram was constructed from 150–200 stimuli of 0.5 ms delivered at 1 Hz with an intensity of 300 μA. Bin width is of 10 ms. Stimulation pulses were delivered at time 0.

other than the 5-HT_{1A}, subtype might be involved. Therefore, to further characterize the receptor mediating the effect of TFMPP and RU 24969, these drugs were tested in mianserin-treated rats (2 mg/kg, i.v.). Mianserin, which unlike (−)propranolol has a more than 100-fold higher affinity for 5-HT_{1D} than for 5-HT_{1B} receptors in the rat (Hamblin et al., 1992), abolished the effect of TFMPP and RU 24969 on the duration of suppression elicited by the stimulation of the 5-HT pathway (Figs. 5C, 6C,D).

DISCUSSION

The present study was based on previous observations showing that the electrical stimulation of the VMT induced a suppression of firing of 5-HT neurons which depends on the availability of 5-HT to activate somatodendritic 5-HT_{1A} autoreceptors (Wang and Aghajanian, 1978). The observation that the suppressant effect of VMT stimulation was blocked by spiperone, a drug with antagonistic activity at 5-HT_{1A} autoreceptors (Blier et al., 1993), further supports this contention. Unlike neurochemical techniques, the paradigm used in the present study provides an indirect measure of extracellular 5-HT. On the other hand, it provides a direct index of the functional consequence of changes in 5-HT availability in the biophase of 5-HT_{1A} autoreceptors. The duration of suppression of firing is proportional to the amount of neurotransmitter released per stimulation-triggered action potential which activates 5-HT_{1A} autoreceptors. Accordingly, longer SIL values were obtained from those neurons in which the latency of suppression of firing was shorter. This observation could be explained by the position of the stimulating electrode relative to the recorded neuron. If the axon of the neuron being recorded is away from the stimulating electrode then the neuron is not directly depolarized and 5-HT release in its immediate vicinity may be minimal. Nevertheless, diffusion of the neurotransmitter from neighbouring activated neurons may still induce a suppression of firing, though less pronounced than if the antidromic spike had been produced directly on the recorded neuron.

The variation in antidromic spike latency observed at different current intensities suggests the existence

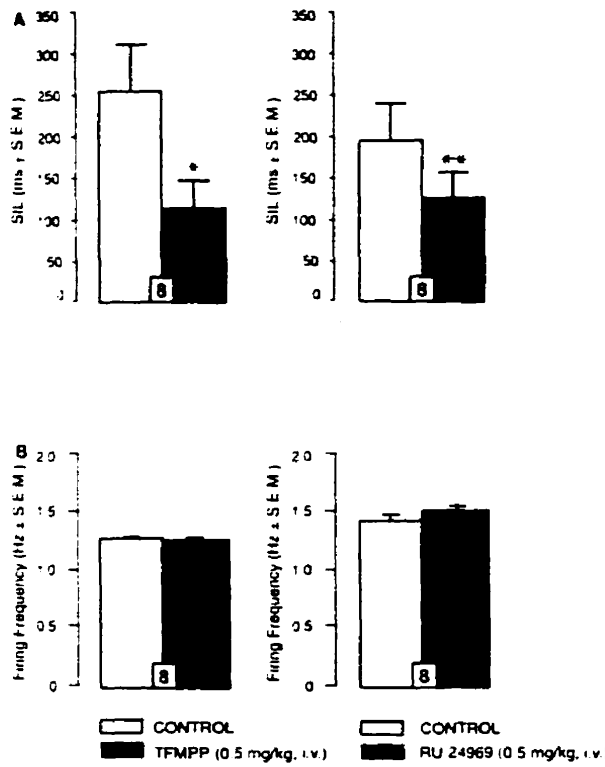

Fig. 3. Duration of the suppression of dorsal raphe 5-HT neuron firing activity produced by the stimulation of the 5-HT pathway prior to (control) and following the intravenous administration of TFMPP and RU 24969 **A.** Effect of TFMPP or RU 24969 on dorsal raphe 5-HT neuron firing frequency **B.** The number of neurons tested (one per rat) is indicated at the bottom of each pair of columns. $^{*}P < 0.02$; $^{**}P < 0.05$ paired Student's t test).

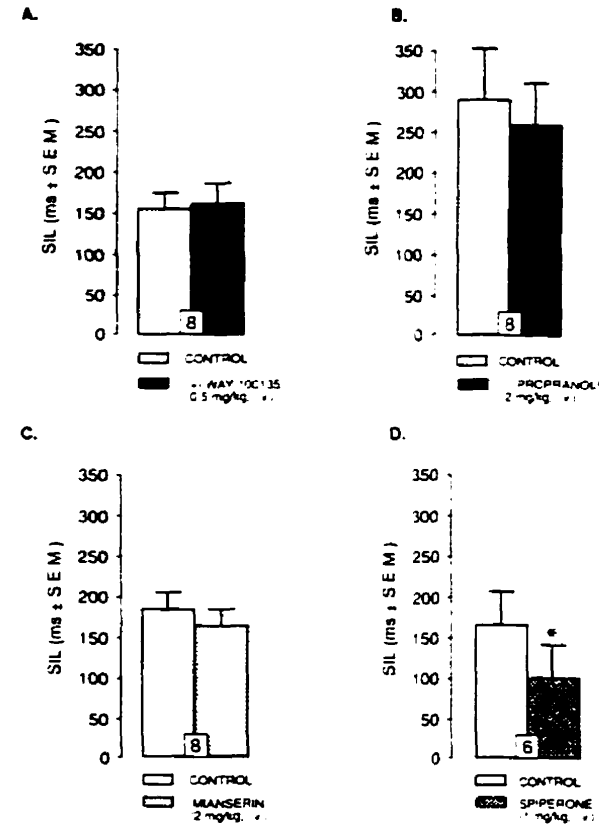

Fig. 4. Duration of the suppression of dorsal raphe 5-HT neuron firing activity produced by the stimulation of the 5-HT pathway prior to (control) and following the intravenous administration of (-)WAY 100135 **(A)**, (-)propranolol **(B)**, mianserin **(C)**, and spiperone **(D)**. The number of neurons tested (one per rat) is indicated at the bottom of each pair of columns. $^{*}P < 0.02$ (paired Student's t test).

of an electrotonic coupling between 5-HT neurons. A fixed delay, secondary to neuronal communication via gap junctions, could explain the quantal nature of latency changes. For a given neuron, an antidromic spike will be elicited within a minimal latency if the stimulus directly activates its axon. If instead of directly activating the recorded neuron the stimulation of the 5-HT pathway activates neighbouring neurons, an antidromic spike may still be elicited in the former, if gap junctions that allow the depolarization to be spread exist. However in this case, the latency of the evoked spike will be longer since it includes not only conduction time but also activation of interposed neurons. The increase in stimulation intensity would thus produce a quantal decrease in latency values because more intense stimuli by-pass intermediary neurons to activate the next neuron in the chain. Progressive activation of interposed neurons, until direct activation of the recorded neuron is achieved, would then explain the quantal reduction in latency values until a minimal fixed latency is reached. Indeed, dendrodendritic contacts and gap junctions have been reported in the dorsal raphe nucleus (Mollgard and Moller, 1975; Park et al., 1982). Whether these anatomical features underlie the physiological interactions observed in this study remains to be determined. The present results are nevertheless consistent with a previous report suggesting a direct coupling between 5-HT neurons (Wang and Aghajanian, 1982). An alternative explanation to this observation could be that changes in latency represent activation of different branches of axons from the recorded cell. However, if this were the case changes in latency would not be necessarily "quantal."

(-)WAY 100135 is a selective antagonist with high affinity for 5-HT_{1A} binding sites (Fletcher et al., 1993; Hoyer, 1991). Unlike WAY 100635, which blocks the suppressant effects of 8-OH-DPAT and of 5-HT itself on 5-HT neuron firing frequency (Fletcher et al., 1994), (-)WAY 100135 does not block the inhibitory action of endogenous 5-HT at the 5-HT_{1A} autoreceptor (Fornal et al., 1994), nor that of microiontophoretically-applied 5-HT onto 5-HT neurons (Haddjeri and Blier, 1994).

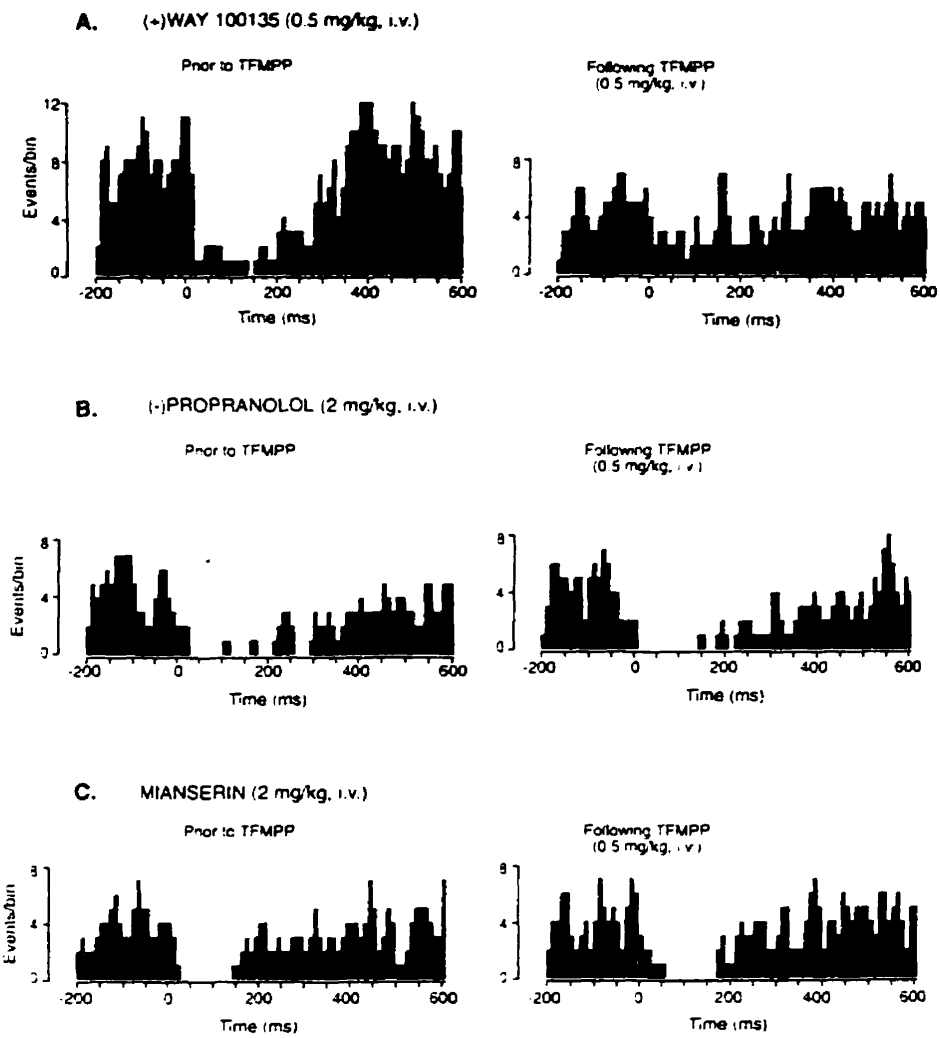

Fig. 5. Peristimulus time histograms obtained from dorsal raphe 5-HT neurons prior to and following the injection of TFMPP in rats that had previously received (−)WAY 100135 (A), (−)propranolol (B) mianserin (C). Each histogram was constructed from 150–200 stimuli of 0.5 ms delivered at 1 Hz with an intensity of 300 μA. Bin width is of 10 ms. Stimulation pulse was delivered at time 0.

Though (−)WAY 100135 (0.5 mg/kg, i.v.) blocks the effect of 8-OH-DPAT on 5-HT neuron firing frequency (Fletcher et al., 1993), the lack of effect of such a dose of (−)WAY 100135 on the duration of suppression of firing elicited by 5-HT pathway stimulation (Figs. 4A and 4B) may be explained by its lack of effect on 5-HT-mediated events. (−)Propranolol has been shown to block the inhibitory effect of microiontophoretically-applied 8-OH-DPAT, but not that of 5-HT (Sprouse and Aghajanian, 1986). Hence its lack of effect on VMT-induced suppression of firing of 5-HT neurons (Fig. 4B) could also be explained by its ineffectiveness to block 5-HT mediated events. In keeping with this interpretation, spiperone, which in contrast to (−)WAY 100135 and to (−)propranolol, has been shown effective in blocking the suppressant effect of microiontophoretically-applied 5-HT onto 5-HT neurons (Blier et al., 1993) was found to decrease SIL values induced by VMT stimulation in the present study (Fig. 4D). Results obtained by Fornal et al. (1994), showing that, in awake cats, spiperone (1 mg/kg, i.v.) markedly increases 5-HT neuron firing activity, while (−)WAY 100135 (0.1, 0.5, and 1 mg/kg, i.v.) has no effect (Metzler et al., 1993), further support the conclusion that, in spite of blocking the effect of synthetic 5-HT_{1A} agonists (Fletcher et al., 1993; Routledge et al., 1993), (−)WAY 100135 does not block the effect of 5-HT itself. Unlike spiperone, (−)WAY 100135 and (−)propranolol were ineffective in blocking

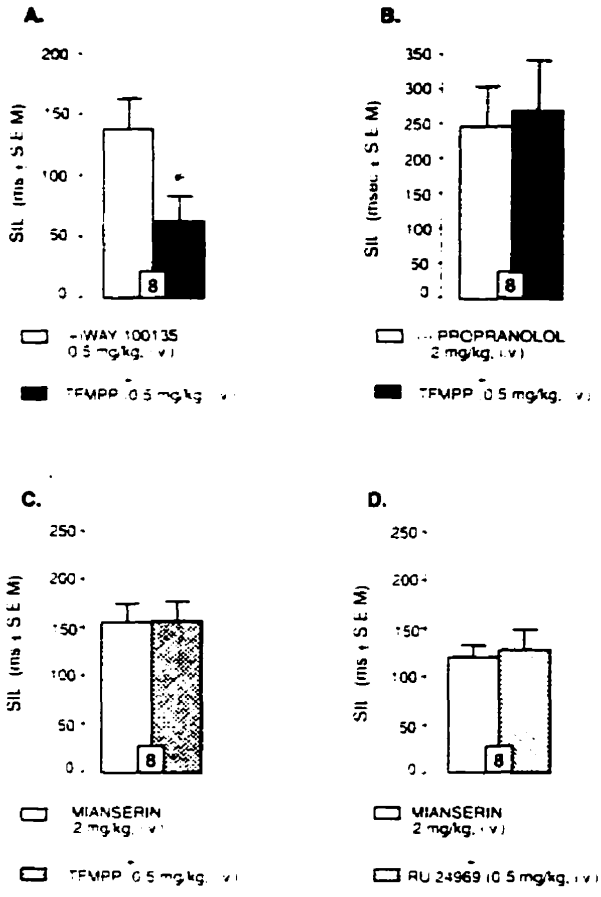

Fig. 6. Duration of the suppression of dorsal raphe 5-HT neuron firing activity produced by the stimulation of the 5-HT pathway in rats pretreated with (−)WAY 100135 prior to and following the intravenous administration of TFMPP (A), in rats pretreated with (−)propranolol prior to and following the intravenous administration of TFMPP (B), in rats pretreated with mianserin prior to and following the intravenous administration of TFMPP (C), in and rats pretreated with mianserin prior to and following the intravenous administration of RU 24969 (D). The number of neurons tested (one per rat) is indicated at the bottom of each pair of columns. *$P < 0.02$ (paired Student's t test).

endogenous 5-HT at 5-HT_{1A} autoreceptors. Indeed, this is an essential property for characterizing the receptor mediating the reduction in SIL values elicited by the systemic injection of TFMPP and RU 24969; (−)WAY 100135 and (−)propranolol, without themselves affecting the inhibitory response to 5-HT pathway stimulation, would have blocked the effect of a synthetic agonist if it were mediated via a 5-HT_{1A} receptor (see below).

Intravenous administration of TFMPP and RU 24969 induced a marked reduction in the suppression of firing elicited by 5-HT pathway activation without altering 5-HT neuron firing activity (Figs. 2, 3A). The lack of effect on 5-HT neuron firing has been previously reported (Crespi et al., 1990; Sprouse and Aghajanian, 1987). Since RU 24969 and TFMPP induce a decrease in dorsal raphe extracellular 5-HT (Blier et al., 1990; Piñeyro et al., 1994a), the firing-independent reduction in SIL values suggests that the amount of extracellular 5-HT available to activate 5-HT_{1A} autoreceptors following the antidromic stimulation of 5-HT neurons was reduced by both agonists. Thus, the receptor involved in this response exerts an inhibitory control on 5-HT release at the level of the dorsal raphe.

The involvement of 5-HT_{1A} receptors in these effects of TFMPP and RU 24969 may be ruled out not only because neither of these compounds induced changes in 5-HT neuron firing activity, but also because (−)WAY 100135, which antagonizes the effect of prototypical 5-HT_{1A} agonists (Fletcher et al., 1993; Routledge et al., 1993), did not change the reduction in SIL values induced by TFMPP. The reduction in SIL values elicited by TFMPP in (−)WAY 100135-pretreated rats (53%), was similar to that observed in controls (56%; Figs. 5A, 6A). The question that remains is which of the other 5-HT receptor subtypes, activated by RU 24969 and TFMPP, might be involved in controlling somatodendritic availability of 5-HT in this paradigm. Given that the 5-$HT_{1A/2C}$ agonist DOI [4-iodo(2,5-dimethoxy)phenylisopropylamine] reduces 5-HT neuron firing activity (Wright et al., 1990), it is unlikely that either 5-HT_{1A} or 5-TH_{2C} receptors might mediate the firing-independent effect of TFMPP and RU 24969, even if the effects of both agonists were blocked by mianserin which has nanomolar affinity for 5-HT_2 sites (Hoyer, 1991; Maj et al., 1978). Furthermore, the fact that (−)propranolol blocks the effect of TFMPP further supports the contention that this is not a 5-HT_{1A}-mediated response. In vitro, (−)propranolol displaces [^{3}H]5-HT from rat 5-HT_{1D} and 5-HT_{1B} sites with a K_i of 1,300 nM and 5 nM, respectively (Hamblin et al., 1992). Moreover, according to Middlemiss and Hutson (1990), a dose range of 1–5 mg/kg of (−)propranolol should produce similar effects to those observed in vitro within a concentration range of 100–1,000 nM. Taking into account that i) the dose of (−)propranolol used was of 2 mg/kg and ii) 1 μM (±)propranolol partially blocked the inhibition of [^{3}H]5-HT release induced by the 5-HT_1 agonist 5-carboxyamidotryptamine in slices of human cortex where autoreceptors are of the 5-HT_{1D} subtype (Galzin et al., 1992), the reversal of the effect of TFMPP by (−)propranolol (Fig. 6B) could be due either to the blockade of 5-HT_{1B} or 5-HT_{1D} receptors. In rat cell clones, however, mianserin readily discriminates 5-HT_{1B} from 5-HT_{1D} receptors. This drug, which displaces [^{3}H]5-HT from 5-HT_{1B} binding sites with a K_i greater than 10,000 nM but from 5-HT_{1D} sites with a K_i of 30 nM (Hamblin et al., 1992), completely blocked the effect of both 5-HT_1 agonists (Fig. 6). Mianserin also blocks α-adrenoceptors (Raiteri et al., 1983); however, since RU 24969 has very weak α-adrenoceptor antagonistic properties (Schlicker et al., 1988) it is unlikely that α-adrenoceptors play a

significant role in the inhibitory effect of TFMPP and RU 24969 on 5-HT release.

Though the present observations do not allow to rule out a possible involvement of 5-HT_{1B} receptors, they indicate that 5-HT_{1D} receptors may play a functional role in controlling extracellular availability of 5-HT in the rat dorsal raphe nucleus. A complete pharmacological characterization of the receptor regulating 5-HT release from the midbrain raphe nuclei has recently been completed, using an in vitro superfusion paradigm. The use of selective 5-HT_{1B} and 5-HT_{1D} agonists (that could not be tested in vivo because they do not cross the blood-brain barrier) confirmed that 5-HT_{1D}, but not 5-HT_{1B}, receptors regulate 5-HT release from rat mesencephalic slices (Piñeyro et al., 1994). Neither the paradigms used in the present or the above mentioned studies allow to determine whether 5-HT_{1D} receptors in the rat mesencephalon are located on 5-HT neurons themselves or on interneurons, nor if they control somatodendritic or axon collateral release of 5-HT. However, given the small number of axon terminals in the dorsal raphe nucleus (Descarries et al., 1982) and the presence of abundant vesicles containing 5-HT in the dendrites of 5-HT neurons (Chazal and Ralston, 1987), somatodendritic release might well be the predominant source of extracellular 5-HT in the raphe nuclei. The observation that 5-HT_{1D} receptors control extracellular somatodendritic availability of 5-HT is consistent with the results obtained by Herrick-Davis and Titeler (1988) showing that 5-HT_{1D} binding sites are present in the rat midbrain where they constitute 42% of total 5-HT_1 binding and with those of Neumaier et al. (1994) showing that the dorsal raphe is one of the rat brain regions in which 5-HT_{1D} mRNA is most prominently expressed. Modulation of 5-HT release in the guinea pig dorsal raphe nucleus has been recently studied by Starkey and Skingle (1994) using fast cyclic voltammetry in slices. Given the lack of selective agonists and antagonists for 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors (Hartig, 1992), these authors were unable to conclude whether one or the two 5-HT_{1D} receptor subtypes control 5-HT release at the somatodendritic level. Since rat 5-HT_{1D} and 5-HT_{1B} receptors are species homologues of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors, respectively (Hartig et al., 1992), our results suggest that in those species where 5-$HT_{1D\alpha\beta}$ receptors exist, the firing-independent modulation of 5-HT release is under the influence of the 5-$HT_{1D\alpha}$ subtype.

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ELSEVIER

5-HT_{1D} Receptors Regulate 5-HT Release in the Rat Raphe Nuclei

In Vivo Voltammetry and In Vitro Superfusion Studies

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The aim of the present study was to characterize the pharmacological profile of 5-hydroxytryptamine (5-HT) receptors modulating 5-HT release in the mesencephalic raphe region. In a first series of experiments, differential normal pulse voltammetry and nafion-coated electrodes were used to measure extracellular 5-HT in the dorsal raphe of anesthesized rats. The intravenous administration of the selective 5-HT_{1A} agonist 8-OH-DPAT (30 μg/kg) and the 5-HT_2 agonist TFMPP (0.5 mg/kg) reduced the 5-hydroxyindole signal by 23% and 18%, respectively. Pretreatment with the 5-HT_{1A} antagonist (−)WAY100135 (0.5 mg/kg IV) 30 minutes before the injection of the agonists, blocked the effect of 8-OH-DPAT but not that of TFMPP. The effect of TFMPP was blocked by (±)mianserin, a drug with high affinity for the rat 5-HT_{1D} receptor, suggesting a role of this receptor subtype in the modulation of 5-HT release at the cell body level of 5-HT neurons. This was confirmed by in vitro superfusion experiments using mesencephalic raphe slices. The prototypical 5-HT_1 agonist 5-carboxy-amidtryptamine (5-CT) and the 5-$HT_{1B/1D}$ agonist sumatriptan (1–1,000 nM) induced a concentration-dependent inhibition of the electrically evoked release of [^{3}H]5-HT from preloaded raphe slices. 8-OH-DPAT (100 nM) produced an inhibitory effect similar to that of sumatriptan (100 nM). The selective 5-HT_{1B} agonist CP 93129 (10–10,000 nM), had no effect in raphe slices, but it dose dependently inhibited [^{3}H]5-HT release from hippocampal slices where autoreceptors are of the 5-HT_{1B} subtype. The inhibitory effect of 5-CT was blocked by the 5-$HT_{1/2}$ antagonist methiothepin (1 μM), the 5-HT_{1A} antagonist S-UH-301 (1 μM), and the 5-$HT_{1B/1D}$ antagonist GR 127935 (1 μM). That of 8-OH-DPAT was blocked by S-UH-301 (1 μM) but not by GR 127935 (1 μM), and that of sumatriptan was blocked by GR 127935 (1 μM) but not by S-UH-301 (1 μM). These results show that, together with 5-HT_{1A} autoreceptors, 5-HT_{1D} receptors negatively modulate the somatodendritic release of 5-HT

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Dorsal and median raphe nuclei give rise to most of the serotoninergic innervation of the forebrain (Anden et al. 1966; Dahlstrom and Fuxe 1964; Ungerstedt 1971). At somatodendritic and terminal levels autoreceptors control 5-HT release (Starke et al. 1989). However, while terminal autoreceptors control regional 5-HT release in projection areas in which they are located (Starke et al. 1989), somatodendritic 5-HT_{1A} autoreceptors, by reducing the firing activity of 5-HT neurons, may influence local 5-HT release not only within the raphe nuclei themselves (Blier et al. 1990; O'Connor and Kruk 1992) but also in the projection areas (Adell et al. 1993; Blier et al. 1990) of those nuclei. Physiologically, the activation of 5-HT_{1A} autoreceptors is determined by the extracellular concentration of 5-HT (Jacobs and Fornal 1991). Hence modulation of 5-HT availability at the somatodendritic level constitutes a major regulatory factor of the efficacy of 5-HT neurotransmission.

