# EFFECT OF CHRONIC PRENATAL ETHANOL EXPOSURE ON NMDA RECEPTOR NUMBER AND AFFINITY FOR [$^3$H]MK-801 IN THE CEREBRAL CORTEX OF THE GUINEA PIG DURING POSTNATAL LIFE

by

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A thesis submitted to the Department of Pharmacology and Toxicology in conformity with the requirements for the degree of Master of Science

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# Abstract

Rajan Kumar Puri: Effect of Chronic Prenatal Ethanol Exposure on NMDA Receptor Number and Affinity for [$^{3}$H]MK-801 in the Cerebral Cortex of the Guinea Pig During Postnatal Life. M.Sc. Thesis, Queen's University, Kingston, Ontario, Canada, September, 1999.

Exposure to ethanol during fetal development can produce severe, permanent neurobehavioural problems in the offspring. A proposed mechanism by which ethanol produces these effects is perturbation of the NMDA receptor system, optimal activation of this system is required for normal brain development and under-activation or over-activation can produce abnormal brain development. Studies have shown that in the near-term guinea pig, chronic prenatal ethanol exposure can increase the number of NMDA receptors in the cerebral cortex and decrease their affinity for [$^{3}$H]MK-801. The objective of this thesis was to test the hypothesis that chronic prenatal ethanol exposure produces persistent changes in the number of *N*-methyl-D-aspartate (NMDA) receptors and/or their affinity for [$^{3}$H]MK-801 in the cerebral cortex during postnatal life. Timed, pregnant Dunkin-Hartley-strain guinea pigs received oral intubation of one of the following regimens, given daily as two equally divided doses 2 hr apart from gestational day (GD) 2 to GD 67 (term, about GD 68): 4 g ethanol / kg maternal body weight; isocaloric-sucrose / pair-feeding; or water. Maternal blood ethanol concentration was measured on GD 57 or 58, 1 hr after the daily dose, and was 235±39 mg/dl (mean±SD). At postnatal day (PD) 10 (pre-weaning), PD 20 (post-weaning) and PD 60 (adulthood), horizontal and vertical spontaneous locomotor activity, body, whole brain and cerebral cortical weights of the offspring were measured. The number ($B_{max}$) of NMDA receptors and their affinity ($K_d$) for [$^{3}$H]MK-801 were determined in cerebral cortical membrane preparations using a radioligand-binding assay followed by saturation isotherm analysis of the data. Chronic prenatal ethanol exposure increased spontaneous locomotor activity, and decreased brain and cerebral cortical weights in offspring at PD 10 and 20 but not PD 60. In contrast, at PD 60, the $B_{max}$ and $K_d$ for [$^{3}$H]MK-801 binding to cerebral cortical NMDA receptors were decreased in the ethanol treatment group compared with the isocaloric-sucrose / pair-fed and water treatment group. There were no changes in [$^{3}$H]MK-801 binding at either of the two younger postnatal ages. Loss of NMDA receptors in the cerebral cortex suggests that chronic prenatal ethanol exposure produces delayed neuronal injury in this brain region.

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# Table of Contents

**Page**

Abstract ............ ii

Acknowledgements ............ iii

Table of Contents ............ iv

List of Tables ............ vii

List of Figures ............ vii

List of Abbreviations and Symbols ............ viii

1. INTRODUCTION

1.1 Statement of the Research Problem ............ 1
1.2 Pharmacology and Toxicology of Ethanol ............ 3

1.2.1 Absorption, Distribution and Elimination of Ethanol in the Adult ............ 3
1.2.2 Ethanol Pharmacokinetics in the Maternal-Fetal Unit ............ 4

1.3 Fetal Alcohol Syndrome (FAS) ............ 6

1.3.1 Manifestations of the FAS ............ 6
1.3.2 Ethanol CNS Teratogenesis ............ 8
1.3.3 Incidence of the FAS ............ 9
1.3.4 The Cerebral Cortex and Hippocampus as Target Sites of Ethanol CNS Teratogenesis ............ 10
1.3.5 Mechanisms of Ethanol CNS Teratogenesis ............ 11

1.3.5.1 Ethanol-Derived Acetaldehyde ............ 12
1.3.5.2 Ethanol-Induced Fetal Hypoxia ............ 13
1.3.5.3 Ethanol-Induced Undernutrition ............ 13
1.3.5.4 Ethanol-Induced Alteration in Prostaglandin Metabolism ............ 14
1.3.5.5 Perturbation of NMDA Receptor-Mediated Synaptic Transmission ............ 15

1.4 Effects of Prenatal Ethanol Exposure on Behaviour ........ 16
1.5 Excitatory Amino Acid Neurotransmission Involving the
L-Glutamate Pathway ........ 18
1.6 NMDA Receptors ........ 20

1.6.1 NMDA Receptor Pharmacology ........ 20
1.6.2 NMDA Receptor Subtypes ........ 22
1.6.3 Role of NMDA Receptors in Neurobehaviour ........ 24
1.6.4 Role of NMDA Receptors in Brain Development ........ 26
1.6.5 Ontogenic Expression of NMDA Receptor Subunit
Subtypes.... ........ 28
1.6.6 Effects of Ethanol on NMDA Receptors in the
Adult Brain ........ 30
1.6.7 Effects of Ethanol on NMDA Receptors in the
Developing Brain ........ 32

1.7 Research Rationale, Hypothesis and Objectives ........ 34

2. MATERIALS AND METHODS

2.1 Chemicals and Solutions ........ 36
2.2 Experimental Animals and Breeding Procedure ........ 36
2.3 Chronic Maternal Treatment Regimens ........ 37
2.4 Gas-Liquid Chromatographic Analysis of Maternal Blood Ethanol
Concentration ........ 38
2.5 Pregnancy Outcome ........ 39
2.6 Measurement of Locomotor Activity ........ 39
2.7 Dissection of the Cerebral Cortex and Hippocampus and Membrane
Preparation ........ 40
2.8 [$^3$H]MK-801 Saturation Binding Assay ........ 40
2.9 Glutamate Activation of the NMDA Receptor Assay ........ 41
2.10 Data Analysis ........ 42

3. RESULTS

3.1 Maternal Blood Ethanol Concentration ........ 43
3.2 Pregnancy Outcome Data ........ 43
3.3 Effect of Chronic Prenatal Ethanol Exposure on Spontaneous
Locomotor Activity ........ 45
3.4 Effect of Chronic Prenatal Ethanol Exposure on Offspring Body,
Brain and Cerebral Cortical Weights ........ 48
3.5 Effect of Chronic Prenatal Ethanol Exposure on Specific [$^3$H]MK-801
Binding in the Cerebral Cortex and Hippocampus ........ 51
3.6 Effect of Chronic Prenatal Ethanol Exposure on NMDA Receptor
Activation by Glutamate in the Cerebral Cortex ........ 57

4. DISCUSSION

4.1 Discussion of Thesis Research ........ 59
4.2 Future Research Directions ........ 66

4.2.1. Effect of Chronic Prenatal Ethanol Exposure on NMDA Receptor Expression in the Adult Cerebellum ........ 66
4.2.2 Effect of Ethanol Exposure During Critical Periods of Development on NMDA Receptor Expression ........ 66
4.2.3 Effect of Chronic Prenatal Ethanol Exposure on NMDA Receptor NR1 and NR2 Subunit Expression ........ 67
4.2.4 Effect of Chronic Prenatal Ethanol Exposure on Cerebral Cortical Neurons ........ 68
4.2.5 Effect of Chronic Prenatal Ethanol Exposure on other Behavioural Parameters ........ 69

References ........ 70

Vita ........ 87

LIST OF TABLES

Table 1. Effect of Chronic Maternal Administration of Ethanol, Isocaloric-Sucrose / Pair Feeding or Water on Pregnancy Outcome of the Guinea Pig ........ 44

Table 2. Body, Whole Brain and Cerebral Cortical Weights at PD 10, 20 and 60 for Offspring of Pregnant Guinea Pigs that received Chronic Oral Administration of Ethanol, Isocaloric-Sucrose / Pair Feeding or Water ........ 49

LIST OF FIGURES

Figure 1A. Cumulative horizontal spontaneous locomotor activity ........ 46

Figure 1B. Cumulative vertical spontaneous locomotor activity ........ 47

Figure 2. Representative saturation isotherm (A) and Scatchard plot (B) .... 52

Figure 3. $B_{max}$ (A) and $K_d$ (B) for [$^3$H]MK-801 binding to cerebral cortical NMDA receptors in the PD 10 offspring ........ 53

Figure 4. $B_{max}$ (A) and $K_d$ (B) for [$^3$H]MK-801 binding to cerebral cortical NMDA receptors in the PD 20 offspring ........ 54

Figure 5. $B_{max}$ (A) and $K_d$ (B) for [$^3$H]MK-801 binding to cerebral cortical NMDA receptors in the PD 60 offspring ........ 55

Figure 6. $B_{max}$ (A) and $K_d$ (B) for [$^3$H]MK-801 binding to hippocampal NMDA receptors in the PD 60 offspring ........ 56

Figure 7. $E_{max}$ (A) and $EC_{50}$ (B) for glutamate stimulated [$^3$H]MK-801 binding to cerebral cortical NMDA receptors in PD 60 offspring ........ 58

# List of Abbreviations

| | |
|---|---|
| *Ad libitum* | at will |
| ANOVA | analysis of variance |
| ARND | alcohol related neurodevelopmental disorder |
| $B_{max}$ | binding site density |
| $Ca^{2+}$ | calcium ion |
| cGMP | cyclic guanosine 3'5'-monophoshate |
| CNS | central nervous system |
| Cyp | cytochrome P450 |
| DNA | deoxyribonucleic acid |
| EAA | excitatory amino acid |
| EDTA | ethylenediaminetetracetic acid |
| e.g. | *exempli gratia*, for example |
| FAE | fetal alcohol effects |
| FAS | fetal alcohol syndrome |
| g | gram (s) |
| *g* | gravitational force |
| GD | gestational day |
| Glu | l-glutamate |
| Gly | glycine |
| hr | hour |
| HEPES | N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid] |
| *in vitro* | in glass |
| *in vivo* | in the living body |
| IQ | intelligence quotient |
| $K_d$ | apparent dissociation constant |
| kg | kilogram |
| L | litres |
| LTP | long-term potentiation |
| M | molar |
| MCi | milicurie |
| Mg | milligram |
| $Mg^{2+}$ | magnesium |
| µg | microgram (s) |
| µm | micrometre (s) |
| min | minute |
| MK-801 | ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclo-hepten-5,10-imine hydrogen maleate |
| [$^3$H]MK-801 | tritiated MK-801 |
| Ml | mililitre(s) |
| mM | milimolar |
| mmol | milimole(s) |
| mRNA | messenger ribonucleic acid |

| | |
|---|---|
| n | number of determinations |
| $Na^+$ | sodium ion |
| nM | nanomolar |
| NMDA | *N*-methyl-D-aspartate |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| PD | postnatal day |
| PG | prostaglandin |
| $PGE_2$ | prostaglandin $E_2$ |
| pH | - base ten logarithm hydrogen ion concentration |
| RNA | ribnucleic acid |
| SD | standard deviation |
| SEM | standard error of the mean |
| w/v | weight per volume |
| °C | degrees centigrade (Celsius) |
| ≥ | greater than or equal to |
| < | less than |
| ≤ | less than or equal to |
| % | percent |
| ± | plus or minus |
| ® | registered trademark |

# 1. INTRODUCTION

## 1.1 Statement of the Research Problem

Despite the knowledge that consumption of ethanol[1] during pregnancy can produce toxic effects on the offspring, prenatal ethanol exposure has persisted since the discovery of alcohol. Ethanol in the maternal blood circulation rapidly distributes across the placenta into the fetal blood circulation, and it has been well established that ethanol can produce teratogenic effects in the developing fetus. The term fetal alcohol syndrome (FAS), was coined to describe the characteristic teratogenic effects that chronic maternal ethanol consumption can produce in offspring (Jones and Smith, 1973). This syndrome is characterized by three major features: growth restriction, craniofacial abnormalities and CNS dysfunction and dysmorphology (Jones and Smith, 1973, Clarren and Smith, 1978, Streissugth et al., 1986). The CNS dysfunction and dysmorphology include intellectual (e.g. mild to moderate mental deficiency), behavioural (e.g. hyperactivity) and neurological (e.g. poor coordination) dysfunction, developmental delay and microencephaly. CNS teratogenicity is considered to be the most debilitating manifestation of the FAS.