Several autoradiographic and binding studies have documented the presence of different 5-HT binding

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sites in the rat raphe nuclei (Herrick-Davis and Titeler 1988; Laporte et al. 1992; Waeber et al. 1988, 1989). However, the role of receptors other than 5-HT_{1A} in modulating 5-HT neurotransmission at the level of the cell body is not as well characterized as that of 5-HT_{1A} autoreceptors. The lack of effect of 1-[3-(trifluoromethyl)-phenylpiperazine (TFMPP) and *m*-chlorophenylpiperazine (*m*CPP) on the firing activity of 5-HT neurons led Sprouse and Aghajanian (1987) to conclude that 5-HT_{1B} receptors were not present on the cell body of 5-HT neurons in the rat brain. More recently it has been demonstrated in vivo, that extracellular availability of 5-HT in the dorsal raphe nucleus of anesthetized rats can be modulated independently of 5-HT neuron firing frequency (Blier et al. 1990) and that 5-HT_{1D} receptors may be involved in this function (Piñeyro et al. 1993, 1994). Therefore, the aim of the present study was to further characterize the pharmacology of 5-HT receptors modulating 5-HT release in the midbrain raphe nuclei of the rat. Furthermore, it was deemed of interest to determine whether the putative 5-HT_{1D} receptors that modulate somatodendritic release of 5-HT were located in the mesencephalic region. An in vitro superfusion paradigm was used to assess the local effects of drugs. Superfusion experiments also allowed a detailed pharmacological characterization of the receptors involved in controlling 5-HT release in the midbrain raphe area, since drugs that cannot be used in vivo because they do not cross the blood-brain barrier could be used in vitro.

MATERIALS AND METHODS

Animals

One hour before starting in vivo voltammetry experiments, Sprague-Dawley rats (225–250 g) were injected with allopurinol (10 mg/kg IP) in order to avoid contribution of uric acid to the voltammetric signal (Blier et al. 1990). All the in vivo experiments were conducted under chloral hydrate anesthesia (initial dose: 400 mg/kg IP). For superfusion experiments rats were sacrificed by decapitation and their brains rapidly removed and dissected on an ice-cold plate. The midbrain raphe region was dissected as described by Kerwin and Pycock (1979) and slices of 0.4 mm thickness were prepared with a McIlwain chopper. Hippocampal slices of similar thickness were also prepared.

Voltammetric Experiments

Extracellular levels of indoleamines were determined by differential normal pulse voltammetry (DNPV) using multifiber pyrrolytic electrodes (20 fibers, 50 μm diameter, 500 μm length). Electrochemical measurements were performed using a Biopulse polarograph (Tacussel, France) with an auxiliary platinum electrode and a reference calomel electrode. The carbon-fiber electrodes were electrochemically pretreated in phosphate buffer saline by applying continuous direct current to generate potentials of +2.85 V, +2.4 V, and +1.6 V for 20 seconds each and were then nafion coated (+4 V, 5 sec). For in vitro and in vivo measurements the following parameters were used: ramp potential of 0 to 700 mV, scan rate of 3 mV/0.4 s, square wave pulse modulation of 50 mV amplitude, period of 90 ms, duration of 40 ms. The electrodes were calibrated in vitro in a phosphate buffer solution (PBS, pH 7.4) containing either 10 nM 5-HT, 10 μM 5-HIAA, or 10 μM uric acid. In vitro the 5-HT 5-HIAA selectivity ratio was 6,000 to 10,000 before and 5,000 to 9,000 after the experiments. With respect to 5-HT uric acid selectivity the ratios before and after the experiment were 8,000 to 12,000 and 10,000 to 15,000, respectively. After calibration the electrodes were stabilized in PBS containing 10 nM 5-HT, and when the peak occurring at 300 mV was stable they were immediately implanted into the dorsal raphe (A = 0.43, L = 0 and V = 0.5, interaural) with a backward angle of 45°. Once the signal was stable in vivo (usually 1.5 hour after implantation), voltammograms were generated for 2.5 hours every 10 minutes. Drugs were administered in the first 30 minutes of this 2.5-hour period. The electrochemical signal was quantified by measuring its height. For each individual animal control values were the mean peak height occurring 30 minutes before drug injection, and subsequent changes were expressed as percentages of the pre-injection values. Following the in vivo experiments, there was an increase in the sensitivity of the electrodes for all compounds tested in vitro. This observation, together with the fact that the selectivity ratio was also maintained after the in vivo experiments, suggests that the nafion coating was not significantly disrupted during implantation into the dorsal raphe nucleus. Statistical analyses were performed by comparing the curves produced under various treatments by means of a two-factor analysis of variance (ANOVA).

Superfusion Experiments

Hippocampal and mesencephalic slices containing dorsal and median raphe nuclei were incubated for 30 minutes at 37°C in Krebs solution containing 20 or 100 nM [^{3}H]5-HT creatinine sulphate (specific activity 22.7 Ci/mmol, NEN Research Products, Mississauga, Canada), respectively, and bubbled with a mixture of 95% O_2 5% CO_2. The composition of the Krebs solution was 118 mM NaCl, 4.7 mM $CaCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 0.004 mM Na_2EDTA, and 0.11 mM ascorbic acid. At the end of the incubation period with [^{3}H]5-HT, one mesencephalic or two hippocampal slices were transferred into

each of the glass chambers and superfused continuously at a rate of 0.5 ml/minute with Krebs solution maintained at 37°C and saturated with the 95% O_2/5% CO_2 mixture. Nineteen consecutive 4-minute fractions were collected starting 60 minutes after the beginning of superfusion. The slices were stimulated twice 8 minutes (S_1) and 44 minutes (S_2) after the end of the washing period. The electrical field generated in the chambers between two platinum electrodes (2 cm apart) had a voltage drop of 5 V/cm. The stimulation parameters used were 360 pulses of 2-ms duration, 30 mA intensity, delivered for 2 minutes at 3 Hz. This stimulation frequency was chosen because it is within the range of the firing rate of 5-HT neurons recorded in freely moving cats (Jacobs 1985). The first stimulation period was always used as a control. The putative antagonists were added 20 minutes before S_1 and remained present thereafter, whereas the agonists were added 20 minutes before S_2 and also remained present until the end of the superfusion. At the end of the experiment slices were solubilized in 0.5 ml Soluene 350 (Packard Instruments, Downers Grove, IL, USA) and radioactivity in the slices and superfusate samples was determined by scintillation spectrometry. The amount of tritium released per 4-minute sample was expressed as a fraction of total tritium contained in the tissue at the start of the respective collection period. The overflow of tritium produced by the electrical stimulation was calculated as the total increase in radioactivity above the basal outflow of tritium determined in the sample immediately preceding the start of stimulation (Sp_1 and Sp_2). In order to assess the drug-induced changes of electrically evoked overlow of radioactivity, S_2 S_1 ratios were calculated, and Sp_2 Sp_1 ratios were examined to determine whether the drugs altered the basal outflow of radioactivity. IC_{50} values for the different agonists were determined by computer analysis (GRAPHPAD, Graphpad Software, San Diego, CA, USA) using concentration-effect curves based on three concentrations of the agonists. The amount of tritium released by electrical stimulation in brain slices under these conditions provides a reliable estimate of the release of tritiated 5-HT (Blier and Bouchard 1993; Moret and Briley 1990). Since 5,7-dihydroxytryptamine lesion abolished reuptake into different cortical subcellular fractions Sette et al. (1983) concluded that in terminal regions such as the frontal cortex [^{3}H]5-HT is captured selectively by 5-HT terminals. It is thus most unlikely that at the concentration used in the present study (100 nM), more than fourfold smaller than the reported K_D of the 5-HT transporter for 5-HT (Barker and Blakely 1995), [^{3}H]5-HT may be captured other than by 5-HT neurons in the dorsal raphe.

The n values refer to the number of superfusion chambers studied. Experiments in which an agonist was compared to the control situation or when the effect of the agonist alone was compared to that of the agonist in the presence of the antagonist, were always run in parallel. Since control results obtained from similar experiments performed on different days did not differ significantly from one another, they were pooled. All results are expressed as means ± SEM. Means were compared using the two-tailed Student's t test and curves by using a two-factor ANOVA. p Values smaller than .05 were considered significant.

Drugs

The following drugs were used: allopurinol purchased from Sigma Chemicals (St. Louis, MO, USA); (±)mianserin; 1-[3-(trifluoromethyl)phenyl]-piperazine (TFMPP), 5-carboxyamidotryptamine, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), (R)-5-fluoro-8-hydroxy-2-(dipropylamino)tetralin (S-UH-301) from Research Biochemicals Incorporated (Wayland, MA, USA); CP 93129 from Pfizer (Groton, CT, USA); GR 127935 and sumatriptan from Glaxo (Greenford, UK); methiothepin maleate from Hoffmann La Roche (Basel, Switzerland); (−)propranolol from Imperial Chemical Industries (Cheshire, UK); (+)WAY100135 from Wyeth Research (Berkshire, UK); NSD 1015 from Aldrich Chemicals (Milwaukee, WI, USA); and ketanserin from Janssen (Beerse, Belgium). Clorgyline was a kind gift from Dr. D. Murphy (NIH Clinical Center), and paroxetine was donated by SmithKline-Beecham (Harlow, UK).

RESULTS

In Vivo Studies

The traces shown in Figure 1 illustrate typical voltammograms obtained in vitro and in vivo. The electrochemical signal was detected in vivo at 286 ± 11 mV (n = 66 electrodes tested) within a range of 200 to 450 mV. The intravenous administration of the decarboxylase inhibitor NSD 1015 (100 mg kg), which inhibits 5-HT and 5-HIAA synthesis, reduced the electrochemical signal by 92% ± 5% over a 2-hour period (n = 4). The monoamine oxidase inhibitor (MAOI) clorgyline (10 mg kg IV) reduced the in vivo signal by 40% ± 6% in the same time (n = 3), indicating that the recorded peak corresponded not only to 5-HT but also to 5-HIAA.

The 5-HT$_1$ agonist TFMPP (0.5 mg kg IV) reduced the 5-hydroxyindole signal by 18% ± 6% over a 2-hour period (Figs. 1A and 2A). 8-OH-DPAT (30 µg kg IV) produced a 23% ± 5% decrease of the signal over the same time (Fig. 2B). The effect of TFMPP was blocked (±)mianserin but not by (+)WAY100135 (Fig. 2A). (+)WAY100135 blocked the effect of 8-OH-DPAT (Fig.

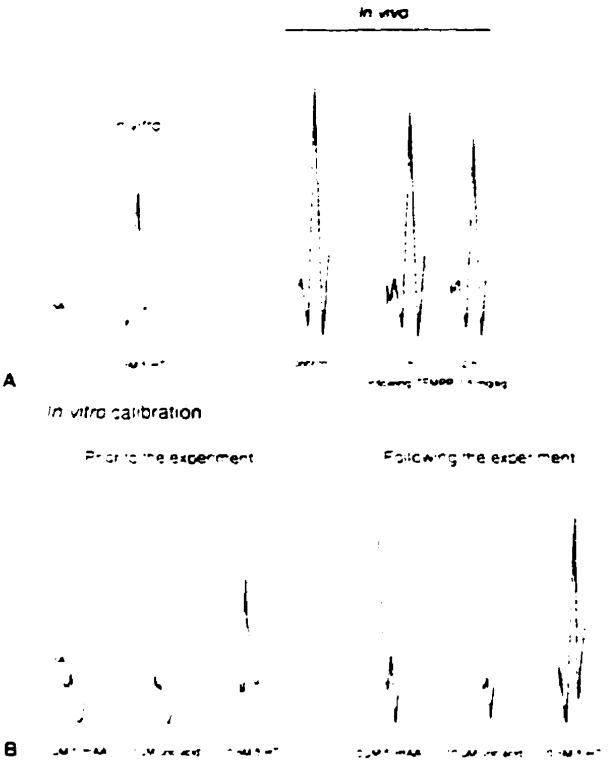

Figure 1. Individual voltammograms obtained with the same electrode (A) voltammograms obtained in vitro and in vivo before and at various intervals following the administration of TFMPP (0.5 mg/kg) and (B) in vitro in phosphate buffer containing either 10 nM 5-HT, 100 mM 5-HIAA, or 100 nM uric acid before and after an in vivo experiment. In vivo voltammograms were obtained every 10 minutes. Those represented in the figure were obtained immediately before as well as 1 hour and 2 hours following the administration of TFMPP.

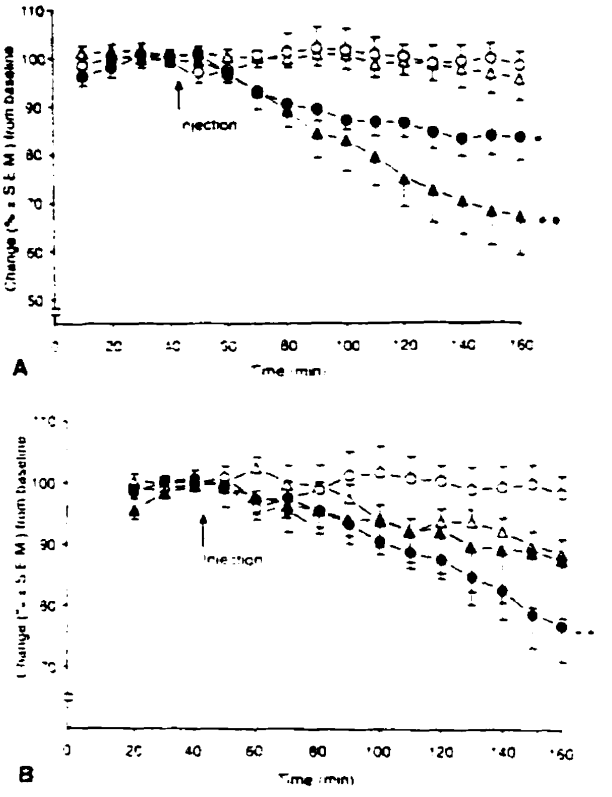

Figure 2. Modifications in the height of the 5-hydroxyindole peak recorded in the dorsal raphe nucleus following the intravenous administration of (A) TFMPP, 0.5 mg/kg (*solid circles*), (±)mianserin, 2 mg/kg, 30 minutes prior to TFMPP, 0.5 mg/kg (open triangles), (+)WAY100135, 0.5 mg/kg, 30 minutes prior to TFMPP, 0.5 mg/kg (*solid circles*), control (*open circles*). (B) 8-OH-DPAT, 30 μg/kg (*solid triangles*), (+)WAY100135, 0.5 mg/kg (open triangles), (+)WAY100135, 0.5 mg/kg, 30 minutes prior to 8-OH-DPAT, 30 μg/kg (*solid triangles*), control (open circles). Each value is expressed as a percentage of baseline ± SEM, the mean of the voltammograms of the 30-minute period before administration of the agonist being taken as 100%. $n = 5$. $^{*}p < .01$, $^{**}p < .001$ using two-factor ANOVA to compare with control curve.

2B). The coadministration of the 5-HT$_{1A}$ antagonist (+)WAY100135 and TFMPP produced greater reduction in baseline values than that produced by TFMPP alone (Fig. 2A), the latter phenomenon being probably due to an intrinsic effect of (+)WAY100135 because by itself this drug reduced the voltammetric signal by 8% ± 5% (Fig. 2B).

In Vitro Studies

The electrically evoked release of [^{3}H]5-HT from raphe slices was almost entirely calcium dependent, as demonstrated by its near-complete suppression by the removal of calcium from the perfusion medium (S_2/S_1 ratios: 1.0 ± 0.1, $n = 10$; and 0.1 ± 0.1, $n = 4$, control and calcium-free superfusion medium, respectively; $p < .001$). On the other hand, spontaneous outflow of tritium remained unchanged following calcium omission (Sp_2/Sp_1 ratios: 0.76 ± 0.02, $n = 10$, and 0.77 ± 0.06, $n = 4$ in slices superfused with normal and calcium-free Krebs, respectively). The differential effect observed on evoked and spontaneous tritium outflow following calcium omission is probably due to the different release mechanisms involved in each case. Indeed, it has been recently suggested that 5-HT may be released via two different mechanisms: (1) calcium-dependent, depolarization-induced vesicular release, and (2) cytoplasmic release, which occurs through the 5-HT carrier, by exchange diffusion and is driven by sodium gradient (Azmitia and Whitaker-Azmitia 1995).

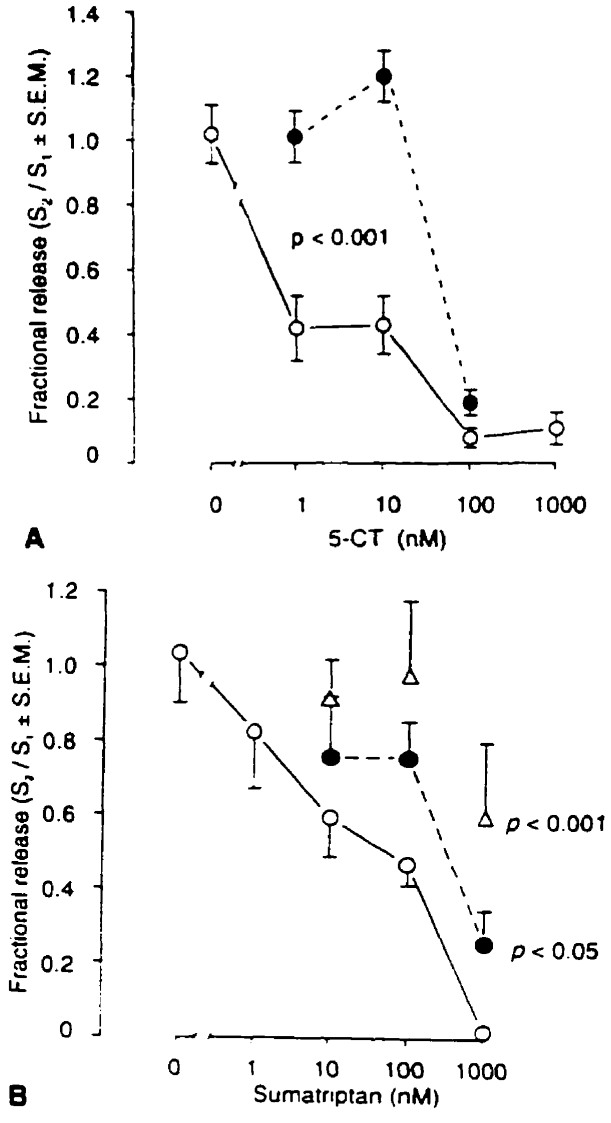

Figure 3. Concentration-effect curves of (**A**) 5-CT introduced 8 minutes before S_2 on the release of tritium elicited by the electrical stimulation of mesencephalic raphe slices preloaded with [^{3}H]5-HT (*open circle*). The *solid circles* show the curve for 5-CT in the presence of 1 μM methiothepin introduced 20 minutes before S_1. (**B**) sumatriptan introduced 8 minutes before S_2 (*open circles*). The filled circles show the curve for sumatriptan in the presence of 1 μM methiothepin and in the presence of GR 127935 (*open triangles*) introduced 20 minutes before S_1. Each point represents the mean ± SEM of 5 to 10 slices obtained from at least 2 to 5 rats. Curves were compared using a two-factor ANOVA.

electrically evoked tritium release from mesencephalic slices (Figs. 3A and 3B). Respective IC_{50} values of 5-CT and sumatriptan, calculated from the dose-response curves, were of 8 nM and 87 nM, indicating that 5-CT is more potent than sumatriptan ($p < .001$, when both curves were compared by ANOVA). Indeed, while a 100-nM concentration of 5-CT produced a near-complete inhibition of [^{3}H]5-HT release (92%), 100 nM of sumatriptan induced a submaximal inhibition (50%). At the same concentration the selective 5-HT$_{1A}$ agonist 8-OH-DPAT also induced a 50% reduction in evoked [^{3}H]5-HT release from mesencephalic slices (Fig. 4). Respective S_2/S_1 ratios for control slices and slices incubated with 100 nM 8-OH-DPAT were 1.0 ± 0.1 (n = 10) and 0.5 ± 0.1 (n = 5; $p < .01$). Except for 5-CT, which at the highest concentration used (1 μM) slightly but significantly reduced the Sp_2/Sp_1 ratio (control Sp_2/Sp_1 ratio: 0.76 ± 0.02, n = 10; 1 μM 5-CT Sp_2/Sp_1 ratio: 0.66 ± 0.02, n = 10; $p < .01$), basal tritium outflow remained unchanged throughout this series of experiments.

Unlike the 5-HT$_{1A}$ agonist 8-OH-DPAT and the 5-HT$_{1B/1D}$ agonist sumatriptan, the selective 5-HT$_{1B}$ agonist CP 93129 (Koe et al. 1992) did not inhibit 5-HT release from mesencephalic slices. Interestingly, the same doses that were ineffective at the cell body level in mesencephalic slices significantly reduced electrically evoked release of tritium in hippocampal slices (Fig. 5), where terminal autoreceptors are known to be of the 5-HT$_{1B}$ subtype (Maura et al. 1986, 1987). Concentrations of CP93129 higher than 100 nM increased spontaneous tritium outflow in hippocampus (Sp_2/Sp_1 ratio for 1 μM: 1.8 ± 0.1, n = 5, $p < .001$ compared to control) and dorsal raphe (respective Sp_2/Sp_1 ratios for 1 μM and 10 μM: 1.0 ± 0.1 and 0.9 ± 0.1, n = 5, $p < .001$ compared to control).

Surprisingly, in a broad concentration range (100 nM-10 μM), TFMPP did not inhibit the electrically evoked release of [^{3}H]5-HT from mesencephalic slices. However, at the highest concentrations assessed (10 μM-1 mM), it produced a dose-dependent increase in spontaneous tritium outflow, suggesting that it could be acting as a displacing agent of the intraneuronal stores of tritium. To test this possibility the effect of TFMPP was assessed in the presence of the selective 5-HT reuptake inhibitor (SSRI) paroxetine (1 μM) in the superfusion medium. Paroxetine, however, did not prevent the effect of TFMPP on spontaneous outflow but, in fact increased the Sp_2/Sp_1 ratio when the drug combination was used. These results are presented in Table 1.

Effect of 5-HT$_1$ Receptor Agonists on [^{3}H]5-HT Release. The 5-HT$_1$ agonist 5-CT and 5-HT$_{1D\ 1B}$ agonist sumatriptan (Hoyer 1991), introduced 8 minutes before S_2, produced a concentration-dependent inhibition of

Effects of 5-HT Antagonists on the Response to 5-HT$_1$ Agonists in the Mesencephalic Slices. Figure 3A shows the concentration-response curve for 5-CT in the absence and presence of methiothepin (1 μM). In the pres-

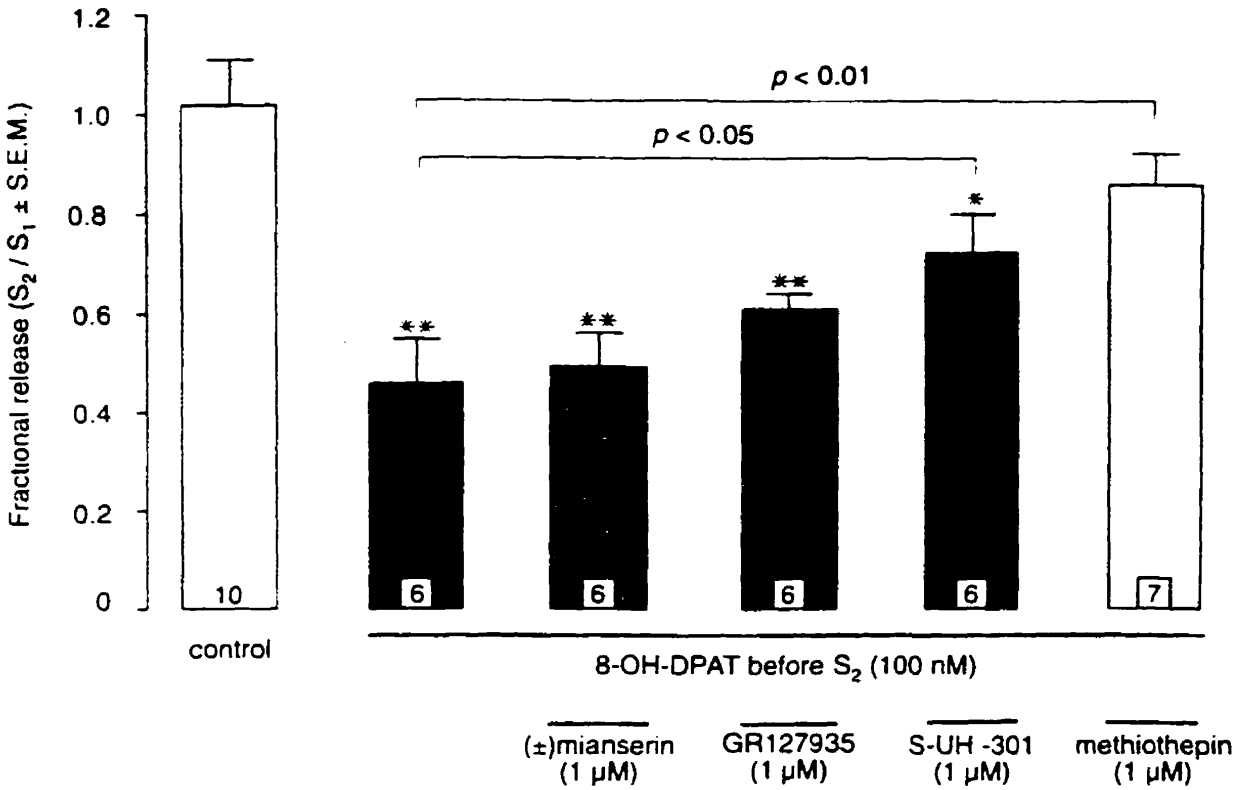

Figure 4. Effect of different 5-HT_1 antagonists on the inhibition of electrically evoked release of tritium produced by 8-OH-DPAT in mesencephalic raphe slices preloaded with [^{3}H]5-HT. The antagonists were introduced 20 minutes before S_1 and remained present in the superfusate until the end of the experiment. 8-OH-DPAT was introduced 20 minutes before S_2 and also remained present until the end of the experiment. Values are expressed as means ± SEM for which the number of experiments per group is given at the bottom of each column. Slices were obtained from three to five rats. The means were compared using a nonpaired Student's *t* test.

ence of methiothepin there was a significant reduction in the inhibition of the evoked [^{3}H]5-HT overflow ($p < .001$, comparing both curves by two-way ANOVA), with an eightfold increase in IC_{50} values (IC_{50} for 5-CT: 8-nM; IC_{50} for 5-CT + methiothepin, 1 μM: 63 nM). The effect of 5-CT (100 nM) was also blocked by the 5-HT_{1A} antagonists S-UH-301 (Fig. 6A; Björk et al. 1991) and (+)WAY100135 (Fletcher et al. 1993, Routledge et al. 1993). Observed S_2/S_1 ratios were 0.08 ± 0.03 for 5-CT (100 nM, $n = 10$) and 0.74 ± 0.06 for 5-CT (100 nM) plus (+)WAY100135 (1 μM; $n = 3$), respectively ($p < .001$). The 5-$HT_{1B\ 1D}$ antagonist GR 127935 (Skingle et al. 1993) and (±)mianserin, a drug with high affinity for the rat 5-HT_{1D} receptor subtype (Hamblin et al. 1992a) also blocked the effect of 5-CT (Fig. 6A).

The effect of 8-OH-DPAT (100 nM) was blocked by methiothepin and S-UH-301, but neither by the 5-$HT_{1B\ 1D}$ receptor antagonist GR 127935 nor by (±)mianserin (Fig. 4). The latter observations thus confirm that at the concentration of 100 nM, inhibition of stimulated [^{3}H]5-HT release by 8-OH-DPAT is a 5-HT_{1A}-mediated effect.

The concentration-effect curve of sumatriptan was shifted to the right by (±)mianserin ($p < .05$) and GR 127935 ($p < .001$; Figure 3B). The calculated IC_{50} values for sumatriptan, sumatriptan + (±)mianserin (1 μM), and sumatriptan + GR 127935 (1 μM) were 85, 227, and 467 nM, respectively. Methiothepin, but not the 5-HT_{1A} antagonist S-UH-301, blocked the effect of 100 nM sumatriptan (Fig. 6B). This observation indicates that the inhibitory effect of sumatriptan on electrically evoked release of [^{3}H]5-HT is not a 5-HT_{1A}-mediated response. The 5-$HT_{1A\ 1B}$ antagonist (−)propranolol, at a concentration of 1 μM, did not block the effect of sumatriptan (100 nM) either (respective S_2/S_1 ratios for sumatriptan and sumatriptan (±)propranolol were 0.4 ± 0.1 and 0.6 ± 0.1 ($n = 9$, $p = .6$). However, at a higher concentration of (−)propranolol (3 μM) the effect of sumatriptan was completely abolished (respective S_2/S_1 ratios for sumatriptan and sumatriptan

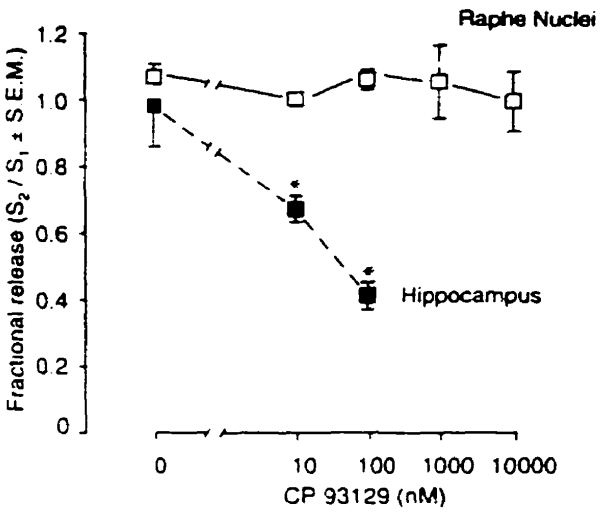

Figure 5. Concentration-effect curve of CP 93129 introduced 8 minutes before S_2 on the release of tritium elicited by the electrical stimulation of mesencephalic raphe slices (*open squares*) or hippocampal slices (*solid squares*) preloaded with [^{3}H]5-HT. Each point represents the mean ± SEM of 5 to 10 slices obtained from at least 2 to 5 rats. * $p < .001$ when compared to the control value using a nonpaired Student's *t* test.

(−)propranolol were 0.4 ± 0.1 and 1.1 ± 0.2 (n = 6, $p < .05$).

The effects of the antagonists themselves on evoked and basal tritium release, were assessed by comparing S_2 and Sp_2 values obtained in experiments in which the antagonist were introduced 20 minutes before S_1, to those obtained in control experiments. Methiothepin, GR 127935, and ketanserin significantly increased the amount of [^{3}H]5-HT released by S_1 as well as spontaneous tritium outflow (Sp_1). Presently ketanserin is the only drug that has been shown to distinguish between 5-HT$_{1Dα}$ and 5-HT$_{1Dβ}$ receptor subtypes (Doménech et

Table 1. Effect of TFMPP on Spontaneous and Evoked Release of [^{3}H]5-HT from Mesencephalic Slices

		S_2 S_1	Sp_2 Sp_1
Control		1 ± 0.1	0.76 ± 0.02
TFMPP	0.1 μM	1.0 ± 0.1	0.77 ± 0.03
	1 μM	0.9 ± 0.1	0.73 ± 0.08
	3 μM	0.9 ± 0.1	0.80 ± 0.03
	10 μM	1.0 ± 0.1	1.07 ± 0.01**
	100 μM	–	2.81 ± 0.31**
	1000 μM	–	9.63 ± 1.97
Paroxetine	(1 μM)		
+ TFMPP	1 μM	1.2 ± 0.1	1.00 ± 0.06**
	10 μM	3.1 ± 0.6*	2.5 ± 0.1**

* $p < .05$ (two-tailed Student's *t* test). ** $p < .001$ (two-tailed Student's *t* test). n = 4 to 10 slices obtained from 2 to 5 rats.

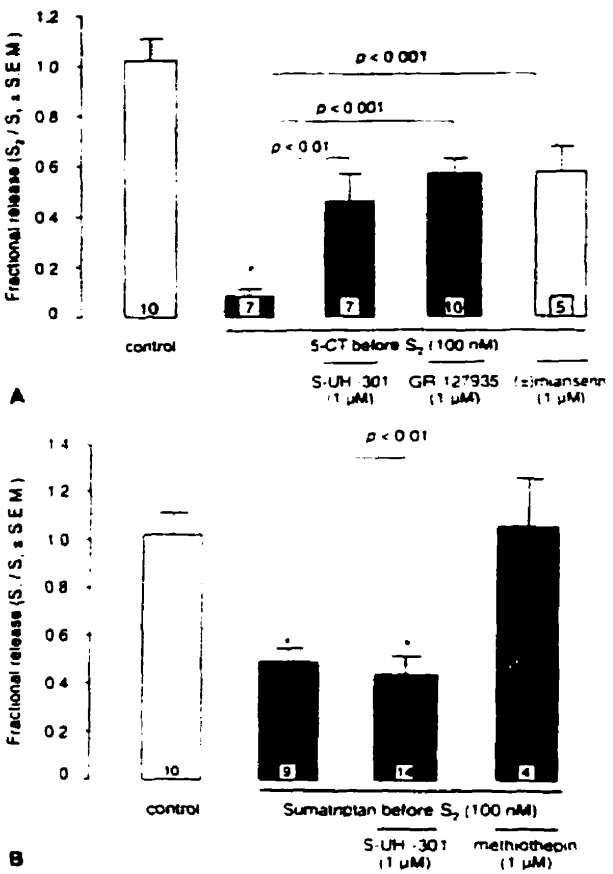

Figure 6. Effect of different 5-HT antagonists on the inhibition of electrically evoked release of tritium produced by (**A**) 5-CT or (**B**) sumatriptan in mesencephalic raphe slices preloaded with [^{3}H]5-HT. The antagonists were introduced 20 minutes before S_1 and remained present in the superfusate until the end of the experiment. 5-CT or sumatriptan were introduced 20 minutes before S_2 and also remained present until the end of the experiment. Values are expressed as means ± SEM for which the number of experiments per group is given at the bottom of each column. Slices were obtained from three to five rats. The means were compared using a nonpaired Student's *t* test.

Table 2. Effects of Putative 5-HT$_{1D}$ Antagonists on Spontaneous Release of [^{3}H]5-HT from Mesencephalic Slices

	Sp_1	S_1
Control	1.50 ± 0.07	1.8 ± 0.2
Methiothepin (1 μM)	2.00 ± 0.09*	6.8 ± 0.9**
GR 127935 (1 μM)	1.73 ± 0.08	4.5 ± 0.2**
Ketanserin (0.3 μM)	3.31 ± 0.15**	4.4 ± 0.5**
Ketanserin (1 μM)	4.31 ± 0.25**	5.8 ± 0.5**
Ketanserin (1 μM) + Paroxetine (1 μM)	6.30 ± 0.20**	

* $p < .01$ (two-tailed Student's *t* test). ** $p < .001$ (two-tailed Student's *t* test). n = 10 to 30 slices obtained from 5 to 15 rats.

al. 1994). Its effect on the inhibition of [^{3}H]5-HT release produced by sumatriptan could not be assessed because it induced a two- to threefold increase in spontaneous tritium overflow. This increase in outflow was not blocked by 1 μM of the SSRI paroxetine, introduced 20 minutes before S_1 (Table 2).

DISCUSSION

In the present study in vivo voltammetry and in vitro superfusion paradigms were used to demonstrate that functional 5-HT$_{1D}$ receptors modulate 5-HT release within the mesencephalic (dorsal and median) raphe nuclei of the rat brain. On the one hand, voltammetry results showed that 5-HT$_1$ receptors other than 5-HT$_{1A}$, and possibly 5-HT$_{1D}$, play a functional role in modulating in vivo the release of 5-HT in the dorsal raphe nucleus of the rat. On the other hand, the in vitro superfusion paradigm was used to complete the pharmacological characterization of the 5-HT receptor subtypes regulating 5-HT release in the mesencephalic raphe nuclei. Moreover, since somatodendritic sites are severed from their afferents in mesencephalic slices, this paradigm also allowed us to confirm that 5-HT release at the cell body level is modulated locally by 5-HT$_{1D}$ receptors. It was not possible, however, to determine whether 5-HT$_{1D}$ receptors in the rat mesencephalon are located on 5-HT neurons themselves or on interneurons, or whether they control somatodendritic or axon collateral release of 5-HT. However, given the small number of axon terminals in the dorsal raphe nucleus (Descarries et al. 1982) and the presence of abundant vesicles containing 5-HT in the dendrites of 5-HT neurons (Chazal and Ralston 1987), somatodendritic release might well be the predominant source of extracellular 5-HT in the raphe nuclei.

The electrochemical results obtained in vitro are consistent with the notion that electrically pretreated carbon-fiber and nafion-coated electrodes do not detect 5-HIAA (Fig. 1A). In the present study a 5,000- to 10,000-fold selectivity for 5-HT compared to 5-HIAA was achieved. For 5-HT uric acid a 8,000- to 15,000-fold selectivity factor was also attained (Fig. 1A). In vivo the administration of the xanthine oxidase inhibitor allopurinol (10 mg kg IP), which brings uric acid to undetectable levels in the brain (Cespuglio et al. 1986) further ensured the lack of contribution of uric acid to the electrochemical signal. Though the in vitro selectivity of the electrodes for 5-HT was still present after the in vivo experiment (see Materials and Methods section and Fig. 1A), during the time they were implanted into the dorsal raphe nucleus these electrodes detected 5-HIAA as well as 5-HT. The shape of the voltammograms obtained from the dorsal raphe in the present study is comparable to that monitored in vivo with DPV and uncoated carbon-fiber electrodes (Crespi 1990). However, the intravenous administration of the MAOI clorgyline produced a much smaller decrease of the voltammetric signal in the present study (40%) than in the latter one (70%). It is noteworthy, however, that the decrease in the voltammetric signal produced by blocking the production of 5-HIAA with clorgyline was smaller than that produced by the decarboxylase inhibitor NSD 1015, which blocks 5-hydroxytryptophan conversion into 5-HT. Moreover, the concentration of extracellular 5-HT in the dorsal raphe calculated from our experiments was of 12 nM, a value that is in perfect agreement with that of 10 nM obtained in vivo from the rat dorsal raphe by Crespi et al. (1988) using single-fiber nafion-coated electrodes. These observations thus indicate that in spite of the fact that in vivo the electrodes detected not only 5-HT but also 5-HIAA, the concentration of 5-HT was not overestimated as would have been the case if 5-HIAA had largely contributed to the signal. On the other hand, results from microdialysis studies suggest that the extracellular somatodendritic concentration of 5-HT would be lower (3–5 nM; Adell et al. 1993; Bel and Artigas 1992). However, a concentration gradient could account for this discrepancy because in the latter studies the dialysis probe was placed in the immediate vicinity (0.5 mm lateral from bregma) of the raphe nuclei while in the present one the tips of the electrodes were positioned directly into the dorsal raphe. In contrast with previous results, nafion-coated electrodes used in the present study detected 5-HIAA as well as 5-HT, which is presumably due to the fact that it is more difficult to coat entirely with nafion multifiber electrodes like the ones used in this study than single-fiber electrodes like those used by Crespi et al. (1988).

At the doses used the nonselective 5-HT$_1$ agonist TFMPP (Schoeffter and Hoyer 1989) and the selective 5-HT$_{1A}$ agonist 8-OH-DPAT produced similar attenuation in the 5-hydroxindole peak recorded from the dorsal raphe nucleus (Fig. 2). The inhibitory effects observed in the present study are consistent with those previously reported by Blier et al. (1990) following systemic administration of 8-OH-DPAT (30 μg kg IV) and RU 24969 (10 mg kg IP). In the Blier et al. (1990) study, however, the reduction in the voltammetric signal 2 hours after the injection of both agonists was of about 50%. Given that nafion-coated electrodes were used in the present study but not in the former, a reduced contribution of 5-HIAA to the voltammetric peak could explain the smaller effect observed. The fact that the 5-HT$_{1A}$ antagonist (+)WAY100135 (Fletcher et al. 1993) blocked the effect of 8-OH-DPAT, but not that of TFMPP, indicates that only 8-OH-DPAT-induced decrease in dorsal raphe extracellular indoles is mediated via 5-HT$_{1A}$ receptors. (+)WAY100135 produced an apparent potentiation of the effect of TFMPP (Fig. 2A). This could be due to an intrinsic effect of (+)WAY100135

because by itself (+)WAY100135 produced a small reduction in the voltammetric signal (8%, NS; Fig. 2B). Two mechanisms could explain the intrinsic activity of (+)WAY100135 observed in the present study: (1) a direct partial agonistic effect at 5-HT$_{1A}$ receptors (Escandon et al. 1994); or (2) an antagonistic activity at α_1-adrenergic receptors (Lanfumey et al. 1993). It is worth noting that at the dose used (0.5 mg/kg IV) in the present study, (+)WAY100135 has been shown to decrease 5-HT neuron firing activity by 20% (Fletcher et al. 1993), in keeping with its putative 5-HT$_{1A}$ partial agonistic action.

(±)Mianserin is a nonselective 5-HT antagonist, its highest affinity being for 5-HT$_{2A}$ (8 nM; Hoyer 1991) and rat 5-HT$_{1D}$ binding sites (30 nM; Hamblin et al. 1992a). This drug readily distinguishes 5-HT$_{1D}$ and 5-HT$_{1B}$ sites in rat cell lines as it binds to the former with an affinity more than 10,000-fold higher than for the latter (Hamblin et al. 1992a). Because TFMPP is an agonist selective for 5-HT$_1$ receptors (Shoeffter and Hoyer 1989), the reduction induced by this drug on extracellular 5-hydroxyindole concentration in the dorsal raphe cannot be ascribed to the stimulation of 5-HT$_{2A}$ receptors, even if its effect is blocked by (±) mianserin. The ineffectiveness of (+)WAY100135 to block the effect of TFMPP ruled out a role for 5-HT$_{1A}$ receptors, and the ability of (±)mianserin to suppress the TFMPP-induced decrease in the voltammetric signal suggests that 5-HT$_{1D}$, rather than 5-HT$_{1B}$, receptors mediate the effect of systemic administration of TFMPP. To test this contention the pharmacological profile of the receptors that regulate somatodendritic 5-HT release was studied in vitro.

The following evidence confirms the presence of 5-HT$_{1A}$ receptors that negatively control 5-HT release within the rat mesencephalic raphe nuclei: (1) the inhibitory effect of the 5-HT$_1$ agonist 5-CT on tritium-evoked release was blocked by 5-HT$_{1A}$ receptor antagonists UH-301 (Fig. 6A) and (+)WAY100135 (Bjork et al. 1991, Fletcher et al. 1993, Routledge et al. 1993); and (2) the 5-HT$_{1A}$ agonist 8-OH-DPAT inhibited tritium release (Fig. 4). These observations are consistent with the suppressant effect of microiontophoretic application of 8-OH-DPAT on the firing activity of 5-HT neurons in the dorsal raphe (Blier et al. 1987). 8-OH-DPAT also has affinity for 5-HT$_{1D}$ receptors (Schoeffter and Hoyer 1990). However, at the concentration used (100 nM) these receptors were not activated because the 5-HT$_{1B/1D}$ antagonist GR 127935 did not block the effect of 8-OH-DPAT (Fig. 4).

Sumatriptan, which binds with nanomolar affinity to 5-HT$_{1D}$ sites (Hoyer 1991), also inhibited [^{3}H]5-HT release from slices containing the mesencephalic raphe nuclei (Fig. 3B), and this effect was blocked by GR 127935 (Fig. 3B). However, sumatriptan has only a 10-fold selectivity in discriminating 5-HT$_{1D}$ from 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors (Hoyer 1991). A role for 5-HT$_{1A}$ receptors in this inhibitory effect of sumatriptan may be ruled out since the 5-HT$_{1A}$ antagonist S-UH-301 (Björk et al. 1991) did not alter the effect of sumatriptan (Fig. 6A). Evidence supporting the involvement of 5-HT$_{1D}$, and not that of 5-HT$_{1B}$, receptors in the inhibitory effect of sumatriptan is twofold: (1) (±)mianserin, which does not significantly bind to 5-HT$_{1B}$ receptors (Hamblin et al. 1992a), blocked this effect of sumatriptan (Fig. 3B), (2) though the selective 5-HT$_{1B}$ agonist CP 93129 (Koe et al. 1992) inhibited 5-HT release from hippocampal slices where 5-HT release is controlled by 5-HT$_{1B}$ receptors (Maura et al. 1986, 1987), this drug was devoid of effect on the evoked release of [^{3}H]5-HT from mesencephalic slices (Table 1). At some of the concentrations used (1 and 10 μM), CP 93129 produced an increase in basal outflow of tritium from mesencephalic slices. It is unlikely, however, that the effect of CP 93129 on basal outflow could account for its lack of effect on electrically evoked release of [^{3}H]5-HT because at the concentration of 100 nM, which did not modify basal outflow (Table 1), it did not affect the evoked release of tritium. The fact that (−)propranolol (1 μM) did not block the effect of sumatriptan (100 nM) further supports the notion that 5-HT$_{1D}$, not 5-HT$_{1B}$, receptors negatively regulate 5-HT release in the raphe nuclei. The effectiveness of higher concentrations (3 μM) to block the effect of sumatriptan (100 nM) could be due to a loss of selectivity. Indeed, (−)propranolol binds to rat 5-HT$_{1D}$ receptors with a K_D of 1,800 nM (Hamblin et al. 1992a). Because methiothepin blocked the effect of sumatriptan (Fig. 6B) and 5-HT$_{1E}$ receptors have only modest affinity for this drug (McAllister et al. 1992; Zgombick et al. 1992), it is improbable that the latter receptors account for the results obtained with sumatriptan. Sumatriptan also binds to 5-HT$_{1F}$ sites; in contrast 5-CT binds only with low affinity to these sites (Glennon and Dukat 1995). It is therefore difficult to reconcile a role of 5-HT$_{1F}$ receptors with the inhibitory effect of 5-CT, which, on the other hand, is blocked by the 5-HT$_{1D/1B}$ antagonist GR 127935 (Fig. 6A).

The increase in [^{3}H]5-HT release observed with methiothepin and GR 127935 (Table 2) is consistent with previous findings (Moret and Briley 1993; Starkey and Skingle 1994; Wilkinson and Middlemiss 1992) and has been attributed to the blockade of autoreceptor activation caused by released 5-HT. Interestingly, the 5-HT$_{1A}$ antagonist S-UH-301 (Bjork et al. 1991) did not produce an increase in tritium-evoked release, although other 5-HT$_{1A}$ antagonists such as (+)WAY100135 have been shown to do so (Starkey and Skingle 1994). This could be explained by the lower affinity of S-UH-301 for the 5-HT$_{1A}$ receptor. Indeed, respective IC$_{50}$ values for S-UH-301 and (+)WAY100135 in displacing [^{3}H]8-OH-DPAT from 5-HT$_{1A}$ receptors are 157 and 34 nM (Cliffe et al. 1993; Cornfield et al. 1991).

The present results are in agreement with those of Starkey and Skingle (1994) showing the presence of functional 5-HT_{1D} receptors in the dorsal raphe of the guinea pig. Molecular biology techniques have revealed that there exist two 5-HT_{1D} receptor subtypes: 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ (Hamblin and Metcalf; Hamblin et al. 1992a, 1992b; Weinshank et al. 1992). The pharmacological profiles of these two receptors are very similar (Hartig et al. 1992), ketanserin being the only compound tested thus far able to differentiate them (Doménech et al. 1994). Because of these similarities, Starkey and Skingle (1994) were unable to conclude whether one or the two 5-HT_{1D} receptor subtypes control 5-HT release at the somatodendritic level. The effect of ketanserin on sumatriptan-induced inhibition of evoked [^{3}H]5-HT release could not be assessed in the present study because at the concentrations tested (0.3 and 1 μM) ketanserin doubled to tripled spontaneous 5-HT outflow, an effect that could not be blocked by the SSRI paroxetine (Table 2). However, since rat 5-HT_{1D} and 5-HT_{1B} receptors are species homologues of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors, respectively (Hartig et al. 1992), it is possible, that in those species where both receptors exist, including humans (Weinshank et al. 1992), it is the 5-$HT_{1D\alpha}$ subtype that would control somatodendritic release of 5-HT.

Consistent with the present findings, Davidson and Stamford (1994) have recently reported that 5-HT_1 receptors, other than 5-HT_{1A} autoreceptors, regulate 5-HT release in the rat dorsal raphe nucleus. The drugs used in the latter study, unlike the present one, did not allow the authors to conclude which 5-HT_1 receptor subtype was involved. We have recently provided evidence that 5-HT_{1A} and 5-HT_{1D} receptors are not functionally redundant because 5-HT_{1A} receptors control 5-HT release in the cell body area by regulating 5-HT neuron-firing activity, whereas 5-HT_{1D} receptors control release in a firing-independent manner (Piñeyro et al. 1993, 1994). Furthermore, Craven et al. (1994) have recently found that GR 127935 does not attenuate 5-HT induced inhibition of firing of 5-HT neurons recorded from guinea pig midbrain slices.