The mechanism by which prenatal ethanol exposure produces these teratogenic effects is not clearly understood. Some potential causes of the FAS include ethanol-derived acetaldehyde toxicity, fetal hypoxia, undernutrition, alterations in prostaglandin metabolism, and perturbation of the L-glutamate-N-methyl-D-aspartate (NMDA) receptor-nitric oxide synthase neuronal system. Of the proposed mechanisms, perturbation of the L-glutamate-NMDA receptor-nitric oxide synthase neuronal system provides a plausible explanation of the CNS teratogenicity produced by chronic prenatal

[1] The terms, ethanol and alcohol, are used interchangeably throughout this thesis.

exposure to ethanol.

The NMDA receptor is an ionotropic, excitatory amino acid receptor that, upon activation, allows the influx of calcium and sodium ions. Optimal functioning of the NMDA receptor population during development is critical as these receptors play an important neurotrophic role, including involvement in neurogenesis, neuronal differentiation and synaptogenesis. In postnatal life, the NMDA receptor participates in other forms of synaptic plasticity, including long-term potentiation, which is considered to be an electrophysiologic correlate of memory. Therefore, prenatal exposure to a chemical agent or pathophysiological insult that alters NMDA receptors of the L-glutamate neuronal system may cause dysmorphology and / or dysfunction in the offspring that manifests in postnatal life.

The cerebral cortex and hippocampus are two brain regions in which ethanol has been shown to have teratogenic effects. The cerebral cortex is important for cognitive function and regulation of behaviour, whereas the hippocampus is important for encoding new information into long-term memory. The goal of this thesis research was to determine whether chronic prenatal ethanol exposure alters NMDA receptor number and / or affinity for [$^{3}$H]MK-801 in these target brain regions during postnatal life.

## 1.2 Pharmacology and Toxicology of Ethanol

Ethanol consumption has been a part of human culture since its discovery by the ancient Egyptians. At low doses, alcohol produces pleasant sedating effects in an adult but at high blood ethanol concentrations, these depressant effects can produce life-threatening respiratory depression. Chronic consumption of ethanol can produce a wide variety of conditions ranging from cirrhosis of the liver to microencephaly or malnutrition.

### 1.2.1 Absorption, Distribution and Elimination of Ethanol in the Adult

Ethanol is rapidly absorbed from both the stomach and the small intestine into the bloodstream (Lim et al., 1993). There is limited first pass elimination by the stomach and liver, consequently, much of the ethanol consumed is absorbed into the bloodstream and circulated throughout the body (Lim et al., 1993, Lieber, 1994). Ethanol is readily able to distribute into cells, and due to its hydrophilicity, the intracellular concentration of ethanol is proportional to the water content of the cell.

Elimination of ethanol from the body occurs predominantly by hepatic biotransformation; the liver is responsible for the biotransformation of 90 to 95% of the body content of ethanol in adult mammalian species (Khanna and Israel, 1980). Alcohol dehydrogenase catalyzes the first step of hepatic biotransformation by oxidizing ethanol to its proximate metabolite, acetaldehyde. The second step in hepatic biotransformation is catalyzed by acetaldehyde dehydrogenase that, via oxidation, converts acetaldehyde to acetate. The ethanol-inducible hepatic cytochrome P450 2E1 enzyme also possesses the ability to catalyze ethanol oxidation (Carpenter et al., 1996, Lasker et al., 1987). This enzyme can play an important role at high ethanol concentrations at which alcohol

dehydrogenase becomes saturated. Catalase is another enzyme which possesses the ability to catalyze the oxidation of ethanol to acetaldehyde (Khanna and Israel, 1980), although the importance of this biotransformation pathway in the disposition of ethanol *in vivo* is not clearly understood.

1.2.2 Ethanol Pharmacokinetics in the Maternal-Fetal Unit

In order for ethanol to have direct effects upon the fetus, it must pass from the maternal circulation across the placenta into the fetal circulation. There is rapid, unimpeded, bi-directional placental transfer of ethanol between the maternal and fetal compartments (Clarke et al., 1985, Brien and Smith, 1991). In a study performed in pregnant monkeys and hamsters, [$^{14}$C] labelled ethanol was injected intravenously into the maternal blood circulation during the last trimester of pregnancy. Within fifteen minutes of administration, the concentration of [$^{14}$C]ethanol was identical in all vascularized tissue of both the mother and the fetus (Idanjaan-Heikkila et al., 1971). These results were replicated in the guinea pig (Clarke et al., 1985) and pregnant ewe (Lafonde et al., 1985). Based on these findings, the maternal blood ethanol concentration appears to be a reliable indicator of the fetal blood ethanol concentration.

Unlike the adult liver, the fetal liver possesses very little alcohol dehydrogenase activity throughout gestation (Card et al., 1989). Chronic prenatal ethanol exposure causes induction of CYP2E1 in the fetal liver. Even with this up-regulation, fetal hepatic microsomal enzymes have limited catalytic ability to oxidize ethanol, approximately 12 to 27% of adult liver microsomal enzyme activity. Consequently, the fetus must rely upon placental transfer of ethanol from the fetal circulation to the maternal circulation and subsequent maternal hepatic biotransformation of ethanol for drug elimination

(Pikkarainen and Rainha, 1967, Clarke et al., 1986a, Clarke at el., 1988). The fetal liver however, does possess aldehyde dehydrogenase activity and is therefore able to metabolize any acetaldehyde that enters the fetal circulation (Clarke et al., 1986a, Clarke at el., 1988). The maternal blood acetaldehyde concentration is a thousand-fold lower than the materanl ethanol concentration and the fetal blood acetaldehyde concentration is ten-fold lower than the maternal acetaldehyde concentration (Clarke et al., 1985). The low level of alcohol dehydrogenase activity and the appreciable aldehyde dehydrogenase activity in the fetal liver acts to protect the fetus from ethanol-derived acetaldehyde.

Studies in pregnant women at 16-18 weeks of gestation (term, about 36 weeks) have shown that there is a time lag in the distribution and elimination of ethanol in the amniotic fluid compared with maternal blood (Brien et al., 1983). Similar results have been obtained in studies conducted with the near-term pregnant ewe (Ng et al., 1982, Brien et al., 1985, Clarke et al., 1988) and guinea pig (Clarke et al., 1986a, Clarke et al., 1986b). Based on these findings, it has been proposed that the amniotic fluid surrounding the fetus may serve as a temporary reservoir for ethanol *in utero* causing prolonged fetal ethanol exposure.

## 1.3 Fetal Alcohol Syndrome (FAS)

### 1.3.1 Manifestations of the FAS

The term Fetal Alcohol Syndrome (FAS), was first used in 1973 to describe the serious post-natal effects of chronic maternal ethanol consumption on offspring (Jones and Smith, 1973). A child diagnosed with the FAS possesses the following symptoms: craniofacial abnormalities (including small palpebral fissures, long, smooth philtrum, narrow upper vermillon); growth restriction (including microcephaly, microencephaly and / or decreased body weight); and CNS dysfunction and dysmorphology (including intellectual deficits, neurological alterations and/or behavioural impairments).

The teratogenic effects of ethanol occur over a continuum. FAS is used to describe a patient who has all three of the aforementioned features of ethanol teratogenicity. In many cases, children who are exposed to ethanol prenatally do not exhibit all of the features of the FAS, but may suffer from one or more of the potential problems associated with chronic prenatal exposure. These children may be diagnosed as having fetal alcohol effects (FAE) (Rosett et al., 1981, Mattson and Riley, 1998) or alcohol-related birth defects (ARBD) (Sokol and Clarren, 1989). Individuals who do not exhibit any of the structural teratogenic effects produced by prenatal ethanol exposure but have some form of CNS dysfunction, may be diagnosed as having alcohol-related neurodevelopmental disorder (ARND) (Abel and Hannigan, 1995, Mattson et al., 1997).

There are several factors that influence the morphological, neurochemical and behavioural alterations induced by prenatal exposure to ethanol. These factors include the developmental stage during which ethanol exposure occurs, the pattern of drinking and the maximum blood ethanol concentration reached during drinking episodes (Goodlett [in

press], Smith et al., 1986, Streissguth et al., 1989, West et al., 1989, Jacobson et al., 1994).

The developmental period during which the fetus is exposed to ethanol is a key determinant of the organ system upon which ethanol has teratogenic effects because many systems develop at different stages of gestation. Exposure to ethanol during the early stages of gestation produces craniofacial and growth abnormalities (Sulik et al., 1981), whereas exposure during late gestation causes CNS functional deficits (Meyer et al., 1990a, Meyer et al., 1990b).

The pattern of maternal ethanol ingestion is another important determinant that influences the teratogenic effects of ethanol in the developing fetus because it directly affects the maximum ethanol concentration. Several studies have shown that consuming smaller doses of ethanol in a binge-type exposure pattern can produce more severe brain damage than a pattern of exposure that involves a greater dose of ethanol consumed over a longer time period (Pierce and West, 1986, Bonthius and West, 1990, Catlin et al., 1992). To date, a threshold has not been established in the human below which maternal consumption of ethanol is safe. In a study by Sisenwein et al (1983), it was reported that a single exposure to ethanol late in gestational life was sufficient to produce CNS dysfunction. For example, mothers who did not consume ethanol throughout gestation, but were given an ethanol infusion to prevent premature labour, had offspring who exhibited a wide variety of neurological problems including decreased intelligence quotient, hyperactivity and behavioural abnormalities (Sisenwein et al., 1983, Steinhausen et al., 1994).

### 1.3.2 Ethanol CNS Teratogenesis

Brain development is a very dynamic process that requires a fine balance of controlling factors such as growth hormones, insulin and physical contact (Shibley and Pennington, 1997, Guerri, 1998). Ethanol exposure can upset this balance resulting in neuronal dysmorphology and dysfunction which, unlike the craniofacial abnormalities and growth restriction observed in children with the FAS, persists into adulthood. It is for this reason that the teratogenic effects of ethanol on the CNS are considered to be the most debilitating characteristics of the FAS.