TFMPP inhibited 5-HT release in vivo but not in vitro (Fig. 2A and Table 2). This is not surprising considering that similar results have been obtained when the effect of RU 24969 was assessed in the rat brain. In vivo RU 24969 decreased 5-HT release in frontal cortex (Blier et al. 1990; Crespi et al. 1990) but in vitro it increased 5-HT release from hippocampal slices (Auerbach et al. 1990). The enhancing effect of RU 24969 and TFMPP on 5-HT release in vitro were abolished by the SSRI fluoxetine. It was concluded from this set of results that at low concentrations both agonists stimulate the terminal autoreceptor and inhibit 5-HT release, whereas at higher concentrations, they interact with the 5-HT carrier (Auerbach et al. 1990). In the present study, the effect of TFMPP on the evoked release of [^{3}H]5-HT was tested over a large range of concentrations, and no significant inhibition was observed. The possibility of an interaction with the 5-HT carrier was also explored; paroxetine did not block the effect of TFMPP on the Sp_2/Sp_1 ratio; in fact, it potentiated the releasing effect of TFMPP. Another explanation for these results could be derived from the observation of Sinton and Fallon (1988) that TFMPP decreases 5-HT neuron-firing activity in the dorsal raphe and stimulates that of 5-HT neurons in the median raphe. Because mesencephalic slices used in the present study contained both nuclei, it is therefore possible that the effects on both nuclei cancel each other out. Alternatively, it should be considered that TFMPP-induced changes in the in vivo voltammograms may have reflected a decrease not only in 5-HT but also in 5-HT metabolism. In fact, systemic administration of RU 24969 has been found to decrease 5-HT release and 5-HT metabolism, monitored in frontal cortex with nafion-coated and uncoated electrodes, respectively (Crespi et al. 1990).

In conclusion, the results obtained in the present study demonstrate the existence of functional 5-HT_{1D} receptors that control the release of 5-HT from mesencephalic raphe nuclei in the rat brain.

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Regulation of 5-Hydroxytryptamine Release from Rat Midbrain Raphe Nuclei by 5-Hydroxytryptamine$_{1D}$ Receptors: Effect of Tetrodotoxin, G Protein Inactivation and Long-Term Antidepressant Administration[1]

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ABSTRACT

Our study was undertaken to characterize the functional properties of 5-hydroxytryptamine (5-HT)$_{1D}$ receptors in the rat midbrain raphe nuclei. In a first series of experiments, designed to assess whether 5-HT$_{1D}$ receptors are coupled to G_{o} proteins, the intracerebral injection of pertussis toxin into the dorsal raphe as well as incubation of midbrain raphe slices with the alkylating agent N-ethyl-maleimide (NEM) reduced the efficacy of the 5-HT$_{1B/1D}$ agonist sumatriptan to inhibit the electrically evoked overflow of [^{3}H]5-HT from preloaded slices. Furthermore, preincubation with NEM also reduced the efficacy with which the 5-HT$_{1B/1D}$ antagonist GR 127935 enhanced evoked overflow of [^{3}H]5-HT. These results indicate that, in rat midbrain raphe nuclei, 5-HT$_{1D}$ receptors are linked to G_{o} proteins. In an attempt to determine whether 5-HT$_{1D}$ receptors are located on 5-HT neurons, the inhibitory effect of sumatriptan and of the nonselective 5-HT agonist 5-carboxyamidotryptamine on K$^+$-evoked overflow of [^{3}H]5-HT was assessed in the presence of the Na$^+$ channel blocker tetrodotoxin. Neither the inhibitory effect of sumatriptan nor that of 5-carboxyamidotryptamine were reduced by the addition of tetrodotoxin to the superfusion medium, suggesting that these 5-HT$_{1D}$ receptors are located on 5-HT neurons and may be considered autoreceptors. In a third series of experiments, rats were treated for 21 days either with the selective 5-HT reuptake inhibitor paroxetine (10 mg/kg/day, s.c.) or the reversible type A monoamine oxidase inhibitor befloxatone (0.75 mg/kg/day, s.c.) and superfusion experiments were performed after a 48-hr washout period. 5-HT$_{1D}$ receptors, similarly to 5-HT$_{1A}$ autoreceptors, desensitize after long-term treatment with a selective 5-HT reuptake inhibitor or a reversible type A monoamine oxidase inhibitor because the efficacy of sumatriptan and of 8-OH-DPAT to inhibit the electrically evoked overflow of [^{3}H]5-HT was reduced after the administration of either drug.

The use of molecular cloning techniques has revealed a great diversity of 5-HT$_1$ receptors (see Beer *et al.*, 1993). This diversity exists not only within the 5-HT$_1$ receptor family, but also within subclasses of 5-HT receptors, particularly the 5-HT$_{1D}$ receptors. In humans, there are two different types of 5-HT$_{1D}$ receptors, 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$, which are encoded by two distinct genes located on different chromosomes (Jin *et al.*, 1991; Demchyshyn *et al.*, 1992; Weinshank *et al.*, 1992). These two receptors are almost indistinguishable from a pharmacological point of view (Doménech *et al.*, 1994). However, in the rat brain, 5-HT$_{1B}$ receptors are the species homologue of the 5-HT$_{1D\beta}$ receptor (Hartig *et al.*, 1992) and have a distinct pharmacological profile different from that of 5-HT$_{1D\alpha}$ receptors (Bruinvels *et al.*, 1993; Hamblin *et al.*, 1992). Radioligand binding studies have shown that, compared to 5-HT$_{1B}$ receptors, 5-HT$_{1D\alpha}$ receptors are sparsely distributed in the rat central nervous system and the former always represent the vast majority of binding sites where 5-HT$_{1B/1D}$ receptors are detected (Bruinvels *et al.*, 1993). In the dorsal raphe, 5-HT$_{1B}$ binding sites, labeled with [^{125}I]cyanopindolol, represent 70% of the sites marked by the 5-HT$_{1B/1D}$ ligand serotonin-5-*O*-carboxymethylglycyl[^{125}I]tyrosinamide (Bruinvels *et al.*, 1993). These findings suggest that the remaining 30% of sites marked by [^{125}I]GTI correspond to 5-HT$_{1D\alpha}$ sites. The presence of 5-HT$_{1D}$ binding sites in the rat midbrain has also been described by Herrick-Davis and Titler (1988) where they were found to account for 42% of 5-HT$_1$ binding sites.

Despite their lower density compared to 5-HT$_{1B}$ binding sites,

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; NEM, N-ethyl-maleimide; SSRI, selective 5-HT reuptake inhibitor; 5-CT, 5-carboxyamidotryptamine; TTX, tetrodotoxin; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; ANOVA, analysis of variation; MAOI, monoamine oxidase inhibitor; SSRI, selective serotonin reuptake blocker; GTI, serotonin 5-O-carboxymethyl-[^{125}I] tyrosinamide.

a functional role has been attributed to 5-HT_{1D} receptors in the rat raphe nuclei (Piñeyro *et al.*, 1994a; 1996a). In preloaded midbrain slices containing median and dorsal raphe nuclei as well as the caudal linear nucleus, the 5-$HT_{1B/1D}$ agonist sumatriptan potently inhibits the electrically-evoked overflow of [^{3}H]5-HT (Piñeyro *et al.*, 1996a). The latter effect cannot be attributed to 5-HT_{1B} receptor activation because it was not mimicked by the selective 5-HT_{1B} agonist CP 93129 (Macor *et al.*, 1990; Piñeyro *et al.*, 1995a). However, the inhibitory effect of sumatriptan was blocked by the 5-$HT_{1B/1D}$ antagonist GR 127935 (Skingle *et al.*, 1993; Piñeyro *et al.*, 1995a) and also by mianserin (Piñeyro *et al.*, 1995a), a drug with high affinity for rat 5-HT_{1D} but not 5-HT_{1B} receptors (Hamblin *et al.*, 1992). Moreover, although 5-HT_{1D} receptor antagonists blocked the effect of sumatriptan, the selective 5-HT_{1A} antagonist S-UH-301 (Björk *et al.*, 1991) did not modify the sumatriptan-mediated inhibition of electrically-evoked overflow of [^{3}H]5-HT. Furthermore, sumatriptan inhibits the evoked [^{3}H]5-HT overflow from midbrain raphe slices of 5-HT_{1B} knock-out mice, an effect which is not blocked by the 5-HT_{1A} antagonist (+)WAY 100135 but by the 5-$HT_{1B/1D}$ antagonist GR 127935 (Piñeyro *et al.*, 1995b). These observations further support the fact that murine 5-HT_{1D} receptors negatively regulate 5-HT release in midbrain raphe nuclei.

A putative role for 5-HT_{1D} receptors as presynaptic heteroreceptors on spiny striatal GABAergic neurons has been recently proposed (Bruinvels *et al.*, 1994). Relatively high levels of 5-$HT_{1D\alpha}$ mRNA have also been found in the locus coeruleus (Bruinvels *et al.*, 1994), suggesting the possibility that this receptor could also be an heteroreceptor on noradrenergic neurons. Moreover, after 5-HT denervation with 5,7-dihydroxytryptamine, the total amount of 5-$HT_{1D\alpha}$ mRNA in the rat dorsal raphe was more than doubled (Neumaier *et al.*, 1994). Although compensatory changes in non-5-HT neurons may account for an increased rate in mRNA synthesis, sprouting of new 5-HT dendritic and axonal processes may also explain such an increase in mRNA content. Functional evidence for the location of release-modulating 5-HT receptors on 5-HT terminals has been previously obtained from superfusion experiments in which 5-HT release was evoked in the presence of tetrodotoxin (see Starke *et al.*, 1989). Tetrodotoxin, by blocking Na^+ channels, prevents the occurrence of Na^+-dependent action potentials. In such circumstances when [^{3}H]5-HT overflow is evoked with high concentrations of potassium, only 5-HT cell bodies or 5-HT axon terminals remain potential targets of drug action (Starke *et al.*, 1989).

Our study was thus undertaken to further characterize the 5-HT_{1D}-mediated response in the rat midbrain raphe nuclei. Three questions were specifically addressed: determine whether 5-HT_{1D} receptors in the midbrain raphe, similar to 5-HT_{1A} autoreceptors, 1) are linked to $G_{i/o}$ proteins, 2) are located on 5-HT neurons and 3) desensitize after long-term treatment with a selective 5-HT reuptake inhibitor or a reversible type-A MAOI. Results from our study have been presented at the XIXth C.I.N.P. Congress (Piñeyro *et al.*, 1994) and at the 24th Annual Meeting of the Society for Neuroscience (Piñeyro *et al.*, 1994a).

Materials and Methods

Treatments. Male Sprague Dawley rats (225–250 g), were implanted s.c., under halothane anesthesia with an osmotic minipump (Alza, Palo Alto, CA) delivering either the SSRI paroxetine (10 mg/kg/day; SmithKline Beecham, Harlow, England), the reversible and selective MAO-A inhibitor befloxatone (0.75 mg/kg/day; Delalande, Rueil-Malmaison, France) or the vehicle used for diluting these drugs (50/50 ethanol and water). After 21 days, the minipumps were removed under halothane anaesthesia and *in vitro* release experiments were carried out 48 hr later, to allow elimination of drugs. Another group of rats was anesthetized with choral hydrate (400 mg/kg, i.p.) and mounted in a stereotaxic frame. Pertussis toxin (Sigma Chemical Co., St. Louis, MO) was dissolved in physiological saline, and 1 μg in 2 μl was injected at rate of 0.4 μl/min into the dorsal raphe at a 45° backward angle to spare the median sinus, (A = 0.43, L = 0 and V = 0.5 with respect to interaural zero). Control rats received an equal volume of the vehicle. Superfusion experiments were carried out 3 to 10 days later. This interval was chosen because it has been previously demonstrated that $G_{i/o}$ protein inactivation in the dorsal raphe takes place during such a time period (Innis and Aghajanian, 1987).

Superfusion experiments. Rats were sacrificed by decapitation and their brains removed and dissected on an ice-cold glass plate. Midbrain slices (0.4-mm thick) containing dorsal and median raphe nuclei as well as caudal linear nucleus, were prepared with a McIlwain chopper and incubated for 30 min at 37°C in Krebs solution containing 100 nM [^{3}H]5-HT creatinine sulphate (specific activity 22.7 Ci/mmol, NEN Research Products, Mississauga, Canada). Kreb's was constantly bubbled with a mixture of 95% O_2:5% CO_2. The composition of the Krebs solution was: 118 mM NaCl, 4.7 mM $CaCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 0.004 mM Na_2EDTA and 0.11 mM ascorbic acid. In some experiments, slices were preincubated in Kreb's solution alone or containing 30 μM of the alkylating agent NEM for 30 min, then washed twice and incubated with [^{3}H]5-HT. At the end of the incubation period with [^{3}H]5-HT, one midbrain slice was transferred into each separate glass chamber and superfused continuously at a rate of 0.5 ml/min with Kreb's solution maintained at 37°C and saturated with the O_2/CO_2 mixture. Nineteen consecutive 4-min fractions were collected starting 60 min after the beginning of superfusion. The slices were stimulated twice 8 min (S_1) and 44 min (S_2) after the end of the washing period. The electrical field generated in the chambers between two platinum electrodes (2-cm apart) had a voltage drop of 5 V/cm. The stimulation parameters used were: 360 pulses of 2 msec duration, 30 mA intensity delivered for 2 min at 3 Hz. This stimulation frequency was chosen because it is within the range of the firing rate of 5-HT neurons recorded in freely moving cats (Jacobs, 1986). For K^+-evoked release, slices were stimulated twice (S_1 and S_2), for 4 min, by changing the regular Kreb's solution to another one containing 35 mM KCl with equimolar reduction in NaCl to maintain isotonicity. The first stimulation period, either for electrical or K^+ stimulation, was always used as control. Except for antagonists that were added 20 min before S_1, all drugs were added 20 min before S_2 and remained present until the end of the superfusion. At the end of the experiment slices were solubilized in 0.5 ml Soluene 350 (Packard Instruments, Downers Grove, IL) and radioactivity in the slices and superfusate samples was determined by scintillation spectrometry. The amount of tritium released per 4-min sample was expressed as a fraction of total tritium contained in the tissue at the start of the respective collection period. The overflow of tritium produced by the electrical stimulation was calculated as the total increase in radioactivity above the basal outflow of tritium determined in the sample immediately preceding the start of stimulation (Sp_1 and Sp_2). To assess the drug-induced changes of evoked overflow of radioactivity, S_2/S_1 ratios were calculated. Sp_2/Sp_1 ratios were also examined to determine whether the drugs altered the basal outflow of radioactivity. The amount of tritium released by electrical stimulation in brain slices under these conditions provides a reliable estimate of the release of tritiated 5-HT (Moret and Briley, 1990; Blier and Bouchard, 1993).

The *ns* refer to the number of superfusion chambers studied. Each

chamber was loaded with one slice and two to four rats were used to calculate the mean for each drug concentration. All results are expressed as means ± S.E.M. To detect treatment effects, concentration-effect curves were constructed by running in parallel experiments with slices obtained from treated rats and the respective controls. Controls for NEM and long-term treatments were pooled in the experimental series with GR 127935 and controls for pertussis and NEM were pooled in the experimental series for sumatriptan. Three to five pairs of rats, treated and control, were used for each drug concentration. When the effect of a given drug was compared in slices obtained from controls to the effect produced in slices obtained following different treatments dose-response curves were compared by two-way ANOVA. When the effect of different drug concentrations was studied within a given group of rats (either control or treated), one-way ANOVA in combination with a *post hoc* test (Neuman-Keuls) was used. Student's *t* test was used whenever two means were compared. *P* values smaller than .05 were considered as significant.

Drugs. The following drugs were used in the superfusion experiments: 8-OH-DPAT and tetrodotoxin (TTX) from Research Biochemicals Incorporated (Natick, MA), GR 127935 and sumatriptan from Glaxo (Greenford, UK) and (+)WAY100135 from Wyeth Research (Berkshire, UK).

Results

Effect of G protein inactivation on 5-HT$_{1A}$- and 5-HT$_{1D}$-mediated inhibition of [^{3}H]5-HT overflow. 8-OH-DPAT (10–100 nM) and sumatriptan (10–1000 nM) reduced the electrically evoked overflow of [^{3}H]5-HT from preloaded midbrain slices (fig. 1). At the dose used in this study, pertussis toxin, which inactivates $G_{i/o}$ proteins by ADP ribosylating their α subunits (Gilman, 1987), has been shown to virtually abolish the inhibitory effect of 5-HT$_{1A}$ agonists on 5-HT neuron firing frequency (Innis and Aghajanian, 1987; Blier *et al.*, 1993). The effect of the pertussis toxin pretreatment on the inhibition of evoked overflow of [^{3}H]5-HT induced by sumatriptan and 8-OH-DPAT in midbrain slices is shown in figure 1. The effect of 8-OH-DPAT was abolished and, that of sumatriptan was significantly attenuated. However, the inhibition produced by 1 μM sumatriptan, although attenuated, was still significant in the pertussis pretreated group (fig. 1B). In slices obtained from rats pretreated with pertussis toxin, 1 μM sumatriptan induced a 47% inhibition although in controls it inhibited [^{3}H]5-HT overflow by 59%.

The next series of experiments was carried out to verify, via an alternative inactivation strategy, whether 5-HT$_{1D}$ receptors are linked to $G_{i/o}$ proteins. Midbrain slices were incubated with the alkylating agent NEM for 30 min at a concentration of 30 μM. *In vitro* preincubation with NEM reduced the effectiveness of sumatriptan to inhibit [^{3}H]5-HT overflow at all concentrations (fig. 2A). Unlike results observed in slices obtained from pertussis toxin pretreated rats, 1 μM sumatriptan did not produce a significant inhibition of [^{3}H]5-HT release in slices preincubated with NEM. In respective control slices run in parallel, 1 μM sumatriptan induced a 59% inhibition of [^{3}H]5-HT release (fig. 2A). To further document the functional coupling of 5-HT$_{1D}$ receptors to $G_{i/o}$ proteins, the effect of the 5-HT$_{1B/1D}$ antagonist GR 127935 was assessed in control slices and in slices preincubated with NEM. An increase in evoked [^{3}H]5-HT overflow from midbrain slices after introducing the 5-HT receptor antagonist GR 127935 has been previously reported in rats (Piñeyro *et al.*, 1995a) and guinea pigs (Starkey and Skingle, 1994). If 5-HT$_{1D}$ receptors are coupled to a $G_{i/o}$ protein, then the alkylating agent NEM should reduce the enhancing effect of the 5-HT$_{1B/1D}$ antagonist GR 127935. In control slices, GR 127935 introduced 20 min before S_2 increased not only spontaneous tritium outflow but also electrically evoked overflow of [^{3}H]5-HT. At the concentrations of 0.3 and 1 μM, GR 127935 induced 64 and 198% increases of S_2/S_1 ratios, respectively (fig. 2B). In slices incubated with NEM, the enhancing effect of 1 μM GR 127935 was markedly reduced (S_2/S_1 ratio was increased only by 32%) (fig. 2B) and spontaneous tritium outflow remained unchanged (table 1).

The release of [^{3}H]5-HT induced by the electrical stimulation during S_1 was greater in slices prepared from rats pretreated with pertussis toxin (56% increase) and in slices preincubated with NEM (51% increase) than in controls processed in parallel (fig. 3). It is noteworthy that in slices in

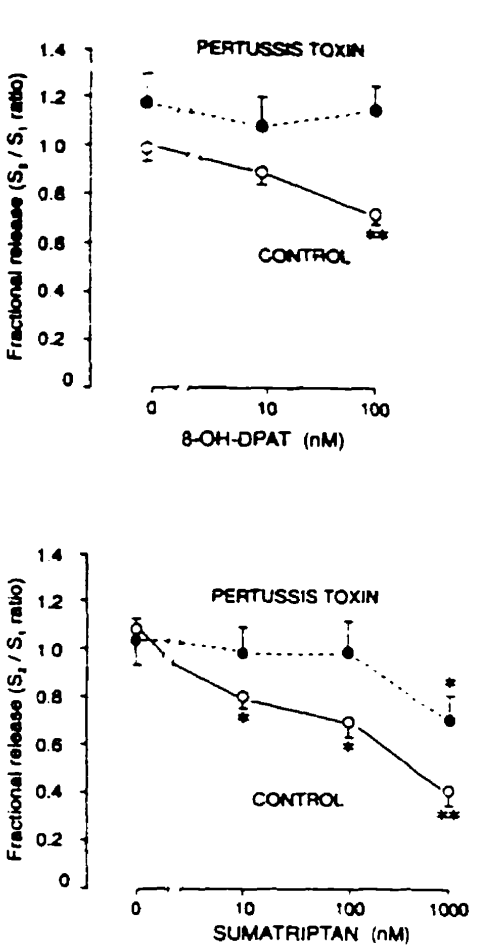

Fig. 1. Inhibition produced by the 5-HT$_{1A}$ agonist 8-OH-DPAT (A) or the 5-HT$_{1D}$ agonist sumatriptan (B) of the evoked release of tritium elicited by electrical stimulation in preloaded midbrain slices prepared from control (open circles) and pertussis-treated rats (1 μg injected intraraphe, solid circles). The experiments were carried out 3 to 10 days after treatment. Ordinates: fraction of total tissue radioactivity released by 360 pulses (30 mA, 2 min, 3 Hz). Values are expressed as the ratio (S_2/S_1) obtained between the second period of stimulation in the presence of agonist (S_2) and the first one carried out in the absence of the agonist (S_1). Each point represents the mean ± S.E.M. of the results obtained in experiments run in parallel using three to four pairs of control and treated rats. Level of statistical significance was calculated using one-way analysis of variance to compare the effect of 8-OH-DPAT (A) or sumatriptan (B) within control or treated groups. ** $P < .01$. * $P < .05$.

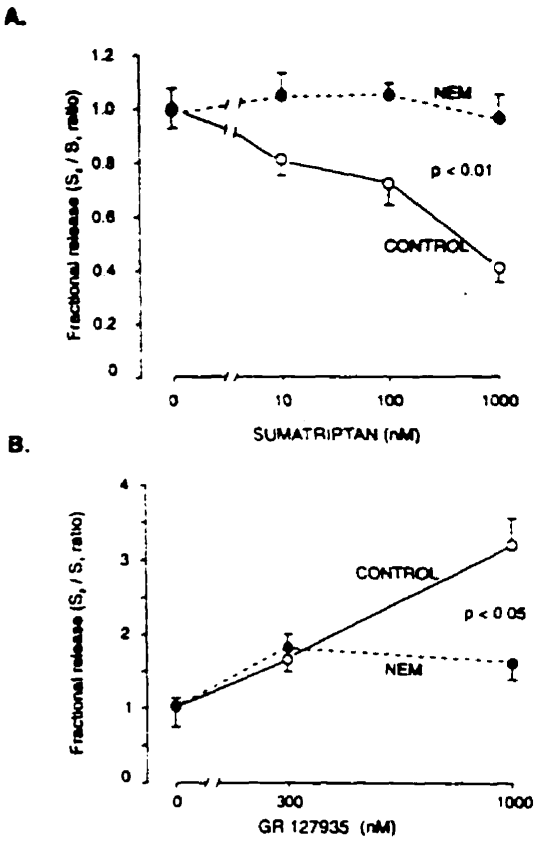

Fig. 2. Inhibition produced by the 5-HT$_{1D}$ agonist sumatriptan (A) or enhancement produced by the 5-HT$_{B/D}$ receptor antagonist GR 127935 (B) of the evoked release of tritium elicited by electrical stimulation of preloaded midbrain control slices (open circles) or slices preincubated with NEM (solid circles). The experiments were carried out immediately after a 30 min incubation period with either Kreb's alone or Kreb's with NEM (30 μM). Ordinates: fraction of total tissue radioactivity released by 360 pulses (30 mA, 2 min, 3 Hz). Each point represents the mean ± S.E.M. of the results obtained in experiments run in parallel for control and treated rats. Three to five pairs of rats were used per point. Statistical significance was calculated using two-way analysis of variance to compare the curves obtained in control and treated groups.

TABLE 1

Effect of GR 127935 on spontaneous outflow of tritium from midbrain slices

	Sp_2/Sp_1		
	No Drug before S_2	0.3 μM GR 127935 before S_2	1 μM GR 127935 before S_2
Control	0.78 ± 0.02	0.96 ± 0.06*	1.08 ± 0.13**
NEM (30 μM)	0.87 ± 0.07	0.75 ± 0.03	0.87 ± 0.04
Paroxetine[a]	0.74 ± 0.04	1.40 ± 0.10**	1.20 ± 0.20*
Befloxatone[b]	0.77 ± 0.02	0.78 ± 0.07	0.94 ± 0.04*

Comparisons were made between different concentrations of GR 127935 and the respective control experiments in which no drug was introduced before S_2. n = 7–15 slices obtained from three to five rats.

[a] Paroxetine (10 mg/kg/day × 21 days + 48 hr washout).

[b] Befloxatone (0.75 mg/kg/day × 21 days + 48 hr washout).

* $P < .01$ using the two-tailed Student's t test.

** $P < .001$ using the two-tailed Student's t test.

which no drugs were added to the superfusion medium during the experiment, radioactivity remaining in the tissue at the end of the experiment was similar in control and pertussis toxin-treated groups (control: 103 ± 7 nCi; n = 12; and pertussis toxin: 94 ± 13 nCi, n = 5), but was significantly reduced in slices preincubated with NEM (control: 103 ± 7 nCi, n = 12; and NEM: 73 ± 9 nCi, n = 5; $P < .05$). The latter observation is in keeping with the observation that spontaneous tritium outflow was significantly increased only in NEM-pretreated slices (table 2).