The developing CNS appears to be vulnerable to ethanol throughout gestation. Ethanol's ability to modify CNS development is closely linked to the period of development during which the exposure occurs, as well as the maximum blood ethanol concentration reached at this time. Specific neuronal populations have characteristic ontogenic profiles during which there are critical periods of vulnerability to ethanol's teratogenic effects (Miller, 1996b). Even a single exposure to ethanol during critical periods of CNS development can produce behavioural alterations that persist throughout postnatal life (Sisenwein et al., 1983).

The teratogenic effects of chronic prenatal ethanol exposure in the developing CNS include intellectual impairment as well as behavioural and neuroanatomical alterations. The FAS is the leading teratogenically induced cause of mental retardation in Western society (Abel and Sokol, 1987) with the average IQ of individuals suffering from the FAS being 66 (Streissguth et al., 1991). It has been shown that prenatal ethanol exposure negatively affects offspring's attention span and reaction time as well as particular intellectual skills such as the ability to solve mathematical problems (Sisenwein et al., 1983, Streissguth et al., 1986, Streissguth et al., 1991).

1.3.3 Incidence of the FAS

The FAS is only observed in the offspring of mothers who are heavy drinkers (defined as the consumption of two or more drinks per day or 14 or more drinks per week throughout gestation) (Abel et al., 1982a, Rosett and Weiner, 1985). In 1982, the incidence of heavy drinking by pregnant women ranged from 2% to 26% depending on the population studied, with the median estimate being 9% of pregnant women. The incidence of the FAS in the Canadian population is estimated to be 2 cases for every 1000 live births (Greene and Wilbee, 1992), with the incidence of fetal alcohol effects being much higher, ranging from 1.7-90.1 cases per 1000 live offspring (Abel, 1982a). The incidence of offspring being born with the FAS among heavy drinking mothers is much higher, with estimates ranging from 2.5-400 cases per 1000 live offspring (Webster, 1989). The incidence of FAE in children born to heavy drinking mothers is approximately 690 cases per 1000 live births (Abel, 1982b).

The developing child's genetic makeup influences his / her sensitivity or resistance to the teratogenic effects of ethanol. Retrospective studies have shown that there is 100% concordance between monozygotic twins and approximately 64% concordance between dizygotic twins (Streissguth and Dahaene, 1993). To date, no single gene has been identified as protecting against or potentiating the teratogenic effects of prenatal ethanol exposure, although certain genes such as Fyn-kinase (a non-receptor type of tyrosine kinase) have been shown to modulate ethanol's teratogenic effects (Miyakawa et al., 1997). Fyn-kinase is co-localized with NMDA receptors and ethanol increases Fyn-kinase mediated phosphorylation of the NMDA receptor, which potentiates NMDA receptor function. This indirect action of ethanol, mediated by increased Fyn-kinase phosphorylation of the receptor, attenuates the direct ethanol-induced inhibition of

NMDA receptor function. Fyn-kinase deficiency potentiates ethanol's ability to inhibit NMDA receptor function, which could increase the magnitude of CNS teratogenesis.

1.3.4 The Cerebral Cortex and Hippocampus as Target Sites of Ethanol CNS Teratogenesis

Experimental animal studies have demonstrated that the cerebral cortex (Fabregues et al., 1985) and the hippocampus (West et al., 1986) are target sites for the teratogenic effects of ethanol.

The main focus of this thesis research was on ethanol CNS teratogenesis in the cerebral cortex, since this brain region supports functions as diverse as cognitive function, regulation of behaviour, perception, memory, language processing and reasoning (Singer, 1996). Autopsies performed on children with the FAS, who died of other causes, revealed neuroanatomical abnormalities in the cerebral cortex such as thinning and disorganization of neurons (Roebuck et al., 1998), severe cerebral dysgenesis and neuronal migration anomalies (Jones and Smith, 1973) and increased rate of holoprosencephaly (failure of the forebrain to divide into 2 hemispheres) (Sulik et al., 1982). Teratogenic effects involving the cerebral cortex also have been observed in animal studies; chronic prenatal ethanol exposure has been shown to produce microencephaly (Chiu et al., 1999), decreased number of neurons (Miller, 1992) and altered neuronal migration (Miller, 1997).

The hippocampus was the other brain region that was examined in this thesis research. The hippocampus, which plays an important role in memory formation and learning, is sensitive to the teratogenic effects of ethanol and many of the signs of intellectual impairment observed in offspring with the FAS can be partially attributed to

hippocampal injury. Studies in the adult guinea pig and rat have shown that chronic prenatal ethanol exposure causes a 25 to 30% decrease in the number of hippocampal CA1 pyramidal neurons in the postnatal animal (Barnes and Walker, 1981, Abdollah et al., 1993).

### 1.3.5 Mechanisms of Ethanol CNS Teratogenesis

The teratogenic effects of ethanol on the CNS are the most debilitating aspect of the FAS and include intellectual, behavioural and neurological impairment as well as anatomical alterations. Unlike the craniofacial abnormalities and growth restriction observed in children with the FAS, the effects on the CNS are persistent and do not dissipate in postnatal life. Chronic prenatal ethanol exposure can modify neuroembryogenesis by altering glial status, neuronal migratory pathways, production and efficacy of trophic factors or cell-to-cell contact.

The period of CNS development known as the brain growth spurt is characterized by a rapid increase in brain weight, synaptogenesis, axonal elongation and dendritic arborization. Chronic prenatal ethanol exposure during this period can alter cellular functioning by interfering with DNA synthesis (Guerri et al., 1990), protein synthesis (Kennedy, 1984), amino acid and glucose uptake (Singh et al., 1986, Karl and Fischer, 1994, Shibly and Pennington, 1997). Ethanol exposure during CNS development also can disrupt signalling pathways such as protein kinase C, protein kinase A, insulin-independent tyrosine kinase and the L-glutamate - NMDA receptor- nitric oxide synthase signal transduction pathway (Shibley and Pennington, 1997).

Prenatal ethanol exposure can alter glial cells that are responsible for modulating the CNS microenvironment and neuronal migration during brain development (Rakic,

1991). It has been demonstrated that ethanol exposure during CNS development alters radial glial fibres. These radial glial fibres are responsible for guiding developing neurons to their target sites and altering the distribution of glial fibres may produce abnormal migration of neurons (Miller, 1993).

There are a number of proposed mechanisms by which chronic prenatal ethanol exposure can produce teratogenic effects in the offspring. The following subsections describe several of the currently proposed mechanisms of ethanol neurobehavioural teratogenesis.

#### 1.3.5.1 Ethanol-Derived Acetaldehyde

Acetaldehyde is the proximate metabolite formed during the oxidation of ethanol and causes toxic effects in both the mother and fetus by inducing the formation of free radicals and protein adducts (Brien and Loomis, 1983, Lieber, 1994). Following consumption of ethanol, the concentration of acetaldehyde in maternal blood is approximately 1000 times lower than the ethanol concentration. The developing fetus lacks alcohol dehydrogenase but has hepatic aldehyde dehydrogenase activity that oxidizes acetaldehyde transferred across the placenta from the maternal blood (Clarke et al., 1986a, Clarke et al., 1986b). The fetal blood acetaldehyde concentration is ten times lower than that of the maternal blood. A number of studies have also shown that the concentration of acetaldehyde required to produce negative effects on the fetus are much higher than those produced after ethanol exposure and that inhibition of aldehyde dehydrogenase does not increase the incidence on ethanol-induced malformations (Webster et al., 1983). In view of this, acetaldehyde may not play a key role in the teratogenic effects produced by maternal consumption of ethanol.

### 1.3.5.2 Ethanol-Induced Fetal Hypoxia

The fetus receives oxygen from the mother via placental transfer; maternal ethanol consumption can cause fetal hypoxia by altering placental blood transfer. *In vitro* incubation of human umbilical vessels with ethanol produces a concentration-dependent contraction of both arteries and veins (Altura et al., 1982). This vasoconstriction however, does not appear to occur *in vivo* (Erskine and Ritchie, 1986). By monitoring instrumented fetal sheep during a maternal infusion of ethanol, it was discovered that mature fetal cerebral hemispheres experience a decrease in blood flow, oxygen delivery and oxygen consumption that may produce focal hypoxia (Richardson et al., 1985). It is not known at this time whether chronic maternal ethanol consumption results in the development of fetal tolerance to the hypoxic effects of ethanol in the cerebral cortex.

### 1.3.5.3 Ethanol-Induced Undernutrition

Consumption of ethanol by pregnant mothers may cause fetal undernutrition due to altered eating habits or by inhibiting placental transfer and fetal absorption of nutrients. Vitamin B6, folate and retinoic acid are essential for optimal brain development and consumption of ethanol interferes with fetal uptake of these nutrients (Ordonez, 1977, Loo, 1980, Dakshinamurti et al., 1985, Chanarin, 1986, Durston et al., 1989). Maternal consumption of ethanol may also impede the transfer of amino acids (Fischer et al., 1983 Michaelis, 1990) and glucose (Snyder et al., 1986) from the mother across the placenta to the fetus. These effects of chronic prenatal ethanol exposure are not consistent across species, some mammals such as rats are highly affected while humans suffer fewer effects (Reynolds and Brien, 1995).

Although undernutrition may cause a decrease in fetal body weight, there are compensatory systems in the developing fetus that can maintain normal brain development. The fetus responds to decreased nutritional state by diverting a greater proportion of available nutrients to the developing brain. This phenomenon is known as the "brain sparing effect" (Patel et al., 1973). In an experiment conducted by Abdollah et al in 1995, it was demonstrated that chronic prenatal ethanol exposure decreased guinea pig fetal brain weight. However, the nutritionally matched control group, which received the same number of calories, did not exhibit any signs of microencephaly (Abdollah and Brien, 1995). This finding indicates that microencephaly is caused by ethanol exposure rather than under-nutrition in the guinea pig.

1.3.5.4 Ethanol-Induced Alteration in Prostaglandin Metabolism

Consumption of ethanol by a pregnant mother increases prostaglandin concentrations in both the maternal and fetal brain (Anton and Randall, 1987). The placenta is the major site of prostaglandin production for the fetus (Mitchell et al., 1985) and can produce an increase in $PGE_2$ concentration in the fetal blood (Bocking et al., 1993). This ethanol-induced rise in $PGE_2$ concentration is important because this prostaglandin has been shown to produce teratogenic effects in a number of mammalian species (Persaud, 1974, Persaud, 1978, Hibelink and Persaud, 1981), including the human (Collins and Mahoney, 1982). In near-term pregnant sheep, maternal infusion of ethanol produced a transient increase in maternal and fetal blood or plasma $PGE_2$ concentrations (Bocking et al., 1993). In the immature ovine fetus, maternal infusion of ethanol did not alter $PGE_2$ concentrations (Bocking et al., 1993) despite the fact that ethanol still had teratogenic effects at this developmental age (Webster, 1989). Based on these results, it

appears that an ethanol-induced increase in prostaglandin production can account for only part of the teratogenic effects of chronic prenatal ethanol exposure.

#### 1.3.5.5 Perturbation of NMDA Receptor-Mediated Synaptic Transmission

The L-glutamate- NMDA receptor- nitric oxide synthase signal transduction system plays an important role in both the developing fetus and the adult (McDonald and Johnston, 1990). During development, this system is responsible for a variety of effects including neuronal survival and differentiation, synaptogenesis and dendritic arborization (McDonald and Johnston, 1990). Consequently, perturbation of this system can disrupt these processes resulting in harmful effects on the developing CNS.

Chronic prenatal ethanol exposure produces suppression of the L-glutamate-NMDA receptor-NOS signal transduction system that appears to persist into adulthood (Diaz-Granados et al., 1997). Experimental evidence indicates that each aspect of this signal transduction system, namely glutamate release, the NMDA receptor population and NOS enzymatic activity is altered by *in utero* ethanol exposure. Both acute and chronic ethanol exposure decreased glutamate release from hippocampal neurons which would decrease the amount of glutamate in the synaptic cleft (Reynolds and Brien, 1994, Savage et al., 1998). Studies have shown that offspring exposed to ethanol during development have fewer NMDA receptors in regions such as the cerebral cortex and hippocampus in postnatal life (Savage et al., 1991, Diaz-Granados et al., 1997). In a study performed by Kimura et al, it was discovered that chronic prenatal ethanol exposure results in decreased NOS enzymatic activity in the hippocampus of the near-term fetal guinea pig (Kimura et al., 1996).

Based on the knowledge that the L-glutamate-NMDA receptor-nitric oxide synthase signal transduction system plays an important role in the development and function of the brain, and that ethanol exposure can alter this system, perturbation of the system may be responsible for many of ethanol's teratogenic effects.

## 1.4 Effects of Prenatal Ethanol Exposure on Behaviour

Epidemiological studies of adolescents and adults who were exposed to ethanol during prenatal life have shown that there is an increased incidence of maladaptive behaviours such as poor judgement, distractibility and difficulty perceiving social cues (Streissguth and Bookstein, 1989, Kelly and Tran, 1997). Other common behavioural alterations produced by prenatal ethanol exposure include severe attention deficits, impulsivity and lack of self-restraint. These traits interfere with scholastic performance during school-age years and work performance during adult life (Streissguth et al., 1986, Streissguth et al., 1995). These behavioural alterations generally persist into adult life and severely limit the ability of these individuals to function productively and integrate in society.

Many of the manifestations of behavioural dysfunction in individuals with ARND can be observed in animal models of ethanol neurobehavioural teratogenicity (Driscoll et al., 1990). Chronic prenatal ethanol exposure produces behavioural alterations such as hyperactivity (Catlin et al., 1992), memory and learning impairments (Klintsova et al., 1998), impaired performance in the radial-arm maze (Reyes et al., 1989, Zimmerberg et al., 1991, Hall et al., 1994), spatial navigation (Goodlett and Peterson, 1995), inhibitory avoidance (Lochry and Riley, 1980, Abel, 1982, Tan et al., 1988) and altered social communication and social recognition in rats (Kelly and Tran, 1997).

Increased locomotor activity can be used as a neurobehavioural index of ethanol CNS teratogenicity because it is the most common behavioural effect produced by chronic prenatal ethanol exposure (Catlin et al., 1992). Experiments performed in the guinea pig have shown that chronic prenatal ethanol exposure produces an increase in locomotor activity that is dose-dependent (Catlin et al., 1992). The mechanism by which chronic prenatal ethanol exposure causes hyperactivity is yet to be determined.

Chronic prenatal ethanol exposure has been shown to decrease synaptic plasticity in the adult brain. Hippocampal slices obtained from adult rats that were chronically exposed to ethanol during prenatal life were less able to form long-term potentiation (LTP) compared with hippocampal slices obtained from control animals (Queen et al., 1993). This ethanol-induced decrease in LTP formation may play an important role in some of the cognitive deficits observed in individuals with the FAS. Prenatal ethanol exposure decreased neuronal plasticity in a brain-region dependent manner, suggesting again that different brain regions exhibit differential sensitivity to the teratogenic effects of prenatal ethanol exposure.

Several studies have examined whether early postnatal behavioural experience can ameliorate some of the behavioural and motor deficits produced by chronic prenatal ethanol exposure. Rearing animals in a complex environment or familiarization with a radial-arm maze improved performance in learning tasks such as the Morris water maze and radial-arm maze (Hannigan et al., 1993, Wainright et al., 1993, Opitz et al., 1997). Preweaning handling of animals exposed to ethanol *in utero* eliminated the deficits in response inhibition compared with offspring that received the same ethanol exposure regimen but were not handled (Gallo and Weinberg, 1982). Although both an enriched environment and preweaning handling can ameliorate the behavioural manifestations of

chronic prenatal ethanol exposure, they do not decrease neuroanatomical alterations such as dendritic spine density in the hippocampal CA1 subfield (Berman et al., 1996).

## 1.5 Excitatory Amino Acid Neurotransmission Involving the L-Glutamate Pathway

L-Glutamate is the most abundant excitatory amino acid (EAA) in the central nervous system (Fonnum, 1984) and has the ability to stimulate almost every neuronal cell in the adult CNS (Curtis and Johnston, 1974). In the presynaptic nerve terminal, glutamate is stored in vesicles; glutamate is released into the synapse following a nerve action potential in a $Ca^{2+}$ dependent fashion (Potashner, 1978, Sandoval and Cotman, 1978, Naito and Ueda, 1983). Glutamate is recovered from the synapse primarily by presynaptic neuronal re-uptake and high-affinity glial uptake systems (Fonnum, 1984).

There are two main classes of EAA receptors: ionotropic and metabotropic. Activation of an ionotropic receptor by glutamate causes the opening of an integral ion channel, whereas metabotropic receptors are linked to G-proteins (Nakanishi, 1994, Pin and Duvoisin, 1995) and influence the activity of phospholipase C, phospholipase $A_2$ or adenylyl cyclase (Sladeczek et al., 1985, Nicoletti et al., 1986, Aramori and Nakanishi, 1992). The three types of ionotropic EAA receptors are: NMDA (N-methyl-D-aspartate), kainate and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole) receptor subtypes (Sommer and Seeburg, 1992, Sprengel and Seeburg, 1993, Hollman and Heinemann, 1994, Bettler and Mulle, 1995).

The L-glutamate-NMDA receptor-nitric oxide synthase pathway is one of the major signal transduction systems in the brain. When an action potential reaches the presynaptic nerve terminal, glutamate is released into the synaptic cleft. Glutamate causes

an initial post-synaptic membrane depolarization by activating the ionotropic non-NMDA receptors. As the membrane potential rises, the voltage-dependent magnesium ion blockade of the NMDA receptor is alleviated. Glutamate then activates NMDA receptors resulting in an influx of $Ca^{2+}$ into the post-synaptic neuron. This rise in intracellular $Ca^{2+}$ concentration activates calmodulin, which then binds to nitric oxide synthase. The $Ca^{2+}$ / calmodulin / nitric oxide synthase complex is enzymatically active and catalyzes the oxidation of L-arginine to L-citrulline and nitric oxide, a free-radical metabolite. Nitric oxide is a gas that is water soluble and can readily diffuse across membranes, thereby producing effects on the surrounding cells such as activation of soluble guanylyl cyclase and increases in intracellular cGMP levels (Dawson and Snyder, 1994). At high concentrations, a peroxynitrate radical formed from nitric oxide can cause toxic effects (MacCall and Valence, 1992).

## 1.6 NMDA Receptors

### 1.6.1 NMDA Receptor Pharmacology

Whereas glutamate interacts with all the EAA receptor subtypes, the NMDA receptor is selectively activated by the synthetic analogue NMDA and the endogenous ligands homocysteate, aspartate and quinolinate (Ganong and Cotman, 1985, Stone and Connick, 1985, Do et al., 1986). Activation of the NMDA receptor causes the opening of a transmembrane ion channel that is highly permeable to calcium and allows the passage of other cations such as sodium and potassium (McDonald and Johnston, 1990).

NMDA receptors have the unique characteristic that the transmembrane ion channel is blocked by $Mg^{2+}$ in a voltage-dependent manner. Activation of the NMDA receptor ion channel requires neurotransmitter binding to the NMDA recognition site and coincident membrane depolarization (via another EAA receptor system) to relieve the voltage-dependent $Mg^{2+}$ blockade (MacDonald et al., 1982, Flatman et al., 1983, Mayer et al., 1984, Nowak et al., 1984, MacDermott et al., 1986).

There are several regulatory sites that modulate NMDA receptor activation. Activation of the NMDA receptor requires not only the binding of glutamate, but also the binding of the essential co-agonist glycine that potentiates the NMDA receptor response by binding at a closely associated site (Johnson and Ascher, 1987, Kleckner and Dingeldine, 1988). There are at least six pharmacologically distinct sites on the NMDA receptor that bind the following ligands or ions: glutamate, glycine, phencyclidine, $Mg^{2+}$, $Zn^{2+}$ and polyamines (Mishra et al., 1992). Drugs such as the dissociative anaestethic ketamine and MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclo-hepten-5,10-imine hydrogen maleate) bind in the ion channel and are thus able to block the influx of cations (Martin and Lodge, 1985, Kemp et al., 1987).

Activation of the NMDA receptor opens the transmembrane ion channel causing an influx of $Ca^{2+}$ ions which may produce an even greater rise in cytoplasmic $Ca^{2+}$ concentration by causing the release of $Ca^{2+}$ from intracellular stores. This NMDA receptor-induced increase in intracellular $Ca^{2+}$ concentration can activate a number of second messenger systems, including protein kinase C and ornithine decarboxylase (ODC) that phosphorylates the NMDA receptor and increases polyamine concentrations, respectively. Phosphorylation of the NMDA receptor decreases the ability of $Mg^{2+}$ to block the transmembrane ion channel and thus potentiates NMDA receptor activation (Chen and Huang, 1992). A rise in polyamine concentration, including spermidine and spermine, can also potentiate NMDA receptor-induced $Ca^{2+}$influx (Siddique et al., 1988, Williams et al., 1991).

Over-activation of NMDA receptors can produce a substantive increase in intracellular $Ca^{2+}$ concentration which causes "excitotoxic" neuronal death. High intracellular $Ca^{2+}$ concentrations can cause the activation of proteolytic and lipolytic enzymes that can produce free radicals in their catalyzed metabolic reactions. These free radicals can damage neuronal DNA and organelles, which may induce neuronal death (Olney and Sharpe, 1969, Choi, 1988). A second mechanism by which increased NMDA receptor activation can cause excitotoxicity is mediated by the large influx of $Na^{+}$ that occurs with opening of the NMDA receptor ion channel. Water follows the $Na^{+}$ gradient into the cell and causes cell swelling or lysis. NMDA receptor-mediated excitotoxicity may cause brain damage following ischemia and play a role in the pathogenesis of chronic neurodegenerative diseases (Choi, 1988, Choi and Rotham, 1990).