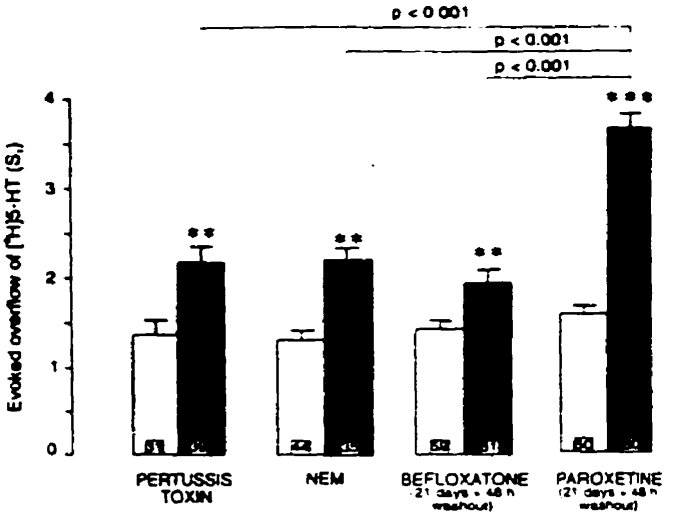

Fig. 3. Effect of G protein inactivation with pertussis toxin or NEM and of long-term administration of paroxetine or befloxatone on the electrically evoked release of tritium in midbrain slices preloaded with [^{3}H]5-HT. Long-term treatments were administered using osmotic minipumps implanted subcutaneously and removed 48 hr before the experiments to allow drug elimination. Ordinates: fraction of the total radioactivity released by 360 pulses (30 mA, 2 min, 3 Hz) during the first period of stimulation before the introduction of any drug in the perfusate (S_1). Experiments were always carried out in pairs of rats: a control and a treated one processed in parallel in the same experiment. Figures at the bottom of each column refer to the number of slices studied per group (five to eight rats per group). Statistical significance was calculated using nonpaired Student's t test to compare control and treated groups. ** $P < .01$; *** $P < .001$.

Effect of tetrodotoxin on the modulation of K$^+$-evoked overflow of [^{3}H]5-HT. In S_1, fractional release of [^{3}H]5-HT induced by K$^+$ stimulation (35 mM K$^+$ for 4 min) was 10 ± 1% (n = 12) of the total amount of radioactivity present in the tissue at the time of the stimulation and the S_2/S_1 ratio was not different from unity. The introduction of the 5-HT$_{1D}$ agonist sumatriptan (1 μM 20 min before S_2) inhibited K$^+$-evoked overflow of [^{3}H]5-HT by 23% (fig. 4). This inhibition was significantly smaller than that produced by the same concentration of sumatriptan on electrically evoked overflow of [^{3}H]5-HT ($P < .01$; fig. 1B). The inhibition of K$^+$-evoked [^{3}H]5-HT overflow observed after incubation with the 5-HT agonist 5-CT (100 nM) was significant when compared to the controls and similar to that observed with 1 μM sumatriptan (fig. 4).

In an attempt to determine whether inhibitory 5-HT$_{1D}$ receptors are located on 5-HT neurons, TTX (1 μM) was added to the superfusion medium after the end of the first period of K$^+$-evoked overflow of [^{3}H]5-HT. The S_2/S_1 and Sp_2/Sp_1 ratios were not altered by TTX, indicating that the toxin did not interfere either with evoked overflow of [^{3}H]5-HT (fig. 4) or spontaneous tritium outflow. Neither the inhibitory effect of sumatriptan (1 μM) nor that of 5-CT (100 nM) were reduced by addition of TTX to the superfusate 20

TABLE 2

Effect of $G_{i/o}$ protein inactivation or long-term antidepressant administration on spontaneous outflow of tritium from midbrain slices

			Sp_1								
Control	Pertussis toxin[a]	P	Control	NEM[b]	P	Control	Paroxetine[c]	P	Control	Befloxatone[d]	P
2.05 ± 0.1 (31)[e]	2.00 ± 0.1 (39)	N.S.	1.76 ± 0.08 (44)	2.03 ± 0.09 (35)	0.03	1.52 ± 0.05 (60)	1.42 ± 0.04 (80)	N.S.	1.47 ± 0.04 (69)	1.46 ± 0.03 (81)	N.S.

[a] Pertussis toxin (1 μg intracerebral dorsal raphe).
[b] NEM: slices were preincubated at 30 μM for 30 min).
[c] Paroxetine (10 mg/kg/day × 21 days + 48 hr washout).
[d] Befloxatone (0.75 mg/kg/day × 21 days + 48 hr washout).
[e] Numbers in parentheses correspond to the number of slices studied.

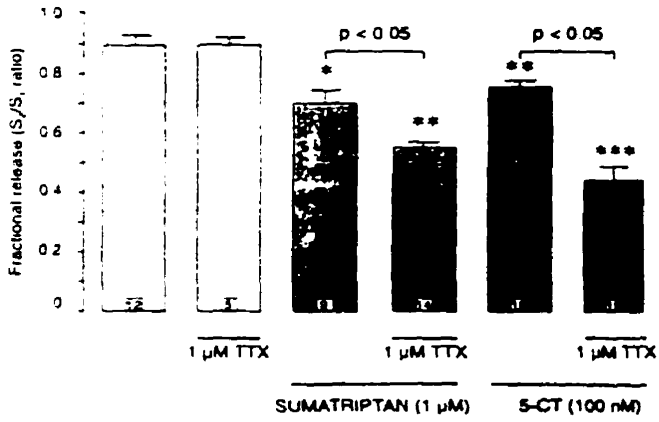

Fig. 4. Effect of tetrodotoxin, sumatriptan and 5-CT introduced 20 min before S_2 on potassium-evoked release of [^{3}H]5-HT from rat midbrain slices. Values are expressed as the ratio (S_2/S_1) obtained between the second period of stimulation in the presence of drugs (S_2) and the first one carried out in the absence of the latter (S_1). Each point represents the mean ± S.E.M. of the results obtained in experiments ran in parallel using three pairs of control and treated rats. Level of statistical significance was calculated using nonpaired Student's *t* test. * $P < .05$; ** $P < .01$ and *** $P < .001$, when compared to controls.

min before S_2. Interestingly, the inhibition of K^+-evoked [^{3}H]5-HT overflow produced by sumatriptan (1 μM) and by 5-CT (100 nM) was more efficacious in the presence than in the absence of TTX (fig. 4).

Effect of long-term paroxetine and befloxatone administration on [^{3}H]5-HT overflow. In the absence of any drug and when compared to controls processed in parallel, the electrically-evoked overflow during S_1 was significantly enhanced by 37 and 132% in the befloxatone and paroxetine groups, respectively (fig. 3). The spontaneous outflow of radioactivity in the sample immediately preceding this first stimulation period (Sp_1) was unchanged by either treatment (table 2). The increase in electrically evoked [^{3}H]5-HT overflow produced by a 21-day treatment with paroxetine was 3.6-fold greater than that produced by 21-day befloxatone administration. The increase induced by befloxatone treatment in turn was not different from that produced by pretreatment with pertussis toxin and the alkylating agent NEM (fig. 3). In slices obtained from paroxetine-treated rats, the radioactivity content at the end of experiments in which no drug was added to the superfusion medium was significantly less than in respective controls (control: 129 ± 7 nCi, $n = 19$; paroxetine: 77 ± 13 nCi, $n = 8$; $P < .05$). In slices obtained from befloxatone-treated rats, final radioactivity content remained unchanged (control: 129 ± 7 nCi, $n = 19$; befloxatone 128 ± 9 nCi, $n = 9$).

Assessment of 5-HT$_{1A}$- and 5-HT$_{1D}$-mediated responses after long-term antidepressant treatments. To assess the sensitivity of somatodendritic 5-HT$_{1A}$ autoreceptors after a 21-day treatment with paroxetine or befloxatone, the effectiveness of the 5-HT$_{1A}$ agonist 8-OH-DPAT (10 and 100 nM) to inhibit electrically evoked overflow of [^{3}H]5-HT was assessed. The capacity of 8-OH-DPAT (100 nM) to inhibit the evoked overflow of [^{3}H]5-HT (fig. 5) was abolished by long-term treatment with paroxetine as well as with befloxatone.

Concentration-effect curves were constructed using sumatriptan (10–1000 nM) to assess the responsiveness of 5-HT$_{1D}$ receptors after prolonged administration of paroxetine or befloxatone. When compared to controls run in parallel, in both paroxetine- and befloxatone-treated groups, there was an attenuation of the efficacy of sumatriptan to inhibit electrically evoked overflow of [^{3}H]5-HT from preloaded midbrain slices (fig. 6). However, at the highest concentration used (1000 nM), sumatriptan more effectively inhibited [^{3}H]5-HT overflow in slices obtained from befloxatone- than paroxetine-treated rats ($P < .01$ comparing the two treated groups by nonpaired Student's *t* test) (fig. 6).

To further assess whether 5-HT$_{1D}$ receptor function is reduced after long-term administration of paroxetine or befloxatone, the efficacy of the 5-HT$_{1D}$ antagonist GR 127935 was examined in midbrain slices prepared from control rats and rats that received the SSRI or the MAOI for 21 days. The rationale for this approach was that if, indeed paroxetine and befloxatone treatments reduced the effectiveness of the 5-HT$_{1D}$-mediated inhibitory control of 5-HT release in the midbrain raphe nuclei, then blockade of the latter receptors should produce a smaller enhancement of electrically evoked [^{3}H]5-HT release in slices prepared from treated rats. GR 127935, at concentrations of 0.3 μM and 1 μM, did in fact produce a smaller enhancement of the evoked overflow of tritium in slices prepared from paroxetine- and befloxatone-treated rats (fig. 7). The attenuation in the effectiveness of GR 127935 to enhance [^{3}H]5-HT overflow induced by the prolonged administration of paroxetine, was not significantly different from that caused by sustained befloxatone treatment ($P = .25$ comparing both curves by two-way ANOVA) (fig. 7). However, in the paroxetine but not the befloxatone-treated group 1 μM GR 127935 produced a significant enhancement (60%, $P < .05$; one-way ANOVA as compared to the respective control).

The evoked overflow of [^{3}H]5-HT (S_1) was significantly greater in midbrain slices obtained from paroxetine treated rats than from rats treated with befloxatone (fig. 3). Inasmuch as desensitization of somatodendritic 5-HT transport-

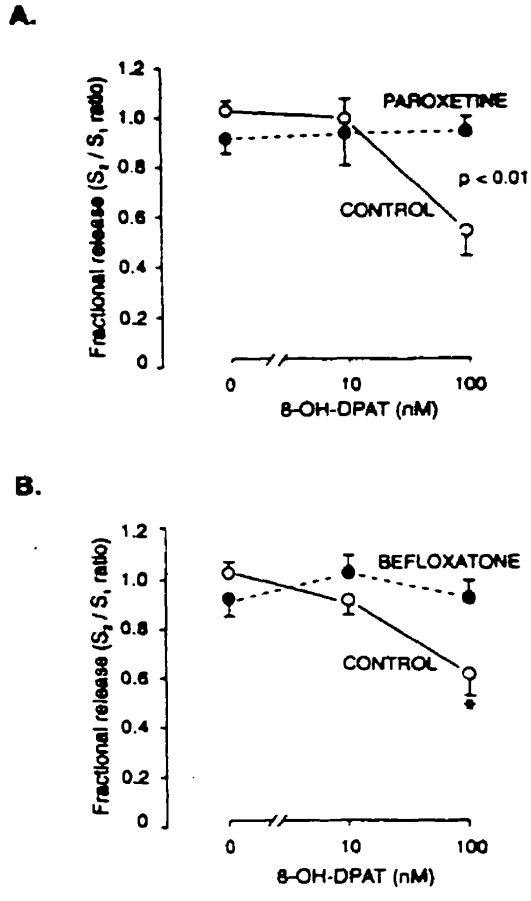

Fig. 5. Inhibition produced by the 5-HT_{1A} agonist 8-OH-DPAT of the evoked release of tritium elicited by electrical stimulation in preloaded midbrain slices prepared from control (open circles) and treated rats (solid circles) with paroxetine (10 mg/kg/day, s.c. × 21 days; A) and befloxatone (0.75 mg/kg/day, s.c., × 21 days; B). The experiments were carried out 48 hr after the removal of the osmotic minipump to allow drug elimination. Ordinates: fraction of total tissue radioactivity released by 360 pulses (30 mA, 2 min, 3 Hz). Values are expressed as the ratio (S_2/S_1) obtained between the second period of stimulation in the presence of agonist (S_2) and the first one carried out in the absence of the agonist (S_1). Each point represents the mean ± S.E.M. of the results obtained in experiments ran in parallel using three to four pairs of control and treated rats. In (A) the level of statistical significance was calculated using two-way analysis of variance to compare the curves obtained in the control and the paroxetine-treated groups. In (B) the level of significance was calculated using one-way analysis of variance to compare the effect of 8-OH-DPAT within the control or the treated group. * $P < .05$.

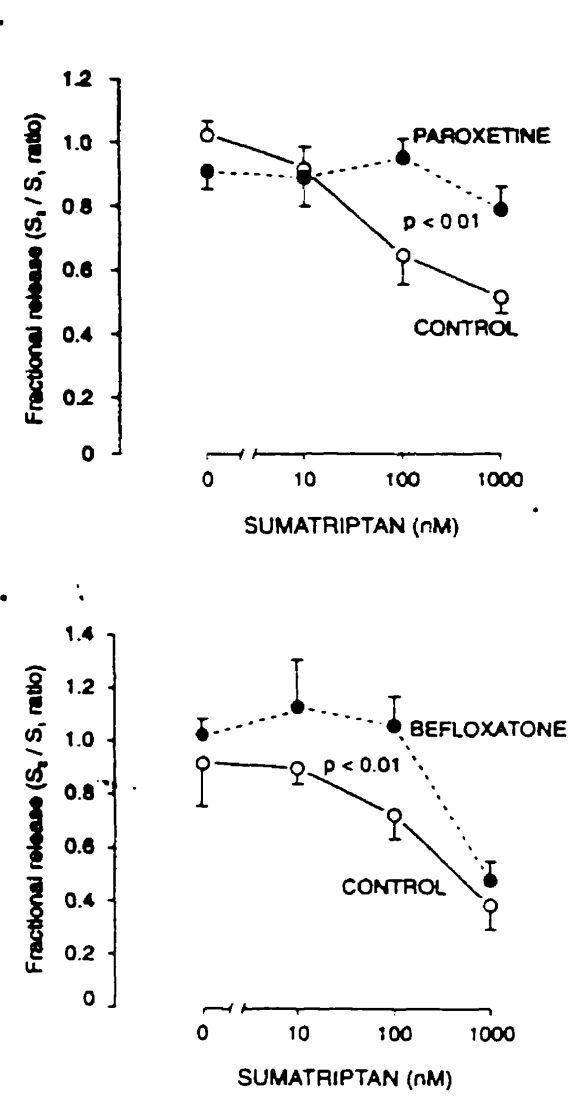

Fig. 6. Inhibition produced by the 5-HT_{1D} agonist sumatriptan of the evoked release of tritium elicited by electrical stimulation in preloaded midbrain slices prepared from control (open circles) and treated rats (solid circles) with paroxetine (A) and befloxatone (B). The experiments were carried out 48 hr after the removal of the osmotic minipump to allow drug elimination. Ordinates: fraction of total tissue radioactivity released by 360 pulses (30 mA, 2 min, 3 Hz). Values are expressed as the ratio (S_2/S_1) obtained between the second period of stimulation in the presence of agonist (S_2) and the first one carried out in the absence of the agonist (S_1). Each point represents the mean ± S.E.M. of the results obtained in experiments run in parallel using three to four pairs of control and treated rats. Level of statistical significance was calculated using 2-way analysis of variance to compare the curves obtained in control and treated groups.

ers has been observed after prolonged SSRI administration (Piñeyro *et al.*, 1994b), the purpose of the next series of experiments was to test whether a differential effect on the 5-HT carrier, could account for the greater enhancement of [^{3}H]5-HT overflow in the paroxetine-treated group. Hence, the effect of 1 μM paroxetine on the evoked overflow of [^{3}H]5-HT, was examined in control slices and in slices obtained from rats that received either paroxetine or befloxatone for 21 days after a 48-hr washout. In slices obtained from rats that had received vehicle, the S_2/S_1 ratio was of unity (fig. 8) when no paroxetine was added to the incubation medium. The addition of paroxetine (1 μM 20 min before S_2) significantly enhanced electrically evoked overflow of [^{3}H]5-HT by 50% (fig. 8). In slices obtained from befloxatone-treated rats, the addition of paroxetine had a similar enhancing effect on 5-HT overflow as in controls (fig. 8). The effect of 1 μM paroxetine was significantly smaller in the paroxetine-treated group than in rats that received either vehicle or befloxatone (fig. 8). In fact, in slices obtained from paroxetine-treated rats, the inclusion of 1 μM paroxetine in the superfusate did not significantly alter evoked overflow of [^{3}H]5-HT (fig. 8).

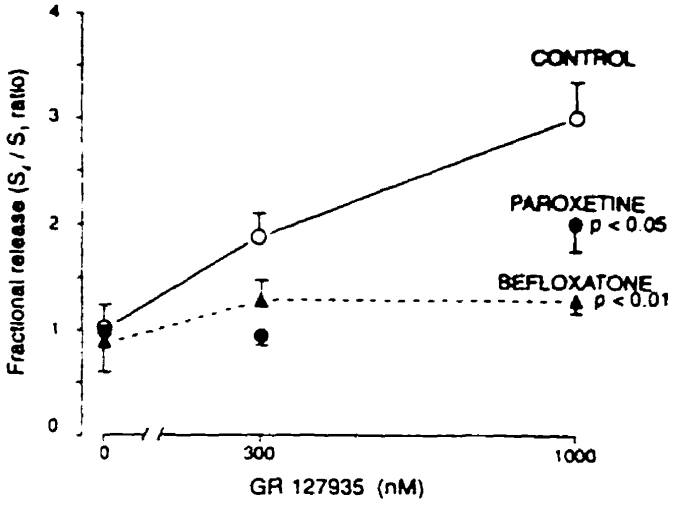

Fig. 7. Enhancement produced by the 5-HT$_{1B/1D}$ receptor antagonist GR 127935 of the evoked release of tritium elicited by electrical stimulation of preloaded midbrain slices obtained from controls (open circles) and from rats treated with paroxetine (solid circles) or befloxatone (solid triangles) for 21 days. The experiments were carried out after a 48-hr washout to allow drug elimination. Ordinates: fraction of total tissue radioactivity released by 360 pulses (30 mA, 2 min, 3 Hz). Values are expressed as the ratio (S_2/S_1) obtained between the second period of stimulation in the presence of the antagonist (S_2) and the first one carried out in the absence of the antagonist (S_1). Each point represents the mean ± S.E.M. of the results obtained in the same experiments using three to five pairs of control and treated rats. Statistical significance was calculated using two-way analysis of variance to compare the curves obtained in control and treated groups.

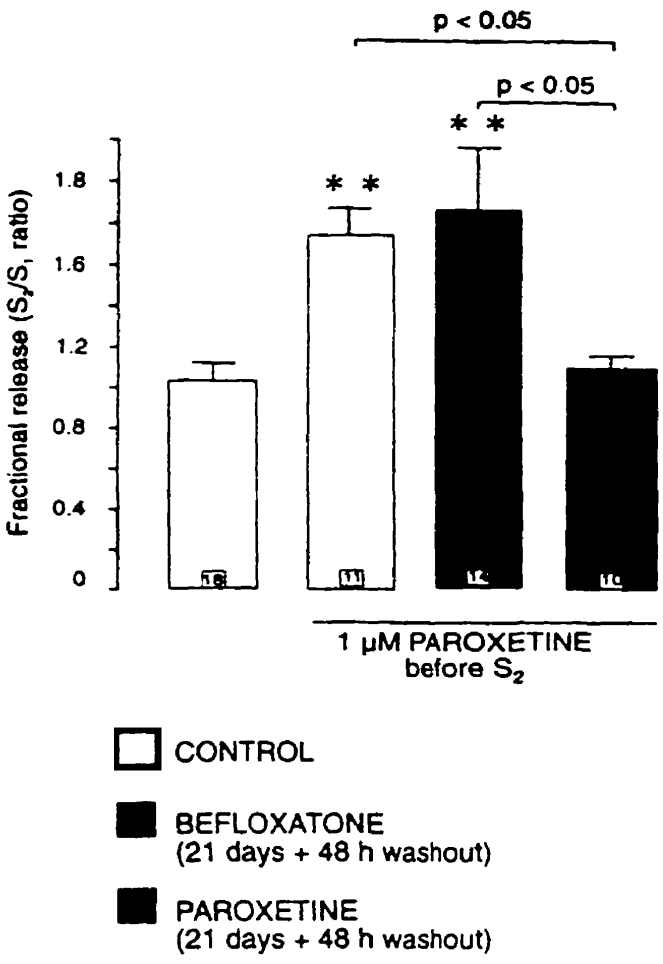

Fig. 8. Enhancement of the evoked release of radioactivity elicited by electrical stimulation in preloaded midbrain slices prepared from rats that had received either vehicle (50/50 saline/ethanol), befloxatone or paroxetine for 21 days. The experiments were carried out after a 48-hr washout period. Ordinates: fraction of total tissue radioactivity released by 360 pulses (30 mA, 2 min, 3 Hz). Values are expressed as the ratio (S_2/S_1) obtained between the second period of stimulation in the presence of the 5-HT reuptake blocker paroxetine (S_2) and the first one carried out in the absence of the agonist (S_1). Each point represents the mean ± S.E.M. of the results obtained in experiments ran in parallel using three pairs of control and treated rats. Level of statistical significance was calculated using nonpaired Student's *t* test to compare control and treated groups. ** $P < .01$.

Discussion

Results obtained in our study may be summarized as follows: 1) $G_{i,o}$ protein inactivation as well as sustained 5-HT reuptake blockade and type A-MAO inhibition lead to enhanced electrically evoked 5-HT release in the rat midbrain raphe nuclei, and 2) blockade of Na^+ channels by TTX did not prevent the inhibitory effect of 5-CT or sumatriptan on K^+-evoked release of [^{3}H]5-HT from midbrain slices.

In the case of G protein inactivation, enhancement of 5-HT release appeared to be due to an attenuation of the effectiveness of 5-HT$_{1A}$ and 5-HT$_{1D}$ receptors to inhibit 5-HT release in rat midbrain raphe nuclei (figs. 1 and 2). Activation of 5-HT$_{1A}$ autoreceptors induces the opening of K^+ channels and hyperpolarizes 5-HT neurons (see Aghajanian *et al.*, 1987). Activation also induces a decrease in high-threshold calcium currents (Pennington and Kelly, 1990). The suppressant effect on high-threshold calcium currents (Pennington *et al.*, 1991) as well as the hyperpolarization, are G protein-mediated effects, the latter being abolished by pretreatment with pertussis toxin (Williams *et al.*, 1988). The reduction in the efficacy of 8-OH-DPAT to inhibit [^{3}H]5-HT release from preloaded slices after intra-raphe injection of pertussis toxin observed in our study is therefore fully consistent with the above-mentioned results, and with *in vivo* (Innis and Aghajanian, 1987; Blier *et al.*, 1993) and *in vitro* observations (Innis *et al.*, 1988) showing that pertussis toxin abolishes the capacity of 5-HT$_{1A}$ agonists to suppress the firing activity of dorsal raphe 5-HT neurons.

There is also considerable evidence suggesting that 5-HT$_{1D}$ receptors are coupled to $G_{i,o}$ proteins. Studies in different species have demonstrated that central 5-HT$_{1D}$ receptors are negatively coupled to adenylyl cyclase activity (Schoeffter *et al.*, 1988; Waeber *et al.*, 1989). Furthermore, it has also been shown that NEM-induced inactivation of 5-HT$_{1D}$ binding in brain membranes is restored by addition of purified $G_{i,o}$ proteins (Stratford *et al.*, 1988), and that in transfected cells inhibition of adenylyl cyclase and elevation of intracellular Ca^{+2} by 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor activation are both significantly attenuated by pretreatment with pertussis toxin (Zgombick *et al.*, 1993). The reduced efficacy of sumatriptan to inhibit [^{3}H]5-HT release from pertussis- or NEM-pretreated slices is in keeping with the latter observations. Although sumatriptan has been reported to have low affinity for 5-HT$_{1A}$ receptors (K_i values varying from 100 to 900 nM; see Zifa and Fillion, 1992), it is unlikely that a reduced 5-HT$_{1A}$-mediated response could account for the decrease in efficacy observed after G protein inactivation. In-

deed, it has been shown that, in the superfusion paradigm, the effect of sumatriptan (100 nM) on [^{3}H]5-HT release is not blocked by the 5-HT$_{1A}$ antagonist S-UH-301 or (+) WAY 100135 (Piñeyro *et al.*, 1995a, 1995b). Conversely, 8-OH-DPAT has affinity for 5-HT$_{1D}$ receptors (Schoeffter and Hoyer, 1990) and its inhibitory action on electrically evoked [^{3}H]5-HT release in rat hippocampus (1000–10000 nM) has been attributed to 5-HT$_{1D}$ receptor stimulation (Limberger *et al.*, 1991). However, in the concentration range used in this study (10–100 nM), 5-HT$_{1D}$ receptors were not activated because it has been previously shown that the 5-HT$_{1B/1D}$ antagonist GR 127935 does not block the inhibitory effect of 8-OH-DPAT (100 nM) on electrically evoked release of [^{3}H]5-HT (Piñeyro *et al.*, 1995a).