1.6.2 NMDA Receptor Subtypes

There are two major types of NMDA receptor subunits, NR1 and NR2. There are eight isoforms of the NMDA receptor NR1 subunit, which are generated by alternate splicing of NR1 RNA (Anatharam et al., 1992, Durand et al., 1992, Nakanishi, 1992a, Nakanishi et al., 1992b Sugihara et al., 1992, Hollman et al., 1993, Zukin and Bennett, 1995). These NR1 subtypes are characterized by the inclusion or deletion of one 5' insert and two 3' inserts (Zhong et al., 1995). Despite the fact that there are eight splice variants, there appears to be little functional difference among them (Bhave et al., 1996). To date, four different subtypes of the NMDA receptor NR2 subunit have been identified: NR2A, 2B, 2C and 2D (Moriyoshi et al., 1991, Monyer et al., 1992, Ishii et al., 1993). Each of these NR2 subunits has a unique pharmacologic profile with its own particular affinities for ligands such as glutamate, glycine, MK-801 and ethanol (Monyer et al., 1992, Williams et al., 1994, Laurie and Seeburg, 1994).

Functional NMDA receptors are believed to be heteromeric proteins made up of either four or five subunits (Laube et al., 1998). Although the subunit composition of native NMDA receptors has not been elucidated, they are likely to be heterooligomers containing various combinations of NR1 and NR2 subunits. Through the use of recombinant NMDA receptors, it has been discovered that receptors composed entirely of NR1 subunits are functionally active, whereas NR2 subunits cannot form functional homomeric receptors. Co-expression of NR1 and NR2 subunits produces receptors that are highly sensitive to NMDA (Monyer et al., 1992, Nakanishi, 1992a, Nakanishi et al., 1992b). Many of the properties of the heteromeric NR1 / NR2 receptor, such as affinity for agonists and antagonists as well as sensitivity to $Mg^{2+}$ and polyamines, depend on the type of NR2 subunit included in the heteromeric complex (Kutsuwada et al., 1992,

Meguro et al., 1992, Monyer et al., 1992, Ishii et al., 1993, Williams, 1993, Williams et al., 1994,).

Studies conducted to examine NR1 mRNA and protein expression demonstrate that the NR1 subunit is expressed ubiquitously in the adult brain (Moriyoshi et al., 1991, Pujic et al., 1993). Conversely, expression of NR2 subunit subtype mRNA has distinct brain regional distributions (Wenzel et al., 1997). NR2A subunit mRNA is expressed throughout the brain, whereas NR2B subunit mRNA is expressed mainly in the forebrain and NR2C subunit mRNA is expressed predominantly in the cerebellum (Monyer et al., 1992, Watanabe et al., 1992, Ishii et al., 1993, Spuhler-Phillips et al., 1997). Based on the regional expression of NR2 subunits, certain brain regions have heightened or diminished sensitivity to NMDA receptor agonists depending on the NMDA receptor isoforms that are present.

1.6.3 Role of NMDA Receptors in Neurobehaviour

In the adult CNS, NMDA receptors play an important role in learning and the formation of new memories. Voltage-dependent blockade of the NMDA receptor ion channel allows the receptor to function as a molecular coincidence detector; NMDA receptor activation requires both membrane depolarization and binding of glutamate in order to cause ion channel opening.

Long-term potentiation (LTP) is a process by which brief repetitive stimuli result in a long-lasting increase in synaptic strength via activation of post-synaptic NMDA receptors (Bliss and Gardners-Merwin, 1973a, Bliss and Lomo, 1973b, Collingridge and Bliss, 1987, Nicoll and Kauer, 1988, Bliss and Collinridge, 1993). It has been shown that the synchronized firing patterns used to create LTP *in vitro* are similar to those produced in an intact animal during learning (Otto et al., 1991). The ability to form LTP is not static throughout life but has been shown to be temporally related to the ontogeny of the NMDA receptor during brain development (McDonald et al., 1989). NMDA receptor blockade has a profound effect on the ability to learn new tasks. Ethanol and other substances that inhibit NMDA receptor function have been shown to inhibit the formation of LTP (Schummers et al., 1997).

Several $Ca^{2+}$ sensitive enzymes have been identified that play a role in converting the possible induction signal, influx of $Ca^{2+}$ through the NMDA receptor, into persistent modifications of synaptic strength. These enzymes include the proteases such as calpain, phosphatases such as calcineurin, phospholipases and protein kinases. To date, research has been predominantly focused on the role of protein kinases, especially protein kinase C (PKC). Inhibition of PKC following tetanus has been shown to block the formation of

LTP. PKC activation alone, however, is not sufficient to induce LTP, but appears to be necessary to form LTP.

Synaptic plasticity, in the form of long-lasting changes in the efficiency of transmission in specific neural circuits, is dependent on NMDA receptor activation (McDonald and Johnston, 1990). Synaptic plasticity involving NMDA receptors is important for many other processes in addition to learning and memory formation. For example, motor pattern generators involved in complex movements such as swallowing and walking require NMDA associated synaptic plasticity.

It has been found that different types of alcohol have different potency for inhibiting the NMDA receptor-activated current and these potencies are linearly related to their ability to produce intoxication (Lovinger et al., 1990). These findings suggest that ethanol-induced inhibition of NMDA receptor-mediated response may contribute to the behaviours observed during ethanol intoxication.

### 1.6.4 Role of NMDA Receptors in Brain Development

During brain development, NMDA receptors play an important neurotrophic role, including involvement in neurogenesis, neuronal differentiation, and synaptogenesis (Aruffo et al., 1987, Pearce et al., 1987). NMDA receptors also play an important role in the coordination of neuronal migration (Komuro and Racik, 1993). Expression of NMDA receptors follows a distinct ontogenic profile; there is transient increased expression of NMDA receptors during the period of rapid neuronal development known as the brain growth spurt. This period of increased NMDA receptor number is temporally related to periods of enhanced synaptic plasticity and consolidation of synaptic connections (McDonald 90). There are regional differences in both NMDA receptor number and subunit composition in the developing brain that depend on the stage of development (Monyer et al., 1994, Abdollah and Brien, 1995, Chiu et al., 1999). In the cerebral cortex of the guinea pig, NMDA receptors can be detected as early as gestational day (GD) 30 in the fetus, but they peak in number at GD 55 (term at GD 68), the end of the brain growth spurt, after which the number of NMDA receptors slowly decreases to adult levels (Mishra et al., 1992). In the hippocampus, the number of NMDA receptors is greater at GD 63 and PD 13 than at GD 50 or PD>60 (Abdollah and Brien, 1995).

The subunit subtype composition of NMDA receptors also depends on the brain region and developmental stage examined. Early in CNS development, NMDA receptor isoforms expressed in brain regions such as the hippocampus exhibit decreased sensitivity to voltage-dependent magnesium blockade, a phenomenon attributed to the expression of specific NMDA receptor subunit subtypes (Ben-Ari et al., 1988, Bowe and Nadler, 1990, Morisett et al., 1990).

NMDA receptor activation plays an important role in promoting neuronal survival during CNS development. Programmed cell death, or apoptosis, occurs during development of the CNS and is responsible for the modeling of neuronal circuitry by pruning redundant neurons (Ikonomidou et al., 1999). The importance of NMDA receptor activation during brain development was illustrated by blocking NMDA receptors and measuring apoptotic neuronal loss in the developing brain. Blockade of NMDA receptors with the non-competitive channel antagonist MK-801 for even a short period during late gestation produced widespread apoptotic neurodegeneration in the developing brain (Ikonomidou et al., 1999). In cerebellar granule cell culture, application of NMDA has been shown to promote cell survival in a dose-dependent manner and this effect can be blocked by co-administration of the NMDA receptor antagonist, MK-801 (Balazs et al., 1988a, Balazs et al., 1988b). These experiments provide strong support for the theory that NMDA receptor activation plays an important neurotrophic role in the developing brain.

### 1.6.5 Ontogenic Expression of NMDA Receptor Subunit Subtypes

Studies of NMDA receptor gene expression in the developing CNS of the rat and mouse have provided a developmental profile of NMDA receptor subunit expression. The results from these studies indicate that brain NR2 subunit mRNA expression is regulated during development in a temporal and region-specific manner (Watanabe et al., 1994).

The developmental time courses of NMDA receptor NR1 and NR2A subunit expression in the rat cerebral cortex and hippocampus are very similar. During the postnatal brain growth spurt, which occurs during the first week of postnatal life in the rat, there is a rapid increase in NR1 and NR2A mRNA, whereas the level of NR2B mRNA remains constant throughout CNS development (Zhong et al., 1995). NR2C mRNA levels were at least an order of magnitude lower than those of NR1, NR2A and NR2B in the cerebral cortex and hippocampus (Zhong et al., 1995).

The cerebellum has a NMDA receptor subunit subtype developmental profile that differs from those of the cerebral cortex and hippocampus (Watanabe et al., 1994). There is a similar increase in NR1 and NR 2A mRNA levels as seen in other brain regions, but in contrast to the cerebral cortex and hippocampus this increase occurs at the end of the brain growth spurt. In the cerebellum, the NR2B mRNA level does not remain constant throughout development but peaks at parturition and then is undetectable approximately one week after the brain growth spurt. As the NR2B mRNA level decreases, there is a concurrent increase in NR2C mRNA levels (Zhong et al., 1995).

The subunit composition of NMDA receptors in the mature cerebral cortex and hippocampus is quite different from that of the cerebellum. The predominant NR2 mRNAs found in the adult rat cerebral cortex and hippocampus are NR2A and NR2B

with only nominal NR2C mRNA expression. This profile is in stark contrast to the cerebellum in which NR2A and NR2C subunit subtypes are highly expressed.

These changes in NMDA receptor subunit composition can be demonstrated by the use of different NMDA receptor ligands. As the developing hippocampus matures, NMDA receptor affinity for ifenprodil decreases (Williams, 1993), whereas receptor affinity for glycine increases (Kleckner and Dingledine, 1991). The sensitivity of hippocampal NMDA receptors to $Mg^{2+}$ blockade also increases during development (Ben-Ari et al., 1988, Bowe and Nadler, 1990, Morrissett et al., 1990, Kleckner and Dingledine, 1991).

### 1.6.6 Effects of Ethanol on NMDA Receptors in the Adult Brain

The effects of ethanol on NMDA receptor ion channel properties are consistent with the euphoric and intoxicating effects in humans (Crews et al., 1996). Acute exposure to ethanol disrupts NMDA receptor ion channel function via interaction at specific hydrophobic sites on channel subunit proteins, and thus decreases the depolarization and influx of $Ca^{2+}$ that are normally associated with NMDA receptor activation (Lovinger et al., 1989, Crews et al., 1996, Diamond and Gordon, 1997).

Chronic consumption of ethanol causes an up-regulation in the expression of NMDA receptors in the CNS. As ethanol suppresses NMDA receptor function, the body tries to maintain a physiological balance by increasing the number of receptors. It is proposed that this increase in NMDA receptor number may be responsible for many of the effects observed during ethanol withdrawl. As ethanol is cleared from the brain, ethanol-induced suppression of NMDA receptor function is alleviated and the increased number of receptors makes post-synaptic neurons sensitive to glutamate. Under these conditions of up-regulation of NMDA receptor number after chronic ethanol exposure, stimulated glutamate release may produce excitotoxicity in the post-synaptic neuron.