The effect of 1 μM sumatriptan was reduced but not abolished by pertussis toxin pretreatment. Inasmuch as pertussis toxin was injected into the dorsal raphe, a diffusion gradient with lower concentrations reaching the median raphe nucleus could account for the incomplete blockade of the effect of 1 μM sumatriptan (fig. 2A). Another possibility to explain the incomplete inactivation of the 5-HT$_{1D}$-mediated response could be due to the fact that 5-HT$_{1D}$ receptors modulate multiple effector pathways, not all of them being inactivated by ADP-ribosylation of α_{o} G-protein subunits. Multifunctional signaling has been described for G protein-coupled receptors monoamine when expressed either endogenously or after transfection into heterologous systems (see Milligan, 1993). Moreover, NEM modifies the activity of G_{o} proteins (Winslow *et al.*, 1987) but also that of other signal-regulating molecules such as phospholipase A_2 (Neve *et al.*, 1995) and different ATPases (Borejdo and Burlacu, 1992; Woodman and Warren, 1991). Hence, the fact that this alkylating agent abolished the inhibitory effect of sumatriptan at all the concentrations tested (10–1000 nM), is in keeping with the assumption that 5-HT$_{1D}$ receptors may modulate multiple signaling pathways in the rat raphe nuclei. Finally, it is possible that a concentration of 1 μM sumatriptan might have stimulated other 5-HT receptors not linked to G_{o}-proteins. Indeed, apart from 5-HT$_{1D}$ receptors, sumatriptan has high affinity for 5-HT$_{1F}$ receptors (Adham *et al.*, 1993) and mRNA for this receptor subtype has been detected in the dorsal raphe nucleus (Bruinvels *et al.*, 1994). However, because the effect of sumatriptan is mimicked by 5-CT (which has low affinity for 5-HT$_{1E,1F}$ receptors; Adham *et al.*, 1993) and the effect of both agonists is blocked by methiothepin (which also has very low affinity for 5-HT$_{1F}$ receptors; Adham *et al.*, 1993) and by the 5-HT$_{1B/1D}$ antagonist GR 127935 (Piñeyro *et al.*, 1995a), it is unlikely that either 5-HT$_{1E/1F}$ receptor subtypes might be involved in the negative regulation of [^{3}H]5-HT release. Furthermore, 5-HT$_{1E}$ as well as 5-HT$_5$ and 5-HT$_7$ bind sumatriptan with very low affinity (Adham *et al.*, 1993; Matthes *et al.*, 1993; Ruat *et al.*, 1993), so it is not likely that they play a role in the inhibitory response elicited by this drug. A role of 5-HT$_{1B}$ receptors in the sumatriptan-mediated response has previously been ruled out (see Piñeyro *et al.*, 1995a, 1995b). Theoretically, it is possible that 1 μM sumatriptan might have stimulated either 5-HT$_4$ or 5-HT$_6$ receptor subtypes that are both positively coupled to adenylate cyclase (see Lucas and Hen, 1995; Monsma *et al.*, 1993). However, no 5-HT$_4$ or 5-HT$_6$ binding sites, or 5-HT$_6$ mRNA, have been found in the raphe nuclei (see Lucas and Hen, 1995). It seems then that if sumatriptan (1 μM) exerted its inhibitory effect via a non-5-HT$_{1D}$ receptor subtype, the latter has not been characterized yet. It is interesting to note, that although the reduction produced by preincubation with NEM of the sumatriptan-mediated effect was greater than that produced by pertussis toxin, both treatments produced a similar enhancement in electrically evoked release of [^{3}H]5-HT (fig. 3).

The 5-HT$_{1B/1D}$ antagonist GR 127935 (Skingle *et al.*, 1993) increased the electrically evoked release of [^{3}H]5-HT in control slices (figs. 2b and fig. 6). This effect has been previously observed not only in rat midbrain raphe nuclei (Piñeyro *et al.*, 1995a), but also in guinea pig dorsal raphe (Starkey and Skingle, 1994). This has been attributed to the blockade of 5-HT receptors, which when activated by endogenous 5-HT, negatively regulate the release of the neurotransmitter. The enhancement in evoked [^{3}H]5-HT release induced by GR 127935 (1 μM) in control slices was 2-fold that observed in slices preincubated with NEM (fig. 2B). The attenuation of the enhancing effect elicited by GR 127935 on electrically evoked release of [^{3}H]5-HT after preincubation with NEM, further suggests that in the rat midbrain raphe 5-HT$_{1D}$ receptors are coupled to G_{o}-proteins (see above). Similar inactivation studies performed in guinea pig midbrain slices indicate that this is also the case for 5-HT$_{1D}$ receptors located in the midbrain raphe nuclei of the latter species (El Mansari *et al.*, 1995).

Our results show that sumatriptan inhibited [^{3}H]5-HT release elicited either by electrical stimulation or by K$^+$-evoked depolarization. However, sumatriptan (1 μM) more effectively inhibited [^{3}H]5-HT release elicited by the former than by the latter method. This was also the case for 5-CT which in our study inhibited K$^+$-evoked release by 17% when at a similar concentration its effect on electrically evoked [^{3}H]5-HT release was about five times higher (Piñeyro *et al.*, 1995a). Furthermore, 8-OH-DPAT in a concentration range of 30 nM to 1 μM was unable to modify K$^+$-evoked release of [^{3}H]5-HT from midbrain slices (Middlemiss, 1987), but in contrast 100 nM of this drug inhibited electrically evoked release of [^{3}H]5-HT. Because in our study K$^+$-evoked release of [^{3}H]5-HT was 7-fold that observed after electrical stimulation, it could be argued that the reduced effectiveness of 5-HT agonists to inhibit [^{3}H]5-HT release elicited by K$^+$-induced depolarization was due to the amount of 5-HT release being too important. However, this explanation is unlikely given that a similar differential effect of 5-HT agonists has been observed in the rat hypothalamus in conditions in which K$^+$- and electrically evoked release of [^{3}H]5-HT were similar (Passarelli *et al.*, 1987).

Inhibition of [^{3}H]5-HT release induced by sumatriptan was not reduced by the presence of TTX in the superfusate (fig. 4). The latter observation therefore indicates that 5-HT$_{1D}$ receptors are autoreceptors located on 5-HT neurons themselves. Thus, the fact that the effect of 5-CT (100 nM) was also not reduced in the presence of TTX further supports the notion that 5-HT$_{1D}$ receptors, as 5-HT$_{1A}$ receptors, are located on 5-HT neurons in the raphe region. It is noteworthy that the inhibition of [^{3}H]5-HT release induced by either sumatriptan or 5-CT was actually enhanced in the presence of TTX. A possible explanation for this observation could be that the blockade of Na$^+$ channels by TTX interfered with stimulatory transynaptic interactions exerted by other neurotransmitter(s) on 5-HT neurons. Hence, in the presence of TTX, the

activation of inhibitory autoreceptors, occurring without any enhancing action of release-stimulating neurotransmitters, may have caused a greater inhibition than that observed when the latter systems were operating (*i.e.*, when no TTX was present). Indeed, GABA (contained in dorsal raphe interneurons; Harandi *et al.*, 1987) and substance P (contained in habenular projections to raphe nuclei; Neckers *et al.*, 1979) have been shown to enhance K^+ induced [^{3}H]5-HT release from midbrain raphe slices (Kerwin and Pycock, 1979). The inactivation of such release-enhancing influences could therefore explain the greater inhibition induced by 5-CT and sumatriptan in the presence of TTX.

Prolonged paroxetine or befloxatone administration lead to an enhancement of electrically evoked release of [^{3}H]5-HT (fig. 3). For both treatments, this effect appears to be due to an attenuation of the capacity of somatodendritic 5-HT$_{1A}$ and 5-HT$_{1D}$ autoreceptors to inhibit [^{3}H]5-HT release from preloaded slices (fig. 5). In the case of 5-HT$_{1A}$ autoreceptors, this is suggested by a decreased effectiveness of 8-OH-DPAT to inhibit electrically evoked [^{3}H]5-HT release after a long-term treatment with either drug. Indeed, these results are in agreement with previous *in vivo* observations from our laboratory showing that prolonged SSRI or MAOI administration induces a shift to the right of the dose-response curve of the somatodendritic autoreceptor agonist LSD (Blier and de Montigny, 1994).

After prolonged SSRI or MAOI administration, not only was there a reduced effectiveness of the agonist sumatriptan to inhibit electrically evoked release of [^{3}H]5-HT, but also the 5-HT$_{1D}$ antagonist GR 127935 produced a smaller enhancement of the amount of neurotransmitter induced by electrical stimulation. Interestingly, in slices prepared from paroxetine-treated rats, the inhibitory effect of sumatriptan on [^{3}H]5-HT release was abolished (fig. 6A), although in slices obtained from befloxatone-treated rats, the highest concentration of sumatriptan used (1000 nM) induced a similar degree of inhibition as in control slices (fig. 6B). A differential effect of prolonged SSRI and MAOI administration has also been observed on terminal 5-HT$_{1D}$ autoreceptors in guinea pig hypothalamus and hippocampus (Blier and Bouchard, 1994), as well as on rat hippocampal 5-HT$_{1B}$ receptors (Blier *et al.*, 1988). Recent findings in our laboratory indicate that this is also the case in guinea pig midbrain raphe, where long-term befloxatone administration failed to produce a desensitization of 5-HT$_{1D}$ receptors although sustained paroxetine treatment effectively did so. However, in the present study the enhancing effect produced by GR 127935 (300 nM) in slices obtained from long-term paroxetine- and befloxatone-treated rats was similarly reduced as compared to controls (fig. 8). The attenuated response observed with 300 nM GR 127935 is in agreement with an induction of desensitization of 5-HT$_{1D}$ receptors to the inhibitory effect of 5-HT. On the other hand, the reason for the difference observed at 1 μM between both treatment groups is not clear. It is possible that the response to GR 127935 might be influenced not only by the occurrence of desensitization of 5-HT$_{1D}$ receptors but also by the nature of the desensitization mechanism involved in either treatment. The different concentrations of 5-HT in the extracellular space after sustained paroxetine or befloxatone administration may also account for this difference. Indeed, the increase in S_1 values was almost four-fold greater in the paroxetine than the befloxatone treatment group.

A desensitization of the 5-HT carrier has been previously observed in rat midbrain raphe nuclei after sustained blockade of the 5-HT transporter (Piñeyro *et al.*, 1994b). In our study, the addition of 1 μM paroxetine in the superfusate increased the electrically evoked release of [^{3}H]5-HT in slices obtained from rats treated either with vehicle or befloxatone for 21 days, but not in slices obtained from long-term paroxetine-treated rats. The reduced effectiveness of paroxetine to increase [^{3}H]5-HT release in slices obtained from paroxetine-treated rats confirms previous results from our laboratory (Piñeyro *et al.*, 1994b; El Mansari *et al.*, 1995), indicating that there is a decreased activity of the 5-HT transporter. The observed differential effect of long-term administration of MAOI and SSRI on the 5-HT transporter has been described by others (Lesch *et al.*, 1993; Kovachich *et al.*, 1992). The fact that both MAOI and SSRI increase extracellular 5-HT (Ferrer and Artigas, 1994; Adell and Artigas, 1991), but only the latter desensitize the 5-HT transporter, rules out the possibility that this effect might be dependent on the extracellular increase of 5-HT. To explain this differential response, Lesch *et al.* (1993) have suggested that heterocyclic and tricyclic antidepressants would exert a direct effect on the regulation of the transcription of the 5-HT transporter gene, although MAOI such as clorgyline would not. In our study, the differential effect on the 5-HT transporter could in fact account for the greater enhancement in [^{3}H]5-HT release from slices obtained from paroxetine-treated rats than those obtained from befloxatone-treated rats. Furthermore, the reduced amount in radioactivity found in slices obtained from paroxetine-treated rats may also be due to the desensitization of the 5-HT transporter, not only because [^{3}H]5-HT release is enhanced, but also because the total amount of radioactivity taken up by the tissue is reduced. Indeed, sustained paroxetine administration reduces [^{3}H]5-HT uptake in midbrain raphe slices by 60% (Piñeyro *et al.*, 1994b). A similar reduction in the amount of radioactivity remaining in the tissue at the end of the experiment was observed in slices preincubated with NEM. However, because NEM does not alter the 5-HT transporter (Blier, 1991) it is improbable that the latter observation might be explained by a reduction of [^{3}H]5-HT uptake. However, the increase in spontaneous tritium overflow produced by NEM (table 2) could account for reduced tissue radioactivity at the end of the experiment. A similar effect of NEM (30 μM) on spontaneous tritium overflow was observed in rat hippocampal slices (Blier, 1991). In the latter study, 30 μM NEM was found to be the optimal concentration that minimally alters spontaneous tritium outflow although inducing G-protein inactivation (Fredholm and Lindgren, 1988).

In conclusion, similarly to 5-HT$_{1A}$ autoreceptors, 5-HT$_{1D}$ receptors that negatively regulate 5-HT release in the rat midbrain raphe nuclei are coupled to $G_{i,o}$ proteins and desensitize after sustained paroxetine or befloxatone administration. Our functional study also suggests that 5-HT$_{1D}$ receptors are located on 5-HT neurons and should therefore be considered as autoreceptors. Further immunocytochemical studies are required to confirm this last contention.

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Regulation of [^{3}H]5-HT release in raphe, frontal cortex and hippocampus of 5-HT$_{1B}$ knock-out mice

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REGULATION of the electrically evoked release of [^{3}H]5-HT was examined in midbrain, frontal cortex and hippocampus preloaded slices obtained from wild-type and 5-HT$_{1B}$ knock-out mice. In the absence of any drug [^{3}H]5-HT released was increased in midbrain and hippocampus but not in frontal cortex slices of [^{3}H]5-HT$_{1B}$ knock out mice. The selective 5-HT$_{1B}$ agonist CP 93129 and the 5-HT$_{1B/1D}$ agonist sumatriptan, inhibited [^{3}H]5-HT release in hippocampus and cortical slices obtained from control mice but had no effect in mutants. In the two projection areas studied, the non-selective 5-HT agonist 5-carboxyamidotryptamine (5-CT) inhibited [^{3}H]5-HT release in both groups of mice, indicating that additional 5-HT receptors, other than 5-HT$_{1B}$, might be involved in the regulation of [^{3}H]5-HT release from 5-HT terminals. In slices containing midbrain raphe nuclei, CP 93129 had no effect in either group. In contrast, sumatriptan inhibited [^{3}H]5-HT release in controls and mutants. The latter effect was blocked by the 5-HT$_{1D}$ antagonist GR 127935, but not the 5-HT$_{1A}$ antagonist (+)WAY 100135, thus suggesting that a 5-HT$_{1D}$-like receptor, possibly 5-HT$_{1D\alpha}$, negatively regulates 5-HT release in mouse midbrain raphe nuclei.

Key words: 5-HT$_{1B}$ receptors; 5-HT$_{1D}$ receptors; 5-HT$_5$ receptors; Autoreceptors; Release; Transgenic mice

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Introduction

Serotonin (5-HT) regulates a wide variety of sensory, motor and behavioural functions, and is thought to be involved in anxiety, impulsivity and depression.[1] It mediates its physiological actions via multiple 5-HT receptor subtypes most of which have been identified by cDNA cloning and belong to the G-protein-coupled receptor superfamily.[2] Among these, 5-HT$_{1B}$ receptors, the rodent homologue of human 5-HT$_{1B\beta}$ receptors,[3] have been classically described as inhibitory autoreceptors implicated in the regulation of 5-HT release in several projection areas such as cortex and hippocampus.[4–6] Although 5-HT$_{1B}$ receptors play a major role in controlling 5-HT release in these projection areas, it has also been reported that other 5-HT receptor subtypes might be involved in the regulation of 5-HT release by axon terminals.[7,8] Even if it is currently accepted that 5-HT$_{1B}$ receptors play a crucial role in controlling 5-HT release in projection areas, their contribution to the regulation of 5-HT release in murine raphe nuclei is not as clear. While the selective 5-HT$_{1B}$ agonist CP 93129 produced no effect on electrically evoked release of [^{3}H]5-HT from preloaded rat midbrain slices, the 5-HT$_{1B/1D}$ agonist sumatriptan inhibited [^{3}H]5-HT release in the latter region.[9] These observations, taken together with the fact that the effect of sumatriptan was blocked by the 5-HT$_{1B/1D}$ antagonist GR 127935, but not by the 5-HT$_{1A}$ antagonist S-UH-301,[9] were interpreted as an indication that 5-HT$_{1D\alpha}$ receptors might play a significant role in modulating 5-HT release in rat midbrain raphe nuclei.

Hence, in the present study, 5-HT$_{1B}$ knock-out mice generated by homologous recombination,[10] were used in order: (i) to determine the role of 5-HT receptors other than 5-HT$_{1B/1D}$ receptors in the regulation of [^{3}H]5-HT release in terminal projection areas, and (ii) to assess the functional role of 5-HT$_{1B}$ receptors in the regulation of [^{3}H]5-HT release from murine midbrain raphe nuclei.

Materials and Methods

Male and female 129/Sv wild-type mice (25–32 g, Taconic Farms, New York, USA) as well as transgenic female mice lacking 5-HT$_{1B}$ receptors (25–32 g, Columbia University, Center for Neurobiology and Behavior, New York, USA) were sacrificed by decapitation and their brains quickly removed for dissection on an ice-cold glass plate. The midbrain was dissected by two transections: rostrally immediately behind the hypothalamus and caudally posterior to the inferior colliculi. The colliculli were removed prior to slice preparation. Slices from midbrain, hippocampus and frontal cortex of 0.4 mm thickness were prepared with a McIlwain chopper and incubated for 30 min at 37°C in Krebs solution

containing either 20 nN [^{3}H]5-HT (specific activity 22.7 Ci mmol^{-1}, NEN Research Products, Mississauga, Canada) for terminal region slices or 100 nM [^{3}H]5-HT for slices containing midbrain raphe nuclei. The superfusate was bubbled with a mixture 95% O_2:5% CO_2. The composition of the Krebs solution was: 118 mM NaCl, 4.7 mM $CaCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 0.004 mM Na_2EDTA and 0.11 mM ascorbic acid. At the end of the 30 min incubation period three hippocampal slices, two slices from frontal cortex or one containing midbrain raphe nuclei were transferred into separate glass chambers and superfused continuously at a rate of 0.5 ml min^{-1} with Krebs solution maintained at 37°C and saturated with O_2/CO_2. Nineteen consecutive 4 min fractions were collected starting 60 min after the beginning of superfusion. The slices were stimulated twice, 8 min (S_1) and 44 min (S_2) after the end of the washing period. The electrical field generated in the chambers between two platinum electrodes (2 cm apart) had a voltage drop of 5 V cm^{-1}. The stimulation parameters used were 360 pulses of 2 ms duration, 30 mA intensity delivered for 2 min at 3 Hz. Antagonists were added 20 min before S_1, and agonists were added 20 min before S_2 and remained present until the end of the superfusion. At the end of the experiments slices were solubilized in 0.5 ml Soluene 350 (Packard Instruments, Downers Grove, Il, USA) and radioactivity in the slices and superfusate samples was determined by scintillation spectrometry. The amount of tritium released per 4 min sample was expressed as a fraction of total tritium contained in the tissue at the start of the respective collection period. The overflow of tritium produced by the electrical stimulation was calculated as the total increase in radioactivity above the basal outflow of tritium determined in the sample immediately preceding the start of stimulation (Sp_1 and Sp_2).

In this report, *n* refers to the number of superfusion chambers studied for each region, all results are expressed as means ± s.e.m., and means for each region were calculated from slices obtained from at least two mice. To assess regulatory mechanisms of [^{3}H]5-HT release in 5-HT_{1B} knock-out mice, experiments were run in parallel using slices obtained from female controls and from female transgenic mice. Experiments for male controls were performed separately. For the three groups of mice studied, superfusion experiments for frontal cortex, hippocampus and midbrain slices from the same mouse were always run in parallel. Means were compared by two-tailed Student's *t*-test. p values <0.05 were considered significant.

Results

Superfusion experiments in wild-type mice: Superfusion experiments were performed using hippocampus, frontal cortex and midbrain raphe slices obtained from wild-type, male and female mice. When no drugs were added, the ratio of electrically evoked release of [^{3}H]5-HT in S_1 and S_2 was not different from unity among groups or regions studied. Basal tritium efflux expressed as Sp_2/Sp_1 was similar in slices obtained from males and females, ranging from 0.93 ± 0.12 in the dorsal raphe to 0.73 ± 0.02 in the hippocampus. The effect of introducing different 5-HT_1 agonists 20 min before S_2 on electrically evoked release of [^{3}H]5-HT was similar in both groups and is summarized in Table 1. The selective 5-HT_{1B} agonist CP 93129 (100 nM) inhibited [^{3}H]5-HT release in cortex and hippocampus, but had no effect in midbrain raphe slices containing somata and dendrites of 5-HT neurones. The non-selective 5-HT agonist 5-CT (100 nM) more potently inhibited [^{3}H]5-HT release in cortex and hippocampus than

Table 1. Effect of 5-HT_1 agonists on electrically evoked release of [^{3}H]5-HT from preloaded hippocampus, cortical and midbrain raphe slices obtained from male and female wild-type mice

	Electrically evoked [^{3}H]5-HT release (S_2/S_1)	
	Female	Male
Hippocampus		
Control	1.10 ± 0.07 (n = 12)	1.02 ± 0.08 (n = 9)
Sumatriptan (100 nM)	0.80 ± 0.07 (n = 7)**	1.01 ± 0.11 (n = 6)
CP 93129 (100 nM)	0.45 ± 0.04 (n = 7)***	0.45 ± 0.08 (n = 7)***
5-CT (100 nM)	0.18 ± 0.08 (n = 6)***	0.22 ± 0.06 (n = 7)***
Frontal cortex		
Control	1.04 ± 0.13 (n = 8)	0.88 ± 0.09 (n = 8)
Sumatriptan (100 nM)	0.73 ± 0.08 (n = 10)*	0.59 ± 0.11 (n = 7)
CP 93129 (100 nM)	0.64 ± 0.12 (n = 7)*	0.68 ± 0.07 (n = 7)
5-CT (100 nM)	0.40 ± 0.05 (n = 7)**	0.40 ± 0.13 (n = 8)***
Midbrain raphe		
Control	1.05 ± 0.12 (n = 6)	0.99 ± 0.09 (n = 7)
Sumatriptan (100 nM)	0.49 ± 0.06 (n = 10)***	0.57 ± 0.09 (n = 9)**
CP 93129 (100 nM)	0.97 ± 0.22 (n = 6)	0.91 ± 0.09 (n = 10)

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with controls, Student's *t*-test.
[^{3}H]5-HT release was evoked twice (S_1, S_2) at an interval of 36 min, for 2 min and the ratio of overflow evoked by S_2: that evoked by S_1 was determined (S_2/S_1). Agonists were introduced 20 min before S_2 and the stimulation parameters used were the following: 30 mA, 2 ms, 3 Hz for 2 min. *n* refers to the number of experiments per group.

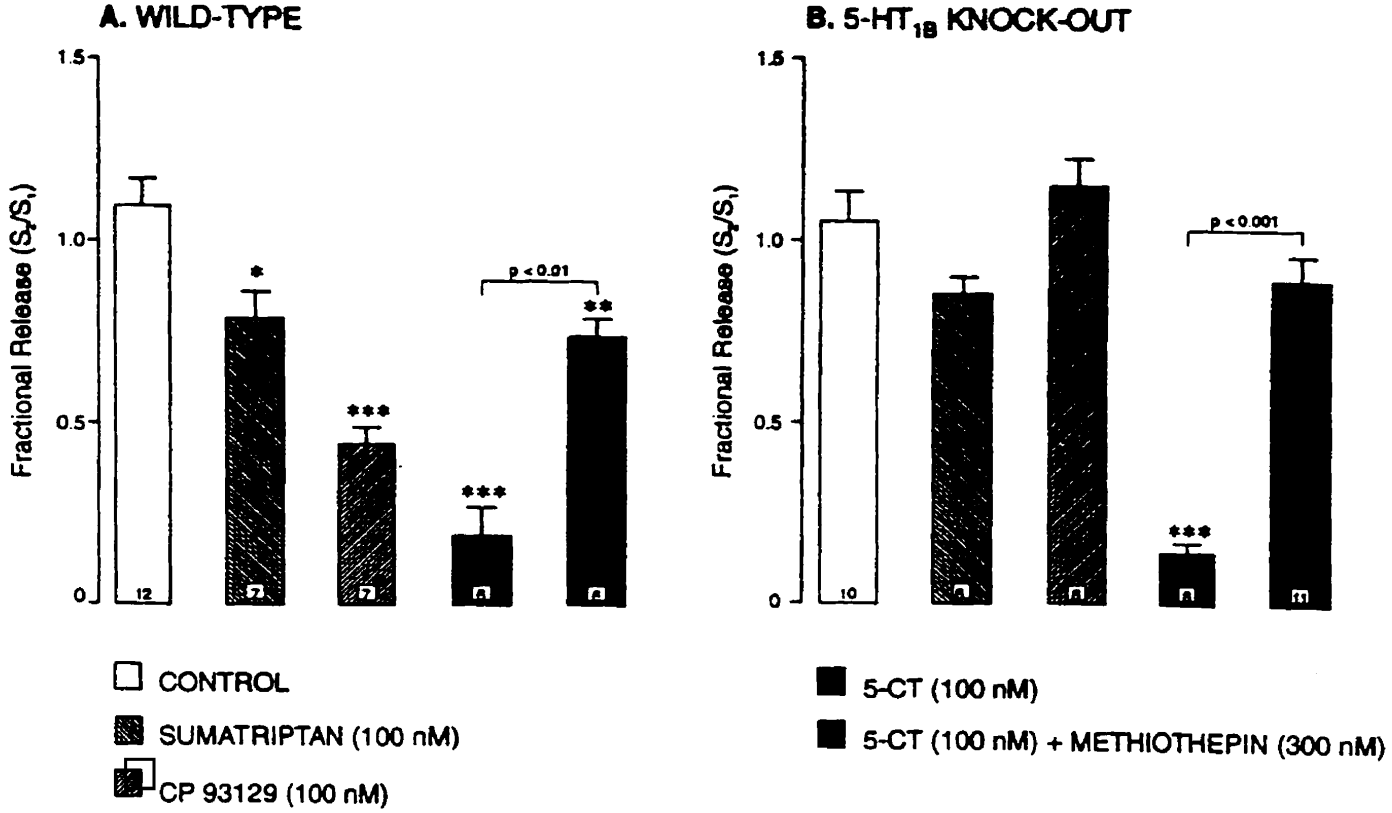

FIG. 1. Effect of different 5-HT agonists and antagonists on the inhibition of electrically evoked release of [^{3}H]5-HT from preloaded hippocampus slices obtained from (A) wild-type and (B) 5-HT$_{1B}$ knock-out mice. Agonists were introduced 20 min before S_2 and also remained present until the end of the experiment. The antagonist methiothepin was introduced 20 min before S_1. Values are expressed as means + s.e.m. for which the number of experiments per group is given at the bottom of each column. For each group, slices were obtained from two mice. The means were compared using Student's *t*-test. $^{**}p < 0.01$; $^{***}p < 0.001$.

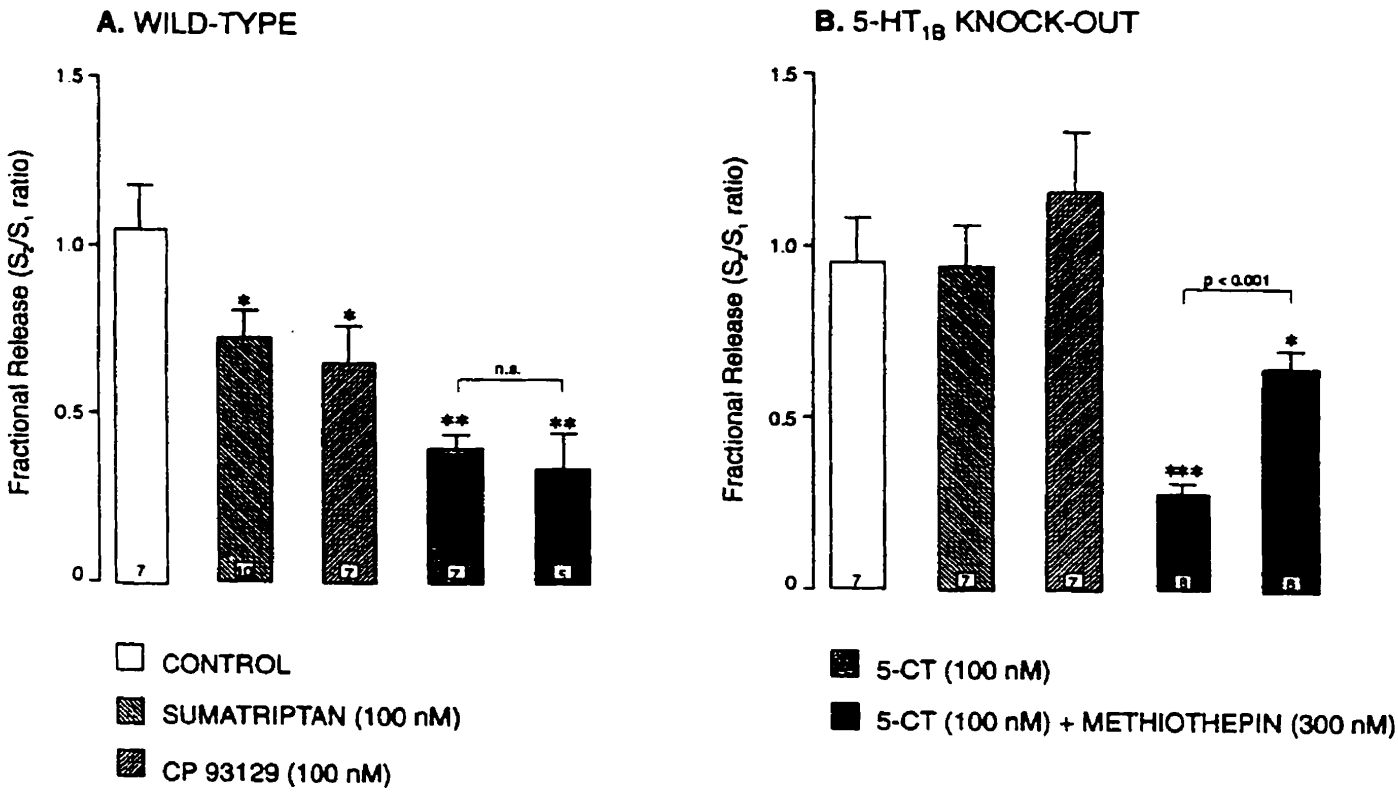

FIG. 2. Effect of different 5-HT agonists and antagonists on the inhibition of electrically evoked release of [^{3}H]5-HT from preloaded frontal cortex slices obtained from (A) wild-type and (B) 5-HT$_{1B}$ knock-out mice. Agonists were introduced 20 min before S_2 and also remained present until the end of the experiment. The antagonist methiothepin was introduced 20 min before S_1. Values are expressed as means + s.e.m. for which the number of experiments per group is given at the bottom of each column. For each group, slices were obtained from two mice. The means were compared using Student's *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

CP 93129 (Figs 1, 2), and the effect of both drugs was similar in males and females (Table 1). Inhibition of electrically evoked release of [^{3}H]5-HT induced by either of these agonists was greater in hippocampus than in cortex ($p < 0.01$, comparing percentage of inhibition produced by CP 93129 or 5-CT in cortex with that produced in hippocampus, using Student's t-test). The 5-HT$_{1B/1D}$ agonist sumatriptan (100 nM) induced a 40% inhibition of electrically evoked release of [^{3}H]5-HT from preloaded midbrain raphe slices but had only a small effect in terminal projection areas.

Superfusion experiments in 5-HT$_{1B}$ knock-out mice: Before the introduction of any drug in the superfusate, i.e. during the first period of electrical stimulation (S_1), fractional release of [^{3}H]5-HT was increased in midbrain and hippocampus slices of 5-HT$_{1B}$ knock-out mice (Table 2). On the other hand, in frontal cortex slices obtained from transgenic mice, electrically evoked release of [^{3}H]5-HT was not different from controls.

As expected, the selective 5-HT$_{1B}$ receptor agonist CP 93129 did not inhibit electrically evoked release of [^{3}H]5-HT in frontal cortex and hippocampus slices obtained from 5-HT$_{1B}$ knock-out mice (Figs 1, 2). This was also the case for sumatriptan since the inhibitory effect observed in slices obtained from controls was absent in slices obtained from transgenic mice (Figs 1, 2). Conversely, the inhibition induced by the non-selective 5-HT agonist 5-CT was similar in control and mutant mice in both terminal regions studied (Figs 1, 2). In mutant, as in controls, 5-CT induced a greater inhibition of [^{3}H]5-HT release in hippocampus than frontal cortex slices ($p < 0.001$, using Student's t-test, Figs 1, 2).

The non-selective 5-HT antagonist methiothepin (300 nM) partially blocked the inhibitory effect of 5-CT (100 nM) in hippocampus slices, but not in frontal cortex slices prepared from controls (Figs 1A, 2A). In slices obtained from transgenic mice, methiothepin abolished the effect of 5-CT in hippocampus but not in frontal cortex where it induced only a partial blockade (Figs 1B, 2B). The effectiveness of methiothepin in blocking the inhibitory effect of 5-CT was significantly greater in frontal cortex slices obtained from transgenic than control mice ($p < 0.001$; Fig. 2).

Introduction of methiothepin in the superfusate 20 min before S_1 produced a significant increase of the electrically evoked release of [^{3}H]5-HT during the first period of electrical stimulation (S_1) in hippocampal slices obtained from control (125 ± 11% increase; $p < 0.001$; $n = 6$) and transgenic (54 ± 13% increase; $p < 0.01$; $n = 11$) mice. In contrast, in frontal cortex slices methiothepin did not induce any change in electrically evoked release of [^{3}H]5-HT either in controls or mutants.

The effects of different 5-HT$_1$ agonists and antagonists were similar in midbrain raphe slices obtained from control and mutant mice (Fig. 3). The 5-HT$_{1B}$ agonist CP 93129 had no effect while the 5-HT$_{1B/1D}$ agonist sumatriptan produced a similar inhibition in both groups. In mutants as well as in controls, the latter effect was blocked by the 5-HT$_{1B/1D}$ antagonist GR 127935 (300 nM) but not by the 5-HT$_{1A}$ antagonist (−)WAY 100135 (1 μM).

Discussion

In the rat, CNS 5-HT release in terminal projection areas is controlled by 5-HT$_{1B}$ autoreceptors.[4–6] The fact that in the present study the 5-HT$_{1B}$ agonist CP 93129 inhibited electrically evoked release of [^{3}H]5-HT both in cortex and hippocampus, together with the obervation that the effect of this agonist was absent in slices obtained from 5-HT$_{1B}$ knock-out mice, indicate that this is also the case in the mouse CNS. Moreover, the 5-HT$_{1B,1D}$ agonist sumatriptan induced a significant inhibitory effect in hippocampus and frontal cortex of control female mice which was no longer present in corresponding slices of mutant

Table 2. Fraction of total tissue radioactivity released by hippocampus, frontal cortex and midbrain raphe slices obtained from control and 5-HT$_{1B}$ knock-out mice

	Electrically evoked release of [^{3}H]5-HT (S_1)		Spontaneous tritium outflow (Sp_1)	
	Control	5-HT$_{1B}$ Knock-out	Control	5-HT$_{1B}$ Knock-out
Hippocampus	1.8 ± 0.2 ($n = 33$)	3.2 ± 0.2** ($n = 35$)	1.74 ± 0.08 ($n = 33$)	2.01 ± 0.07* ($n = 35$)
Frontal cortex	1.7 ± 0.2 ($n = 32$)	2.1 ± 0.2 ($n = 30$)	1.69 ± 0.06 ($n = 32$)	1.86 ± 0.09 ($n = 30$)
Midbrain raphe	1.4 ± 0.1 ($n = 23$)	2.8 ± 0.3** ($n = 24$)	1.70 ± 0.05 ($n = 24$)	1.77 ± 0.06 ($n = 23$)

*$p < 0.01$, **$p < 0.001$ using non-paired Student's t-test.
S_1 is the fraction of total tissue radioactivity released by 360 pulses (30 mA, 2 ms, 3 Hz) in the first period of stimulation before the introduction of any drug in the perfusate.
Spontaneous outflow of radioactivity (Sp_1) refers to the fraction of total tissue radioactivity released during the 4 min preceding the first stimulation.

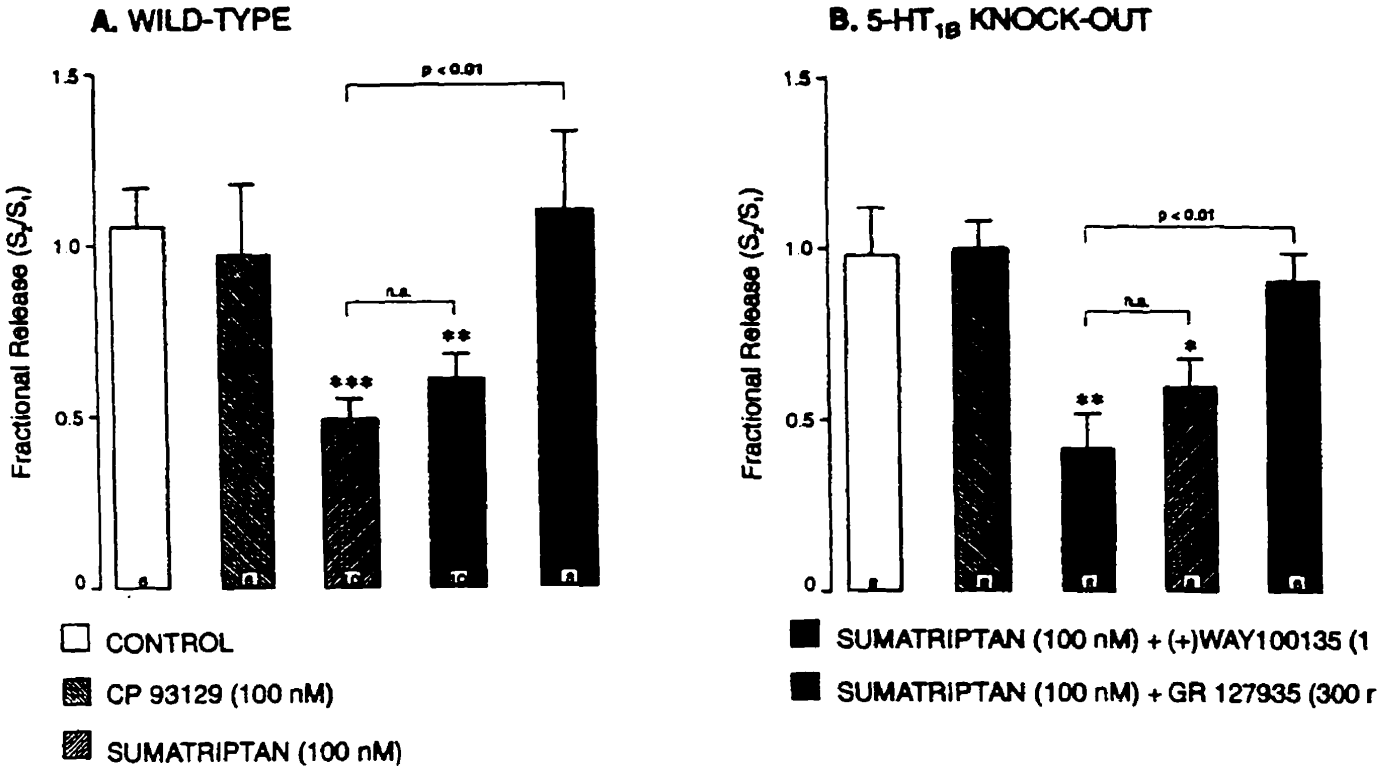

FIG. 3. Effect of different 5-HT$_1$ agonists and antagonists on the inhibition of electrically evoked release of [^{3}H]5-HT from preloaded midbrain raphe slices obtained from (A) wild-type and (B) 5-HT$_{1B}$ knock-out mice. Agonists were introduced 20 min before S_2 and also remained present until the end of the experiment. The antagonists were introduced 20 min before S_1. Values are expressed as means + s.e.m. for which the number of experiments per group is given at the bottom of each column. For each group, slices were obtained from two mice. The means were compared using Student's *t*-test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

female mice run in parallel (Table 1; Fig. 1A). The inhibition induced by sumatriptan in control mice was probably mediated by 5-HT$_{1B}$ receptors, because it would not have disappeared in mutants had it been mediated by 5-HT$_{1D}$ receptors.

In hippocampus, as well as in frontal cortex of controls, the non-selective 5-HT agonist 5-CT induced a greater inhibition of electrically evoked release of [^{3}H]5-HT than the selective 5-HT$_{1B}$ agonist CP 93129 (Figs 1A, 2A). This could be due to the fact that CP 93129 binds with lower affinity and stimulates 5-HT$_{1B}$ receptors with a weaker potency than 5-CT[11-13] or, alternatively, it is possible that 5-CT may have induced its inhibitory effect via multiple 5-HT receptors. Indeed, 5-CT binds with very high affinity (low nanomolar range) not only to 5-HT$_{1B}$ receptors,[11,12] but also to 5-HT$_{1A}$,[12] 5-HT$_{1D}$,[11] 5-HT$_5$,[14] and 5-HT$_7$ receptors.[15,16] It is unlikely that 5-CT induced the effects observed in cortex and hippocampus via 5-HT$_{1A}$ receptors since they have not been described as 5-HT release-controlling receptors in projection areas. An effect of 5-CT via 5-HT$_{1D}$ receptors may also be ruled out based on the results obtained with sumatriptan (see above). Other 5-HT$_1$ receptor subtypes such as 5-HT$_{1E}$ and 5-HT$_{1F}$ are probably not involved in the inhibitory effect of 5-CT because they have a very low affinity for this drug (K_d values in the high μM range[17,18]). However, the possibility that 5-CT could have induced the observed inhibition of [^{3}H]5-HT release either via 5-HT$_5$, 5-HT$_7$ or other 5-HT receptors not yet characterized cannot be excluded. Further pharmacological studies with appropriate 5-HT$_{5/7}$ selective drugs, unavailable for the time being, are needed to prove this suggestion. Interestingly, similar observations to those reported in the present study were obtained using a plasma protein extravasation paradigm. In 5-HT$_{1B}$ knock-out mice, 5-CT, but not sumatriptan or CP 93129, blocked plasma protein extravasation caused by trigeminal stimulation. It was therefore concluded that such an effect was mediated by a non-5-HT$_{1B-1D-1F}$ receptor subtype.[19] It is also worth noting that 5-HT$_{5B}$ and 5-HT$_7$ mRNA have been found in the dorsal raphe nuclei, suggesting that either of these two receptor subtypes may be expressed as 5-HT autoreceptors on 5-HT neurons.[14,15]

The regulation of 5-HT release in projection areas was further characterized by studying the effects of methiothepin in terminal projection areas of control and mutant mice. The main results of this experimental series may be summarized as follows. First, methiothepin (300 nM) blocked the effect of 5-CT in hippocampus (Fig. 1A) but not in cortical slices (Fig. 2A) obtained from the same female wild-type mice.

Second, the addition of methiothepin to the superfusate induced an increase in electrically evoked release of [^{3}H]5-HT in hippocampus but not in cortex of both control and transgenic mice. These observations could be interpreted as regional differences in the population of 5-HT receptors which control 5-HT release in cortex and hippocampus. The latter interpretation is further supported by the fact that the electrically evoked release of [^{3}H]5-HT in the absence of any drug (i.e. during S_1) was increased in hippocampus but not in frontal cortex slices obtained from 5-HT_{1B} knock-out mice and by the observation that in mutants, as well as in controls, 5-CT induced a greater inhibition of [^{3}H]5-HT release in hippocampus than frontal cortex slices

Though methiothepin binds with high affinity to 5-HT_{1B} receptors,[11] it also binds to other receptor subtypes putatively involved in regulation of 5-HT release at the terminal level, with the following order of potency: 5-$HT_7 \geq$5-HT_{1B}>5-HT_5. Given that methiothepin was less potent in blocking the effect of 5-CT in cortex than in hippocampus, it could be then speculated that the receptor subtype with the least affinity for this drug (5-HT_5) predominates in the former region while those that present a higher affinity for methiothepin (5-$HT_{1B/7}$) predominate in the latter region. Methiothepin is one of the most potent antagonists of terminal 5-HT_{1B} autoreceptors,[20] and yet at a concentration of 300 nM it was still unable to block the inhibitory action of 5-CT (100 nM) in frontal cortex of wild-type female mice (Fig. 2A). Similarly in midbrain raphe slices, where 5-HT_{1B} receptors do not seem to be implicated in the inhibition induced by 5-CT on [^{3}H]5-HT release (see below), methiothepin (1 μM) was ineffective in blocking the effect of 5-CT (100 nM).[9] This observation further suggests that in frontal cortex the predominant receptor population activated by 5-CT is not of the 5-HT_{1B} subtype, as seems to be the case in hippocampus. If, in fact, 5-HT_{1B} receptors were the predominant receptor population that regulates [^{3}H]5-HT release in hippocampus but not in cortex, then the enhanced electrically evoked release of [^{3}H]5-HT is not surprising because removal of 5-HT_{1B} sites would surely alter the regulation of neurotransmitter release in the region where the latter predominate. It is also noteworthy that methiothepin more effectively blocked the effect of 5-CT in slices obtained from controls than mutants (Figs 1, 2). The reason for this change in efficacy remains unclear, though it might be related to changes not only in the type but also in the sensitivity of 5-HT receptors controlling [^{3}H]5-HT release in mutants, compared with controls.

Unlike in the projection areas, the effect of 5-HT agonists and antagonists in the midbrain raphe was similar in wild type and mutant mice (Table 1, Fig. 3). The inhibition of evoked release of [^{3}H]5-HT induced by the 5-$HT_{1B/1D}$ agonist sumatriptan and the lack of effect of the selective 5-HT_{1B} agonist CP 93129 suggest that 5-HT_{1D}, rather than 5-HT_{1B} receptors, negatively control 5-HT release in mouse midbrain raphe nuclei. This idea is further supported by the fact that the effect of sumatriptan was also present in 5-HT_{1B} knock-out mice. On the other hand it is unlikely that the lack of effect of CP 93129 in both groups of mice could be attributed to the use of an insufficient concentration of drug since 100 nM of CP 93129 induced significant inhibition in hippocampus and cortex. Furthermore, the use of higher concentrations of CP 93129 did not seem appropriate since at higher concentrations the drug has been shown to induce a large displacement of [^{3}H]5-HT increasing its basal overflow,[9] making it difficult to interpret alterations of evoked release of the neurotransmitter. In contrast with our present and previous observations,[9] Davidson and Stamford[21] have recently reported that CP 93129 (300 nM) significantly inhibited 5-HT release in rat dorsal raphe slices as measured by fast cyclic voltammetry. Although this discrepancy could be due to the use of different experimental paradigms, the possibility that the concentration of CP 93129 (300 nM) used in the latter study[21] might have altered spontaneous outflow of the transmitter cannot be excluded since the authors did not report basal 5-HT outflow in the presence of the drug.

Sumatriptan has affinity not only for 5-HT_{1D} and 5-HT_{1B}, but also for 5-HT_{1A} receptors, to which it binds with 25-fold lower affinity than to 5-HT_{1B}.[12] However, the fact that the effect of sumatriptan was blocked by the 5-$HT_{1B/1D}$ receptor antagonist GR 127935,[22] but not by the selective 5-HT_{1A} antagonist (−)WAY 100135,[23] rules out the possibility that the observed inhibition might have been due to 5-HT_{1A} receptor activation. This also confirms the role of 5-HT_{1D} receptors in the negative regulation of 5-HT release in midbrain raphe nuclei. Similar results have been reported in rat midbrain slices,[9] suggesting that this is a common regulatory property of 5-HT neurotransmission in murine brain. The presence of mRNA for 5-$HT_{1D\alpha}$ receptors in rat dorsal raphe neurones[11] together with the present results, strongly suggest that 5-$HT_{1D\alpha}$ receptors play a regulatory role in 5-HT neurotransmission in rodent species.

The only difference observed between wild-type and mutant mice at the somatodendritic level was that, in the absence of any drug, the electrically evoked release of [^{3}H]5-HT was enhanced by 100% in mutants. This observation is most interesting since 5-HT_{1D} but not 5-HT_{1B} receptors seem to control 5-HT release in midbrain raphe nuclei (see above). Moreover, it further supports the idea that the mechanisms regulating [^{3}H]5-HT release are region-

specific and undergo different adaptative changes to cope with the lack of release-controlling 5-HT$_{1B}$ autoreceptors.

Conclusion

The results obtained in the present study confirm previous findings that 5-HT$_{1D}$ receptors negatively regulate 5-HT release in murine raphe nuclei[9] and indicate that that in addition to 5-HT$_{1B}$ receptors another receptor sybtype regulates 5-HT release in projection areas, such as cortex and hippocampus. The possibility that 5-HT$_{5/7}$ receptors might be involved has been suggested since it best explains the present series of observations. However, further studies, including dose–response curves for different agonists and antagonists, are needed to test this possibility.