The sensitivity of NMDA receptors to the suppressant effects of ethanol is dependent on the subunit composition (Masood et al., 1994, Harris et al., 1995). The rank order for inhibition of NMDA receptor activation by ethanol is NR1/2B and NR1/2A > NR1/2C > NR1/2D (Spuhler-Phillips et al., 1997). Subunit dependent inhibition of NMDA receptors by ethanol correlates well with regional sensitivity to ethanol in the mature CNS: hippocampus > forebrain >> cerebellum >> brain stem (Randoll et al., 1996).

In the mature CNS, chronic consumption of ethanol alters the subunit composition of NMDA receptors. Chronic consumption of ethanol did not alter the amount of NR1 subunit protein in the cerebral cortex, hippocampus or cerebellum but does increase NR2A and 2B mRNA in both the cerebral cortex and hippocampus (Follesa and Tieku, 1994). These results indicate that NR2 gene expression is regulated by ethanol, whereas NR1 gene expression is insensitive. There are a few explanations for why there is an increase in the number of functional NMDA receptors following chronic ethanol exposure despite the fact there is no change in NR1 subunit protein. There may be functional receptors in the non-treated brain that are strictly composed of NR1 subunits, and following chronic ethanol exposure, NR2 subunits are added to these receptors. Another explanation is that the number of NR1 subunits in the NMDA receptor decreases following chronic ethanol exposure, ie instead of 3 NR1 subunits per NMDA receptor there are 2 NR1 subunits per NMDA receptor.

## 1.6.7 Effects of Ethanol on NMDA Receptors in the Developing Brain

Normal CNS development requires an optimal level of NMDA receptor activation; under-activation may delay or disrupt development, whereas over-activitation can produce excitotoxic brain damage (McDonald and Johnston, 1990). The developing brain is sensitive to the neurotoxic effects of ethanol, especially during the period of rapid brain development known as the brain growth spurt. The effects of chronic prenatal ethanol exposure on NMDA receptors are dependent on the brain region and developmental age examined.

In the near-term fetal guinea pig, chronic prenatal ethanol exposure produces an increase in the number of NMDA receptors in the cerebral cortex and a decrease in NMDA receptor affinity for the NMDA receptor antagonist, MK-801 (Chiu et al., 1999). In an earlier experiment using the same treatment protocol, it was discovered that at the same gestational age, there was a decrease in the number of NMDA receptors in the hippocampus with no alteration in receptor affinity for glutamate (Abdollah and Brien, 1995).

Several studies have been conducted to examine the long-term effects of chronic prenatal ethanol exposure on NMDA receptors in various brain regions. Prenatal ethanol exposure throughout gestation produced a decrease in the number of NMDA receptors in the adult (postnatal day 45-180) rat hippocampus (Savage et al., 1991), but had no effect on AMPA, kainate or metabotropic glutamate receptors (Queen et al., 1993). It was also discovered that hippocampal slices from adult rats that received chronic prenatal ethanol exposure had decreased stimulated glutamate release (Savage et al., 1998) and decreased the ability to form LTP (Queen et al., 1993). It is hypothesized that this decrease in LTP is also related to the decrease in the number of hippocampal NMDA receptors. It has been

reported that ethanol exposure during late gestation or early postnatal life (the brain growth spurt in the rat) produced a decrease in the number of NMDA receptors in a combined rat hippocampal / cerebral cortical membrane preparation at PD 20-23 (Diaz-Granados et al., 1997).

Chronic *in utero* ethanol exposure can alter the expression of NMDA receptor subunits in the fetal brain. Chronic prenatal ethanol exposure increases NR1 and NR2B protein expression (Kumari and Ticku, 1998). Interestingly, the mechanism by which ethanol increases NR1 and NR2B protein expression is different. Ethanol enhances the transcription of NR2B subunit DNA, whereas it has no effect on NR1 transcription. Rather than increasing the amount of mRNA encoding the NR1 subunit, ethanol appears to stabilize NR1 mRNA, increasing its half-life from 16 hours to 24 hours (Kumari and Ticku, 1998). Increasing the half-life of the NR1 subunit mRNA increases the number of times that each NR1 subunit mRNA is translated to protein. Although ethanol selectively affects the stability and transcription of NR1 and NR2B subunits, the final outcome is the same: increased synthesis of NR1 and NR2B subunit proteins and increased number of NMDA receptors.

## 1.7 Research Rationale, Hypothesis and Objectives

NMDA receptors play important roles in both the developing and mature CNS. During CNS development, NMDA receptors are involved in promoting neuronal survival, migration and differentiation, whereas in the mature CNS, NMDA receptors play a key role in the regulation of behaviour and cognitive function. Based on the fact that normal CNS development and function require optimal activation of NMDA receptors, it has been hypothesized that the neurobehavioural teratogenic effects of chronic prenatal ethanol exposure are due to perturbation of NMDA receptor expression.

Several studies have shown that prenatal exposure to ethanol throughout gestation produces alterations in the NMDA receptor population in a variety of brain regions of the offspring. In the near-term fetal guinea pig, chronic prenatal ethanol exposure selectively alters NMDA receptor population in the cerebral cortex and hippocampus; there is an increased number of NMDA receptors in the cerebral cortex and a decreased number in the hippocampus. Other studies examining the long-term effects of ethanol exposure during brain development in the adult rat have demonstrated that there is a decrease in the number of NMDA receptors in the hippocampus as well as in a combined hippocampal / cerebral cortical preparation.

The goal of this thesis research was to test the hypothesis that chronic prenatal ethanol exposure produces an alteration in the cerebral cortical NMDA receptor population of the guinea pig that persists during postnatal life and is correlated with the hyperactivity and brain growth restriction of the offspring. The research objective was to determine the effects in postnatal offspring of chronic prenatal ethanol exposure on behaviour, morphology and neurochemistry. Behavioural changes were determined by

measuring spontaneous locomotor activity, whereas brain and brain regional weights were used as a gross index of dysmorphology. The neurochemical parameters that were examined included the number of NMDA receptors, their affinity for MK-801 and their sensitivity to glutamate. The guinea pig was chosen as the experimental animal due to the fact that it experiences a prenatal brain growth spurt during the third-trimester equivalent of gestation (Dobbing and Sands, 1970, Dobbing and Sands, 1979). Brain development of the guinea pig is more similar to that of the human than is the case for other rodent species, including the rat, which have a postnatal brain growth spurt. The chronic ethanol regimen used in this thesis research has been shown to produce consistent CNS teratogenicity in the guinea pig, manifesting as increased locomotor activity and brain growth restriction.

# 2. MATERIALS AND METHODS

## 2.1 Chemicals and Solutions

HEPES (N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid]), EDTA (ethylenediaminetetraacetic acid) disodium salt and Trizma® base (Tris) were purchased from Sigma Chemical Co. (St. Louis, MO). [$^3$H]MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen maleate) (22.5 Ci /mmol, 97% radiochemical purity) was supplied by Dupont-New England Nuclear (Lachine, QC). Scintiverse® scintillation fluid was obtained from Fisher Scientific (Unionville, ON). Ethanol was purchased from Commercial Alcohols (Montreal, QC). A protein-dye-binding assay kit, based on the method of Bradford (1976), was purchased from Bio-Rad Laboratories (Mississauga, ON). All other chemicals were purchased from a variety of commercial suppliers and were at least reagent-grade quality. Aqueous chemical solutions were prepared using deionized water obtained from a Nanopure® water purification system (Barnstead Sybron, Boston, MA), except for the aqueous ethanol and sucrose solutions, which were prepared using tap water. Deionized ultra-filtered bottled water used for the glycine NMDA receptor activation assay was purchased from Fisher Scientific (Napean ON).

## 2.2 Experimental Animals and Breeding Procedure

Nulliparous female Dunkin-Hartley-strain guinea pigs (500-600 g body weight; Charles River Canada Inc., St. Constant, QC) were bred with male Dunkin-Hartley-strain guinea pigs using an established procedure (Elvidge, 1972). The last day of full vaginal-membrane opening was defined as gestational day (GD) 0. On GD 1, the pregnant

animals were housed individually in stainless-steel wire cages in a room with an ambient temperature of 23°C and a 12 hr light/12 hr dark cycle with lights on at 0800 hr. All animals were monitored daily for general health and pregnancy status, and were cared for according to the principles and guidelines of the Canadian Council on Animal Care. This experimental protocol was approved by the Queen's University Animal Care Committee.

### 2.3 Chronic Maternal Treatment Regimens

On GD 2, each pregnant guinea pig was randomly assigned to receive one of the following three treatment regimens up to and including GD 67 (term, about GD 68). Each pregnant animal in the ethanol group received 4 g ethanol / kg maternal body weight / day as an aqueous ethanol solution (30% v/v) with *ad libitum* access to food (Purina Guinea Pig Chow 5025®) and water. This ethanol regimen was selected because it does not produce maternal or embryonic / fetal demise, but does cause behavioural changes, including hyperactivity in the offspring (Abdollah et al., 1993). Each pregnant guinea pig in the isocaloric-sucrose / pair-fed group was given an aqueous sucrose solution (42% w/v) that was isocaloric and isovolumetric to the daily ethanol dose administered to the respective paired animal in the ethanol group; received food in the amount consumed by the paired ethanol-treated pregnant guinea pig on each day of gestation; and had *ad libitum* access to water. Each pregnant animal in the water group received tap water that was isovolumetric to the aqueous ethanol solution and had *ad libitum* access to food and water. Each daily treatment was administered by intubation into the oral cavity as two equally divided doses, with the first dose given between 1000 and 1130 hr and the second dose given 2 hr later.

2.4 Gas-Liquid Chromatographic Analysis of Maternal Blood Ethanol Concentration

On GD 57 or 58, maternal blood was collected from an ear blood vessel at 1 hr after the second divided dose of ethanol, sucrose or water for the quantitation of ethanol concentration by an established procedure (Steenaart et al., 1985). A 100 µl aliquot of blood was added to 900 µl of a saline solution containing 3.4% w/v perchloric acid, 65µg/ml sodium azide, 76 mg/ml thiourea and 3.0 mg/ml 1-propanol (internal standard). The deproteinized sample was centrifuged at 13,000 x g for 1 min in order to precipitate the denatured proteins. A 200 µl aliquot of the supernatant was placed in a hypovial; nitrogen gas was used to displace the ambient air; and the vial was then rapidly sealed with a rubber septum. These samples were stored at -70°C until the ethanol concentration was quantified using an established gas-liquid chromatographic procedure with a column consisting of 5% Carbowax 20M as the stationary phase, nitrogen gas as the carrier gas and a flame ionization detector (Steenart et al., 1985). The maternal blood ethanol concentration provides an index of fetal exposure to ethanol because the maternal and fetal blood ethanol concentrations are very similar after equilibrium distribution of ethanol has been achieved in the maternal-fetal unit (Brien and Smith, 1991). The maternal blood sample was obtained 1 hr after oral administration of the second divided dose of 4 g ethanol / kg maternal body weight because the apparent maximal blood ethanol concentration in the maternal-fetal unit occurs at about this time (Clarke et al., 1986a, Clarke et al., 1986b). GD 57 or 58 was the gestational age selected for blood sampling, which allowed comparison of the maternal blood ethanol concentration data of this study with those of our previous investigations (Abdollah et al., 1993, Abdollah and Brein, 1995, Chiu et al., 1999).

## 2.5 Pregnancy Outcome

At birth, designated postnatal day (PD) 0, the litter size and number of live offspring were recorded. On PD 1, each individual offspring was weighed, and the entire litter and mother were moved into a large plastic bin with shaved wood chips as bedding. The offspring were monitored daily for health, and perinatal death was defined as death that occurred at parturition or during the first 10 days of postnatal life. The offspring were weaned at PD 17. Male and female littermates were separated at PD 22, before sexual maturation, and were housed in groups of up to four in plastic bins.

## 2.6 Measurement of Locomotor Activity

On each of PD 10 (pre-weaning), 20 (post-weaning) and 60 (adulthood), the locomotor activity of randomly selected individual offspring was determined in an Opto-Varimex® instrument (Columbus Instruments, Columbus, OH), which uses infrared beams to quantitate motion in an open field. One set of beams was set at 5 cm above the floor of the apparatus and measured horizontal locomotor activity (ambulatory exploratory activity), whereas another set of beams set at 14 cm height, recorded vertical locomotor activity (climbing and rearing). Each offspring was placed in the 42 cm x 42 cm x 21 cm unit for 1 hr in a quiet, stimulus-free environment and cumulative horizontal and vertical locomotor activity measurements were recorded at 10 min intervals. The animals were studied for 1 hr because cumulative locomotor activity normally has approached a plateau by the end of this time period (Abdollah et al., 1993).

## 2.7 Dissection of the Cerebral Cortex and Hippocampus and Membrane Preparation

On the next day after the measurement of locomotor activity at PD 10, 20 or 60, randomly selected individual offspring were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and then were euthanized by decapitation. The brain was excised and weighed. The cerebral cortex was dissected, frozen in liquid nitrogen, weighed after freezing, and then stored at -70°C for up to 30 days. The cerebral cortex was thawed and then homogenized in ice-cold homogenizing buffer (300 mM sucrose, 10 mM Tris, 2 mM EDTA, pH 7.3) to give a 5% w/v homogenate which was centrifuged at 1,000 x g for 10 min at 4°C. The pellet was discarded, and the supernatant was centrifuged at 20,000 x g for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in the homogenizing buffer to give a protein concentration in the range of 10-15 mg protein / ml suspension. This crude membrane preparation was divided into 300-μl aliquots, stored at -70°C and then analyzed within 2 months.

## 2.8 [$^3$H]MK-801 Saturation Binding Assay

To determine NMDA receptor number ($B_{max}$) and affinity ($K_d$) for [$^3$H]MK-801 in the cerebral cortical membrane preparation, saturation isotherm analysis was performed. A 300 μl aliquot of the membrane preparation was thawed, diluted to 1 ml with binding assay buffer (30 mM HEPES, 1 mM EDTA, pH 7.0), incubated for 30 min at 37°C, and then centrifuged at 13,000 x g for 10 min at 4°C. The cerebral cortical membrane pellet then was washed three times by suspension in 1 ml of binding assay buffer and centrifugation at 13,000 x g for 10 min at 4°C. The final membrane preparation, suspended in binding assay buffer, had a protein concentration in the range

of 1 to 2 μg protein / μl suspension, as determined using a protein-dye-binding method with bovine serum albumin as the standard (Bradford, 1976).

The radioligand-membrane binding assay was conducted using a 50-μl aliquot of the membrane preparation (50 to 100 μg of protein), saturating concentrations of L-glutamate and glycine (100 μM of each amino acid), assay buffer and [$^3$H]MK-801, ranging in concentration from 0.5 to 40 nM, in a final volume of 0.5 ml. Incubation was conducted at 37°C for 3 hr to allow equilibrium binding to occur. The binding assay was terminated by vacuum filtration of the reaction mixture through a Whatman® GF/B glass fibre filter, which then was rinsed twice with 5-ml aliquots of ice-cold binding assay buffer. The radioactivity retained on the filter was quantitated by liquid scintillation spectrometry using 5 ml of Scintiverse® scintillation fluid and a Beckman LS 3800 liquid scintillation counter. Non-specific binding of [$^3$H]MK-801 was determined in a separate set of samples incubated with 10 μM non-radiolabelled MK-801.

2.9 Glutamate Activation of the NMDA Receptor Assay

In order to determine the ability of glutamate to modulate NMDA receptor function, an activation assay was performed using saturation analysis. An aliquot of the cerebral cortical membrane preparation, used to determine receptor number and affinity for [$^3$H]MK-801, was washed and suspended as previously described. The activation assay was conducted using a 50-μl aliquot of the membrane preparation (50 to 100 μg of protein), a sub-maximal concentration of glycine (50 nM), assay buffer, a constant concentration of [$^3$H]MK-801 (5nM) and glutamate concentration, ranging from 1 nM to 100 μM, in a final volume of 0.5 ml. The other assay conditions were identical to those

used in the $[^3H]$MK-801 binding assay. The reaction mixture was incubated for 3 hr at 37°C after which the assay was terminated by rapid vacuum filtration. The amount of radioactivity retained on the filter was quantitated using liquid scintillation spectrometry.

2.10 Data Analysis

Saturation isotherm plots of cerebral cortical $[^3H]$MK-801 specific binding of individual offspring were analyzed by non-linear regression. Body, brain, and cerebral cortical weights, NMDA receptor number ($B_{max}$) and affinity ($K_d$) for $[^3H]$MK-801 for the ethanol, isocaloric-sucrose / pair-fed and water treatment groups are presented as group means ± SD. Homogeneity of variance of the data for each parameter was assessed using Bartlett's test before conducting parametric analysis. After this initial test of the data, one-way randomized-design analysis of variance (ANOVA) was performed, followed by Newman-Keuls multiple-comparisons test for a significant F statistic ($p \leq 0.05$). Two groups of data were considered to be statistically different when $p < 0.05$. The cumulative horizontal and vertical locomotor activity data for the three treatment groups at PD 10, 20 and 60 were analyzed using a two-way, randomized design, ANOVA. The cumulative horizontal and vertical locomotor activity data for the three treatment groups at PD 10, 20 and 60 were also analyzed at the individual 10 min time intervals using one-way ANOVA followed by Newman-Keuls multiple-comparisons test for a significant F statistic ($p < 0.05$). The behavioural data are reported as group means ± SEM.

## 3. RESULTS

### 3.1 Maternal Blood Ethanol Concentration

The chronic oral ethanol regimen produced a maternal blood ethanol concentration of 235±39 mg / dl (51.1±8.5 mM, n=8) at 1 hr after the second divided dose on GD 57 -58. This value was comparable with the maternal blood ethanol concentration data of our previous studies (Abdollah et al., 1993, Abdollah and Brein, 1995, Chiu et al., 1999). There was no measurable ethanol in the blood of the pregnant animals in the isocaloric-sucrose / pair-fed or water treatment groups.

### 3.2 Pregnancy Outcome Data

The pregnancy outcome data for the ethanol, isocaloric-sucrose / pair-fed, and water treatment groups are presented in Table 1. There was no maternal demise or spontaneous abortion and the length of gestation and litter size were not altered. Four of the 22 offspring that received chronic prenatal ethanol exposure died shortly after birth as did two of the 24 offspring in the water treatment group; there was no perinatal death in 23 offspring of the isocaloric-sucrose / pair-fed. The offspring of the ethanol-treated and isocaloric-sucrose / pair-fed treated pregnant guinea pigs had lower birth weight compared with the water-treated pregnant animals ($p<0.05$). There was no difference in the numbers of male and female littermates among the three treatment groups.

Table 1. Effect of Chronic Maternal Administration of Ethanol, Isocaloric-Sucrose / Pair Feeding or Water on Pregnancy Outcome of the Guinea Pig *

| Pregnancy Outcome Variable | Treatment | | |
|---|---|---|---|
| | Ethanol (8) | Sucrose (8) | Water (7) |
| Maternal death | 0 | 0 | 0 |
| Spontaneous abortion | 0 | 0 | 0 |
| Length of gestation (days) | 68.3±1.3 | 66.4±2.1 | 66.7±1.4 |
| Litter size | 3.3±0.9 | 3.3±1.0 | 3.9±1.1 |
| Perinatal death | 4 | 0 | 2 |
| Individual offspring birth weight (g) | 84.4±12.0[a] (18) | 88.5±13.5[a] (23) | 98.9±11.1[b] (20) |
| % Male littermates | 65.6±36.3 | 41.7±39.6 | 29.8±35.0 |
| % Female littermates | 34.4±36.3 | 58.3±36.6 | 70.2±35.0 |

* The number of pregnant guinea pigs / litters is reported in parentheses, except for the individual offspring birth weight for which the number of individual offspring is reported in parentheses. Maternal death, spontaneous abortion and perinatal death are reported as the number of occurrences. The data for the other variables are reported as group means ± SD. There was a statistically significant decrease in offspring birth weight in the ethanol and isocaloric-sucrose / pair-fed treatment groups compared to the water treatment group. Group means with different letters are statistically different ($p \leq 0.05$) from each other.

### 3.3 Effect of Chronic Prenatal Ethanol Exposure on Spontaneous Locomotor Activity

The cumulative horizontal and vertical spontaneous locomotor activity data of the offspring in the three treatment groups at PD 10, 20 and 60 are presented in Figure 1A and 1B. There was no effect of gender on spontaneous locomotor activity in any treatment group at any of the postnatal ages examined (data not shown). Consequently, the data of the male and female offspring in each treatment group were combined for the purpose of further analysis. Two-way ANOVAs performed at PD 10 and PD 20 demonstrated that there were time and treatment effects. One-way ANOVA demonstrated, that at PD 10, the ethanol-treated offspring exhibited increased horizontal spontaneous locomotor activity at each of the ten minute intervals and vertical spontaneous locomotor activity at the 30, 40, 50 and 60 minute intervals compared with the isocaloric-sucrose / pair-fed and water-treated offspring ($p<0.05$). At PD 20, one-way ANOVA demonstrated that the ethanol-treated offspring exhibited increased vertical spontaneous locomotor activity at the 10, 20 and 30 minute intervals, but not horizontal spontaneous locomotor activity compared with the two control groups ($p<0.05$). At PD 60, two-way ANOVA demonstrated that there was a treatment effect but no time effect. At PD 60, there was no difference at any of the ten minute time intervals in either horizontal or vertical spontaneous locomotor activity among the three treatment groups.