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GENERAL DISCUSSION, SUMMARY AND CONCLUSION

The main findings of the present research endeavour may be summarized as follows: i) the 5-HTT on 5-HT neurons is down-regulated following prolonged administration of the selective SSRI paroxetine, ii) the acute electrophysiological effects of the tricyclic drug tianeptine are not mediated by an effect on the 5-HTT, iii) its long-term effects are not compatible with an enhancement of the efficacy of 5-HT synaptic transmission in rat hippocampus and, in this projection area, its sustained administration does not induce long-lasting plastic changes on the 5-HTT, iv) 5-HT release in the rat dorsal raphe may be controlled independently of 5-HT_{1A} receptor activation, v) 5-HT_{1D} receptors negatively regulate 5-HT release in rat and mice midbrain nuclei; and vi) that multiple 5-HT receptor subtypes may be involved in the regulation of 5-HT release in terminal projection areas.

Three main lines of evidence indicate a reduction in 5-HTT uptake activity in rat hippocampus following prolonged administration of the SSRI paroxetine: a) a decrease in the density of 5-HTT sites, b) a tolerance to its *in vivo* electrophysiological effects and c) a decrease of the *in vitro* [^{3}H]5-HT uptake capacity. A reduction in the total number [^{3}H]paroxetine binding sites in cortical and hippocampal membranes and a decrease in the amount of [^{3}H]5-HT taken up by dorsal raphe slices indicate that the plasticity of the 5-HTT occurs not only in multiple projection areas but also in the cell body and dendrites of 5-HT neuron (Piñeyro et al., 1994). Superfusion experiments in midbrain raphe slices provide additional evidence of the functional consequences of somatodendritic 5-HTT down-regulation: following prolonged administration of paroxetine, the electrically evoked release of [^{3}H]5-HT (during S_1) in rat and guinea-pig midbrain raphe is much higher than

that observed following sustained befloxatone treatment (El Mansari and Blier, 1996; Piñeyro and Blier, 1996). That the enhanced somatodendritic output of [^{3}H]5-HT is due to 5-HTT transporter desensitization was demonstrated by the fact that superfusion of midbrain raphe slices with medium containing 1 μM paroxetine (introduced 20 minutes before S_2) produced a 50% increase in slices obtained from saline- or befloxatone treated rats but remained unchanged in slices obtained from rats that had received paroxetine for 21 days. Furthermore, in frontal cortex of long-term paroxetine treated guinea-pigs there is no 5-HT_{1D} autoreceptor desensitization (unlike in the hippocampus and hypothalamus), and electrically evoked [^{3}H]5-HT release is still enhanced (Blier and Bouchard, 1994; El Mansari and Blier, 1996). This is due to a desensitization of the 5-HTT that was demonstrated by the reduced effectiveness of the same dose of paroxetine to inhibit [^{3}H]5-HT uptake in frontal cortex slices of guinea-pigs treated with the SSRI for 21 days as compared to two days (Blier and Bouchard, 1994).

In spite of being an extended phenomenom desensitization of 5-HT uptake activity is not ubiquitous. The same paroxetine treatment and similar paradigms that were used to demonstrate desensitization in frontal cortex did not induce any significant changes in hypothalamus (Blier and Bouchard, 1994) and orbito-frontal cortex (El Mansari et al., 1995). Regional differences in the adaptability of the 5-HTT could be explained on the basis of differences in regional predominance of distinct regulatory pathways. Though regional differences in plasticity could theoretically explain divergent observations on the adaptability of the 5-HT transporter following sustained 5-HT reuptake blockade reported in the literature (see introduction, Table 7), in most cases this explanation seems unlikely since the majority of studies have been performed either in hippocampus or frontal cortex.

A necessary condition for desentization of 5-HT uptake activity to occur seems to be a high degree of 5-HT uptake inhibition: a dose of 10 mg/kg, s.c. of paroxetine which produce 60-100% inhibition of 5-HT uptake across different brain regions of rats and guinea-pigs and has been found to consistently induce a decrease in 5-HTT function (Piñeyro et al., 1994; Blier and Bouchard, 1994; El Mansari et al., 1995, El Mansari and Blier, 1996). On the other hand, even if plasma levels of a reuptake blocker are kept stable by the use of minipumps (see discussion on this point in the last subsection of the introduction and article I, *pg: 3044*), but the degree of blockade is not high enough, no changes in 5-HTT function will be observed. Administration of fluoxetine at a dose that produced 30% blockade of 5-HT reuptake (5 mg/kg, s.c.), even if administered via osmotic minipumps for 8 weeks did not produce any change in 5-HT reuptake in the orbito frontal cortex (El Mansari et al., 1995). Another important issue that may have played a role for numerous negative results in the study of the 5-HTT following its sustained blockade is the extreme sensitivity of the desensitization response to the assay conditions. As illustrated in Figure 11 of article I, the decrease in the total amount of radioactivity taken up by hippocampal slices is seen when slices are preincubated with 100 nM but not 5 nM [^{3}H]5-HT and conversely a decrease in the efficacy of paroxetine to inhibit 5-HT uptake is seen with preincubation at 5 nM but not 100 nM concentration of [^{3}H]5-HT. Moreover, when preincubation with the radiolabelled neurotransmitter at 20 nM was used, no change in 5-HTT activity was detected.

The role of a down-regulation of the 5-HTT in the antidepressant response to SSRIs makes sense from a teleological point of view since the consequence of a reduction in the number of transporters (and blockade of the remaining ones) could imply an enhancement of the efficacy of 5-HT synaptic transmission, secondary to an increase

in 5-HT remaining in the synapse following each action potential. On the other hand, the antidepressant response may be achieved within the first week of treatment when an SSRI is combined with pindolol (Artigas et al., 1994; 1996; Blier and Bergeron, 1995; Isaac et al., 1996). Given that within this same time period the transporter is not desensitized (El Mansari et al., 1995), it does not seem that its down-regulation is an essential component for the rapid antidepressant effect. On the other hand, it may account for part of the slower but progressive improvement (Blier and Bergeron, 1995; Isaac et al., 1996) in the following weeks of treatment.

An increase in CA_3 pyramidal firing was the only effect observed following the acute (intravenous or microiontophoretic) administration of tianeptine in our study. The activating effect of tianeptine on the firing activity of dorsal hippocampus pyramidal neurons was not modified by 5,7-DHT lesion and thus it was concluded that it does not depend on the integrity of 5-HT terminals and the 5-HTT on them (Piñeyro et al., 1995a). Though it could be argued that the lesion may have not been complete, the lack of any change in this stimulating effect following a treatment that has been shown to destroy more than 90% of hippocampal 5-HT terminals (de Souza and Kuyatt, 1987) is an unexpected response from a drug claimed to increase hippocampal pyramidal neuron firing activity secondary to an increase in 5-HT uptake activity (Dresse and Scuvée-Moreau; 1988). However, it should be noticed that Dresse and Scuvée-Moreau (1988) inferred that the enhancing effect demonstrated for tianeptine in *ex vivo* uptake studies could account for the *in vivo* increase in CA_1 pyramidal neuron firing and the reduction in the effect of chlomipramine observed following the systemic administration of tianeptine. In fact, most if not all of the *in vivo* studies that have been performed to determine the mechanism of action underlying the neurochemical or behavioural effects

of tianeptine have been indirect (see introduction, section on uptake, tianeptine). Furthermore, since the initial studies in which tianeptine was believed to specifically enhance 5-HT uptake, other pharmacological effects of the drug have been described (see introduction, *e.g:* Bolaños-Jiménez et al., 1993 and Frankfurt et al., 1995, cited therein) and it is then possible that some of its previously described effects are not exclusively due to its 5-HT uptake enhancing properties.

Following its prolonged administration, tianeptine was found to reduce the recovery time from microiotophoretic applications of 5-HT and interfere with the action of the SSRI paroxetine, both actions compatible with increased 5-HT uptake. However, more direct *ex vivo* biochemical studies, did not demonstrate increased [^{3}H]5-HT uptake in hippocampal slices (Piñeyro et al., 1995a) and, none of the *in vivo* effects compatible with an increase in 5-HT upatke seemed to correlate with an enhancement in the efficacy of 5-HT synaptic transmission in rat hippocampus. Moreover, the amount of tianeptine and its metabolites that were found in the brain following 20 mg/kg/d x 14 days (via osmotic minipump) were irregular. This was the same dose regimen that produced a faster recovery of CA, pyramidal neurons from 5-HT applications, and reduced the effectiveness of paroxetine to prolong 5-HT-induced inhibition of CA_3 neuron firing but did not enhance the local efficacy of 5-HT neurotransmission (Piñeyro et al., 1995b). In the literature, results from *ex vivo* uptake and binding studies in the brain following prolonged tianeptine administration have been variable (see introduction), a possible explanation could be an irregular penetration across the blood brain barrier. Such an explanation is indeed compatible with the lack of effect of sustained tianeptine administration on hippocampal [^{3}H]cyanoimipramine binding parameters observed in the present study. On the other hand, the mechanism responsible for the observed increase in cortical 5-HTT

number remains unclear.

Evidence indicating that 5-HT release in the rat dorsal raphe may be controlled independently of 5-HT_{1A} receptor activation was gathered using electrophysiological and neurochemical techniques. The first paradigm allowed to indirectly infer the amount of extracellular 5-HT available to stimulate 5-HT_{1A} somatodendritic autoreceptors, by measuring changes in 5-HT neuron firing activity; functional consequence of 5-HT_{1A} receptor activation. That the changes in 5-HT neuron firing were mediated by 5-HT activating 5-HT_{1A} receptors were proven by Wang and Aghajanian (1977), and in article IV of this study. The fact that 5-HT_1 agonists TFMPP and RU 24969 were shown to reduce the duration of suppression of 5-HT neuron firing evoked by 5-HT pathway stimulation, without modifying the basal electrical activity of these neurons, was interpreted indication that TFMPP and RU 24969 could reduce extracellular 5-HT in the dorsal raphe nuclei without activating 5-HT_{1A} receptors. This assumption was supported by a) voltammetry studies in which the injection of TFMPP was shown to reduce the 5-hydroxyindole signal in the DRN and b) by the fact that (+) WAY 100135 blocked neither the electrophysiological or neurochemical effects of TFMPP (articles IV and V).

Neither of the *in vivo* systemic approaches allowed a detailed study of the pharmacology of non-5HT_{1A} receptors controlling 5-HT release in the midbrain raphe nuclei. Superfusion studies performed in midbrain raphe slices of rats and mice indicated that 5-HT_{1D} receptors negatively regulate 5-HT release in rodent brain in this area. The accumulated evidence may be separated into three categories. **1. Observations that directly support a role for 5-HT_{1D} receptors include**: i) the prototypical 5-HT_1 agonist 5-CT induced a concentration-dependent inhibition of the electrically-evoked release of [^{3}H]5-HT from preloaded rat raphe slices which was partly blocked by the 5-$HT_{1B/1D}$ antagonist

GR 127935 and by mianserin, which in rats has a very high selectivity for 5-HT_{1D} receptors; ii) the 5-$HT_{1B/1D}$ agonist sumatriptan inhibited electrically-evoked release of [^{3}H]5-HT from rat and wild-type mice raphe slices, and this effect was inhibited by mianserin and GR 127935, iii) midbrain slices from 5-HT_{1B} knock-out mice maintained a similar pharmacological response as that observed in wild-types and iv) the sumatriptan response has the same functional characteristics as 5-HT_{1D} receptor: it is mediated by NEM-, pertussis toxin-sensible, $G_{i/o}$-protein-coupled receptors. **2.** Observations that directly rule out other specific receptor subtypes include: i) the fact that the effect of sumatriptan was not blocked by S-UH-301 nor by (+) WAY 100135 excludes a possible non-selective activation of 5-HT_{1A} receptors and ii) in spite of its inhibitory effect on evoked [^{3}]5-HT release in terminal regions, the lack of effect of the selective 5-HT_{1B} agonist CP 93129 to inhibit evoked release from rat and mice midbrain slices rules out the possibility that 5-HT_{1B} may have been involved in the sumatriptan-mediated response in midbrain raphe. **3.** Findings that allow to **rule out** a role of other receptor subtypes in the observed inhibition of 5-HT release from raphe slices include: i) 5-HT_{1E} and 5-HT_{1F} receptors have low affinity for 5-CT, ii) the former subtype does not bind sumatriptan with high affinity, iii) the latter receptor subtype has low affinity for methiothepin and does not bind propranolol, both of which blocked the effect of sumatriptan; hence, in spite of the fact that sumatriptan binds to 5-HT_{1F} receptors it is unlikely that these or the 5-HT_{1E} subtype may have mediated the sumatriptan-induced inhibition. iv) Sumatriptan and GR 127935 have very low affinity for 5-$HT_{5A/5B}$ receptors, v) the affinity of sumatriptan for 5-HT_7 receptors is also considerably low (though higher than for 5-HT_5), vi) if indeed 5-HT_7 receptors would had played an important role in inhibiting 5-HT release in midbrain nuclei the inhibitory effect of 8-OH-DPAT (that binds with higher affinity to 5-HT_7 than 5-HT_{1D}

receptors) would have been greater (due to summatory effect) and not similar, to that of sumatriptan; hence, even if mRNA for both 5-$HT_{5/7}$ receptor subtypes is present in the DRN, the 5-HT_{1D} receptor was deemed a better choice that would contemplate the whole set of observations. (Note: see introduction Table 7 for affinity values).

In contrast with our observations on the role of 5-HT_{1B} receptors in controlling 5-HT release in the DRN Davidson and Stamford (1995), using a higher concentration of CP 93129, have reported that it inhibited *in vitro* 5-HT release from rat DRN. However, in the british study, the effect of CP 93129 was blocked at scattered time points during a continuous superfusion with isamoltane. The effectiveness of blockade was judged by a significant difference on these points and not by an ANOVA on the time effect curves of CP 93129 vs CP 93129 + isamoltane. Another difference between our study and that of Davidson and Stamford (1995) was the stimulation protocol used. In our study, stimulation parameters were set to mimick 5-HT neuron firing frequency during wakefulness (1 Hz), while in the latter pseudo-single pulse stimulations (100 Hz) were used. It has been extensively documented in the introduction that different stimulation parameters, producing different concentration of endogeneous 5-HT in the biophase of 5-HT receptors, may produce different results even if all other experimental variables are kept constant. If indeed 5-HT_{1B} receptors play any regulatory role in rat midbrain raphe, it would probably involve terminal 5-HT release while 5-HT_{1D} receptors would control its larger somatodendritic counterpart (see introduction). Since 5-HT terminals are certainly not abundant in DRN and MRN (see introduction), the previous interpretation would contemplate various observations: i) that the effect of tonic activation of this receptor even following an SSRI seems negligible (see Figure 4 of the introduction), ii) that the effect of 5-HT_{1B} receptor activation in this area may be negligible when extracellular 5-HT

concentrations are high, like would be expected during wakefulness, iii) it would be in keeping with the fact that 5-HT_{1B} receptors are terminal autoreceptors in rat brain and finally, iv) could explain the observation that even if binding studies show a predominance of 5-HT_{1B} receptors over the 5-HT_{1D} subtype, in our paradigm, the activation of the latter produces an effect that is not produced by the former.

Concerning the actual location of the 5-HT_{1D} receptors, the fact that they are functional in midbrain raphe slices indicate that they may be present within the midbrain raphe nuclei. Indeed, in this paradigm the raphe nuclei are separated from their afferent sources so the possibility that the receptors in question are located on cell bodies of any of the extra-raphe afferents to the 5-HT nuclei may be ruled out. Furthermore, since addition of TTX to the perfusion medium of midbrain raphe slices does not modify the inhibitory effect of sumatriptan on the K^+-evoked release of [^{3}H]5-HT (Piñeyro and Blier, 1996a) 5-$HT_{1D\alpha}$ receptors have been proposed to be autoreceptors. This suggests that 5-HT_{1D} receptors in raphe nuclei, as the 5-HT_{1A} subtype, are located on 5-HT neurons. However, direct immunocytochemical studies using 5-HT_{1D} receptor antibodies should be used to confirm this hypothesis. It is also worth noting that there is no direct evidence that proves that the effects observed *in vivo* are caused by the activation of the receptors activated *in vitro*. If indeed the response mediated by TFMPP and RU 24969 in the electrophysiological paradigm were secondary to 5-HT_{1D} **autoreceptor** activation, it would be expected that none of the drugs would have changed the frequency to the initiation of the suppression elicited by 5-HT pathway stimulation. The recording conditions in our study were not set up to address this issue so no conclusion may be drawn regarding this point.

The possible role of 5-HT_{1D} autoreceptors in the antidepressant response has

been analyzed in the introduction. With regards to the human brain, it has been claimed for some time, that the blockade of inhibitory terminal 5-HT_{1D} autoreceptors could be one of the alternative strategies to induce a faster anidepressant effect. When this hypothesis was tested in freely moving guinea-pigs, it was observed that for the 5-HT_{1D} antagonist GR 127935 to produce an increase in cortical 5-HT release previous blockade of 5-HT_{1A} autoreceptors was necessary (see introduction; Figure 4). This observation may be interpreted by incorporating the notion of a somatodendritic inhibitory 5-HT_{1D} receptor that when blocked (in freely-moving animals) induces an increase in somatodendritic availability of 5-HT and activates 5-HT_{1A} autoreceptors. On the other hand, if the animals are anesthesized 5-HT neurons are not firing at high frequency and an increase in extracellular 5-HT may not induce further changes. Furthermore, since the basal firing frequency is slow in anesthesized animals, the amount of 5-HT released by spontaneous cell depolarization is also low making it possible that the blockade of an inhibitory somatodendritic 5-HT_{1D} autoreceptor has no important consequences given the fact that it is not being activated. Hence, low 5-HT neuron firing activity as well as low somatodendritic 5-HT release during anesthesia could account for the lack of change in guinea-pig 5-HT neuron-firing frequency observed by Sprouse et al., (1995) following GR 127935 administration. These results are in contrast with the decrease in 5-HT release observed by Price et al., (1994) in cortex of freely moving guinea-pigs following the administration of this 5-HT_{1D} receptor antagonist. If we keep in mind that the expected effect of terminal 5-HT_{1D} receptor blockade would be an increase in 5-HT release in projection areas, then the observed opposite effect indicates that the local intraraphe increase in extracellular 5-HT, secondary to somatodendritic 5-HT_{1D} receptor blockade overrides the terminal effect. The corollary to this observation would be that only a

selective blockade of terminal 5-HT_{1D} receptors may acutely enhance 5-HT release. Theoretically then, activation of somatodendritic 5-HT_{1D} receptor would have a similar enhancing effect. Thus, the possibility of applying 5-HT_{1D} drugs as an alternative antidepressant strategy relies on the development of terminal vs somatodendritic specific ligands. If indeed, as proposed above (see introduction) in humans as in rodents, somatodendritic receptors are of the 5-$HT_{1D\alpha}$ subtype and terminal receptors are of the 5-$HT_{1D\beta}$ subtype this migh be possible in the near future.

An alternative strategy to produce a faster antidepressant response would be to develop antagonists for terminal autoreceptors that do not play a significant role in regulating 5-HT neuron activity at the somatodendritic level. Results from superfusion studies in 5-HT_{1B} knock-out mice do indeed provide evidence that a non-homogeneous terminal autoreceptor population exists: non-5-HT_{1B} receptors continue to control 5-HT release in terminal projection areas in the absence of 5-HT_{1B} receptors. Furthermore, it is possible that the combination of autoreceptor subtypes in the terminal autoreceptor populations in different projection areas (*eg:* cortex and hippocampus) are not the same. This would not be surprising given that each of the different 5-HT nuclei contribute in a different manner to the innervation of distinct terminal fields (see introduction). It is possible then to speculate that in the future new antidepressant drugs will become not only more efficacious and rapid as a result of being not only receptor- but also structure-specific.

In conclusion, the present research endeavour demonstrates there is a desensitization of the 5-HT transporter following administration of paroxetine, an adaptative response that may contribute to long-term antidepressant effects of SSRIs. The role of 5-HT_{1D} receptors in the regulation of 5-HT availability in the biophase of

somatodendritic autoreceptors and the possibility that more than one 5-HT receptor subtype controls 5-HT release in terminal projection areas, were also assessed. Given the importance of autoregulation of 5-HT neurotransmission in the antidepressant response, it is hoped these findings will contribute to the future development of new antidepressant drugs or combination strategies for the treatment of depression.

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B. Physiological aspects of the 5-HT system

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iii) 5-HT reuptake

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General discussion, summary and conclusion

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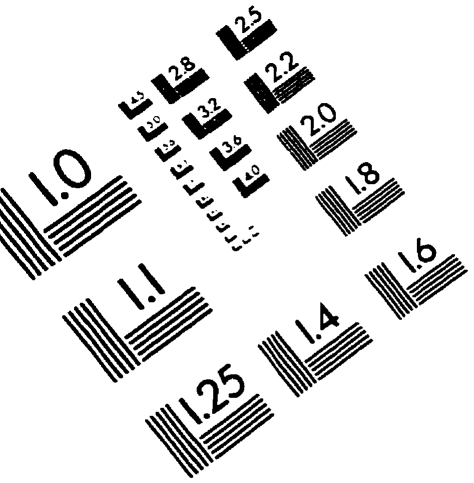
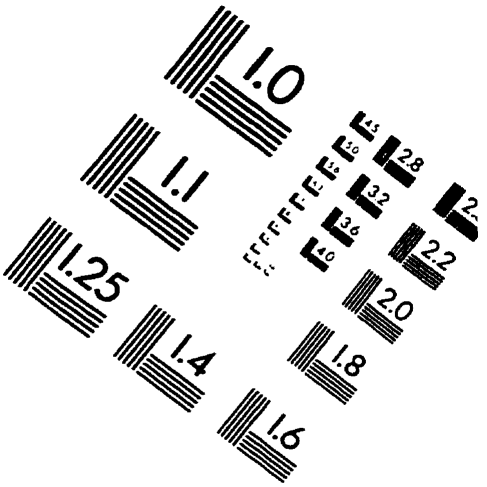
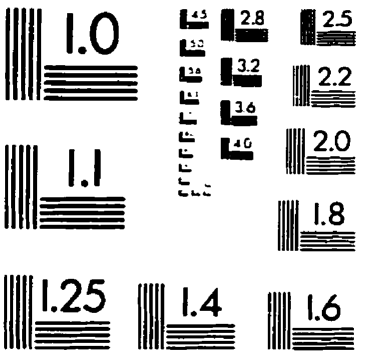
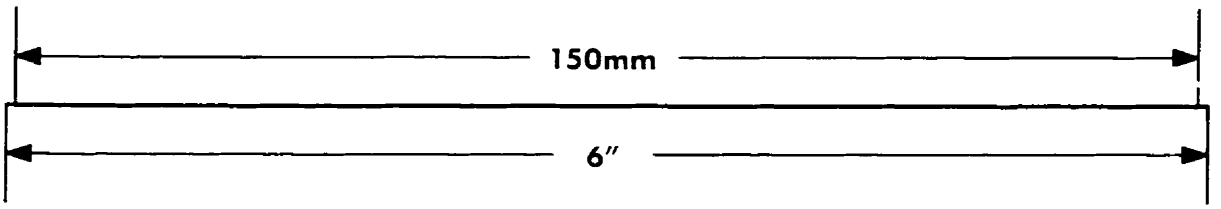
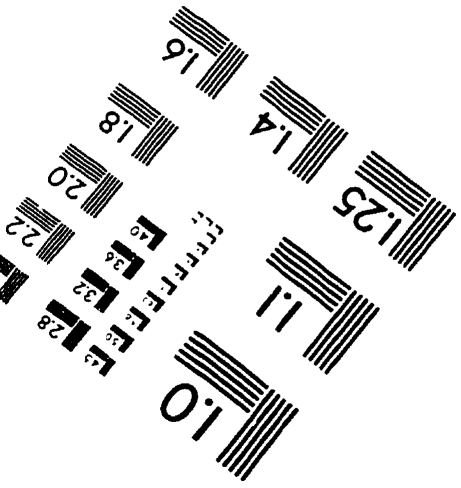
IMAGE EVALUATION TEST TARGET (QA-3)

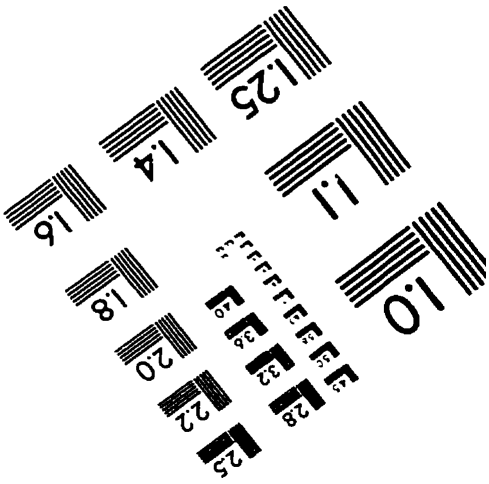












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