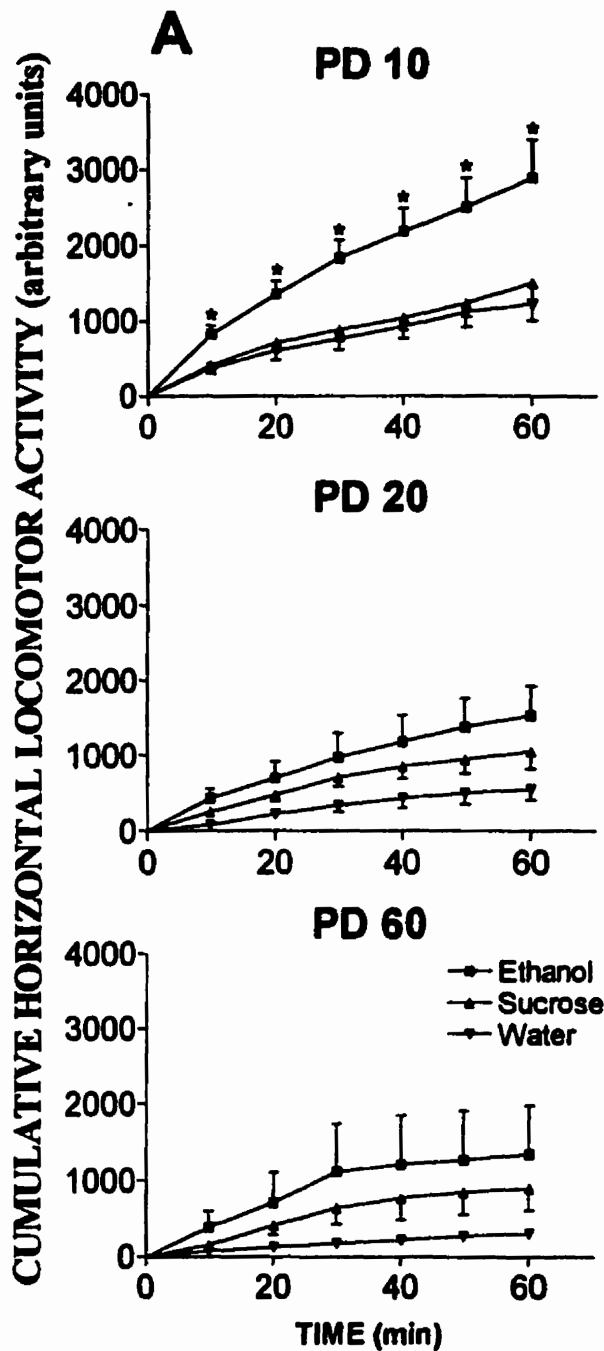



Figure 1A. Cumulative horizontal spontaneous locomotor activity for a 60-min period on PD 10, 20 and 60 for offspring of pregnant guinea pigs that received chronic daily oral administration of 4 g ethanol / kg maternal body weight (n=18 offspring from 8 litters at PD 10, n=12 offspring from 5 litters at PD 20, n=6 offspring from 3 litters at PD 60); isocaloric-sucrose / pair feeding (n=23 offspring from 8 litters at PD 10, n=17 offspring from 5 litters at PD 20, n=11 offspring from 3 litters at PD 60); or water (n=20 offspring from 7 litters at PD 10, n=14 offspring from 4 litters at PD 20, n=8 offspring from 2 litters at PD 60). The data are presented as the mean ± SEM of the individual littermate values. At the time points indicated, ethanol treated offspring had higher spontaneous locomotor activity than both isocaloric sucrose/pair-fed and water treated offspring (* $p \leq 0.05$).

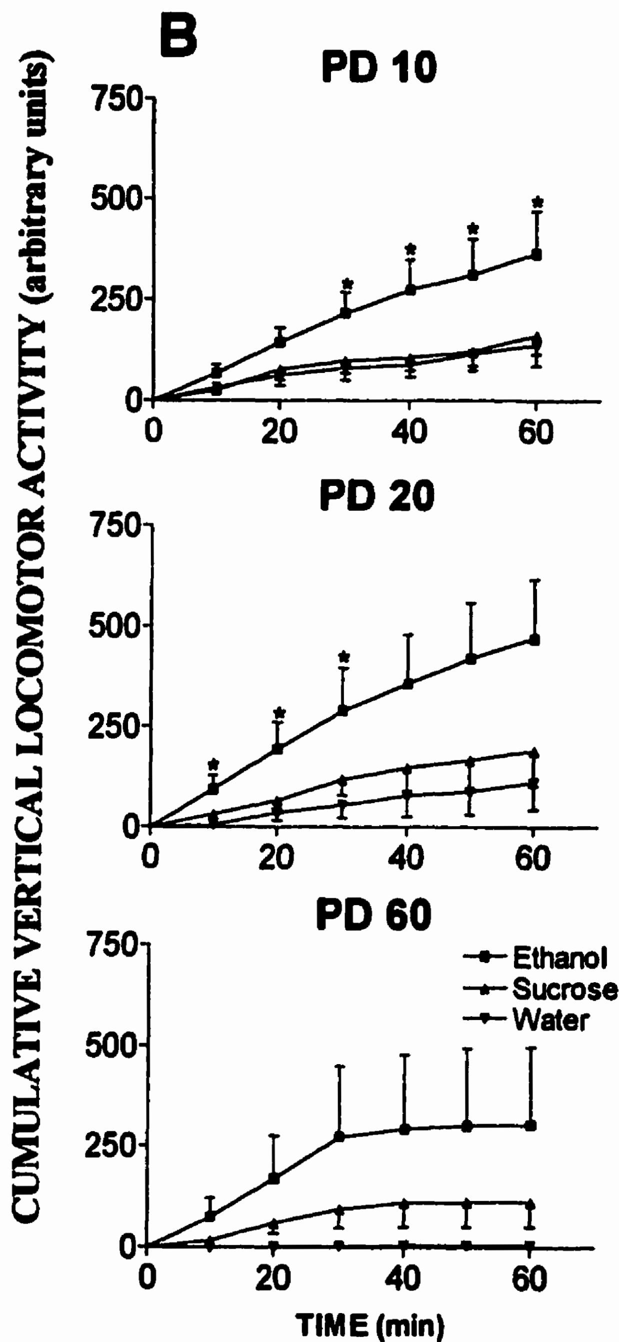



Figure 1B. Cumulative vertical spontaneous locomotor activity for a 60-min period on PD 10, 20 and 60 for offspring of pregnant guinea pigs that received chronic daily oral administration of 4 g ethanol / kg maternal body weight (n=18 offspring from 8 litters at PD 10, n=12 offspring from 5 litters at PD 20, n=6 offspring from 3 litters at PD 60); isocaloric-sucrose / pair feeding (n=23 offspring from 8 litters at PD 10, n=17 offspring from 5 litters at PD 20, n=11 offspring from 3 litters at PD 60); or water (n=20 offspring from 7 litters at PD 10, n=14 offspring from 4 litters at PD 20, n=8 offspring from 2 litters at PD 60). The data are presented as the mean ± SEM of the individual littermate values. At the time points indicated, ethanol treated offspring had higher spontaneous locomotor activity than both isocaloric sucrose/pair-fed and water treated offspring (* $p \leq 0.05$).

## 3.4 Effect of Chronic Prenatal Ethanol Exposure on Offspring Body, Brain and Cerebral Cortical Weights

Offspring body, brain and cerebral cortical weights at PD 10, 20 and 60 are presented in Table 2. There was no effect of gender on any of these measured parameters in any treatment group at any of the postnatal ages examined (data not shown). Consequently, the data of the male and female offspring in each treatment group were combined for the purpose of further analysis. The offspring in the ethanol and the isocaloric-sucrose / pair-fed treatment groups had lower body weight at parturition than the water treatment group ($p<0.05$). Ten days after birth, the body weights of the offspring in both the ethanol and isocaloric-sucrose / pair-fed groups was similar to the water control group. At each of PD 10 and 20, the offspring that received chronic prenatal ethanol exposure had decreased brain weight and cerebral cortical weight compared with the isocaloric-sucrose / pair-fed and water treatment groups ($p<0.05$). At PD 60, the ethanol-treated offspring had decreased brain weight compared with the offspring of the water treatment group and decreased cerebral cortical weight compared with the offspring of the isocaloric-sucrose / pair-fed treatment group ($p<0.05$).

Table 2. Body, Brain and Cerebral Cortical Weights at PD 10, 20 and 60 for Offspring of Pregnant Guinea Pigs that received Chronic Oral Administration of Ethanol, Isocaloric-Sucrose / Pair Feeding or Water *

| Postnatal Age | Variable | Treatment | | |
|---|---|---|---|---|
| | | Ethanol | Sucrose | Water |
| PD 10 | Body weight (g) | 181.8±15.8 (6) | 185.0±13.2 (6) | 166.5±34.6 (6) |
| | Brain weight (g) | 2.60±0.14$^{a}$ | 2.89±0.29$^{b}$ | 2.90±0.20$^{b}$ |
| | Cerebral cortical weight (g) | 1.52±0.10$^{a}$ | 1.72±0.17$^{b}$ | 1.70±0.14$^{b}$ |
| PD 20 | Body weight (g) | 238.2±29.8$^{a}$ (6) | 193.0±22.9$^{b}$ (6) | 258.7±27.2$^{a}$ (6) |
| | Brain weight (g) | 2.78±0.15$^{a}$ | 3.01±0.19$^{b}$ | 3.44±0.15$^{c}$ |
| | Cerebral cortical weight (g) | 1.49±0.09$^{a}$ | 1.66±0.12$^{b}$ | 1.82±0.11$^{c}$ |
| PD 60 | Body weight (g) | 575.2±84.7 (6) | 537.6±74.5 (11) | 615.9±77.0 (8) |
| | Brain weight (g) | 3.44±0.16$^{a}$ | 3.70±0.24$^{a,b}$ | 3.88±0.36$^{b}$ |
| | Cerebral cortical weight (g) | 1.83±0.19$^{a}$ | 2.23±0.33$^{b}$ | 2.10±0.15$^{a,b}$ |

* The number of offspring that were studied is reported in parentheses. The data are presented as group means ± SD. There were statistically significant decreases in brain and cerebral cortical weights at PD 10; in body, brain and cerebral cortical weights at PD 20; and in brain and cerebral cortical weights at PD 60. Group means with different letters are statistically different ($p \leq 0.05$) from each other.

## 3.5 Effect of Chronic Prenatal Ethanol Exposure on Specific [$^3$H]MK-801 Binding in the Cerebral Cortex and Hippocampus

Representative saturation isotherm and Scatchard plots for specific [$^3$H]MK-801 binding in the cerebral cortical membrane preparation of individual offspring at PD 60 for the three treatment groups are presented in Figure 2. These data are consistent with a single binding site for [$^3$H]MK-801 on the cerebral cortical NMDA receptors of the offspring of the chronic ethanol, isocaloric-sucrose / pair-fed and water treatment groups, using saturating concentrations of L-glutamate and glycine. There was no effect of gender on the radioligand-binding characteristics of [$^3$H]MK-801 in any treatment group at any of the postnatal ages examined (data not shown). Consequently, the data of the male and female offspring in each treatment group were combined for the purpose of further analysis. At each of PD 10 and 20, there was no difference in the NMDA receptor number ($B_{max}$) or affinity ($K_d$) for [$^3$H]MK-801 in the cerebral cortex Figures 3 & 4. The data for $B_{max}$ of the cerebral cortical NMDA receptors and their $K_d$ for [$^3$H]MK-801 of the PD 60 offspring in the three treatment groups are presented in Figure 5. Chronic prenatal ethanol exposure decreased both the $B_{max}$ and $K_d$ values compared with the isocaloric-sucrose / pair-fed and water treatment groups ($p<0.05$). At PD 60, there was no difference in the NMDA receptor number ($B_{max}$) or affinity ($K_d$) for [$^3$H]MK-801 in the hippocampus of the offspring in the three treatment groups (Figure 6).

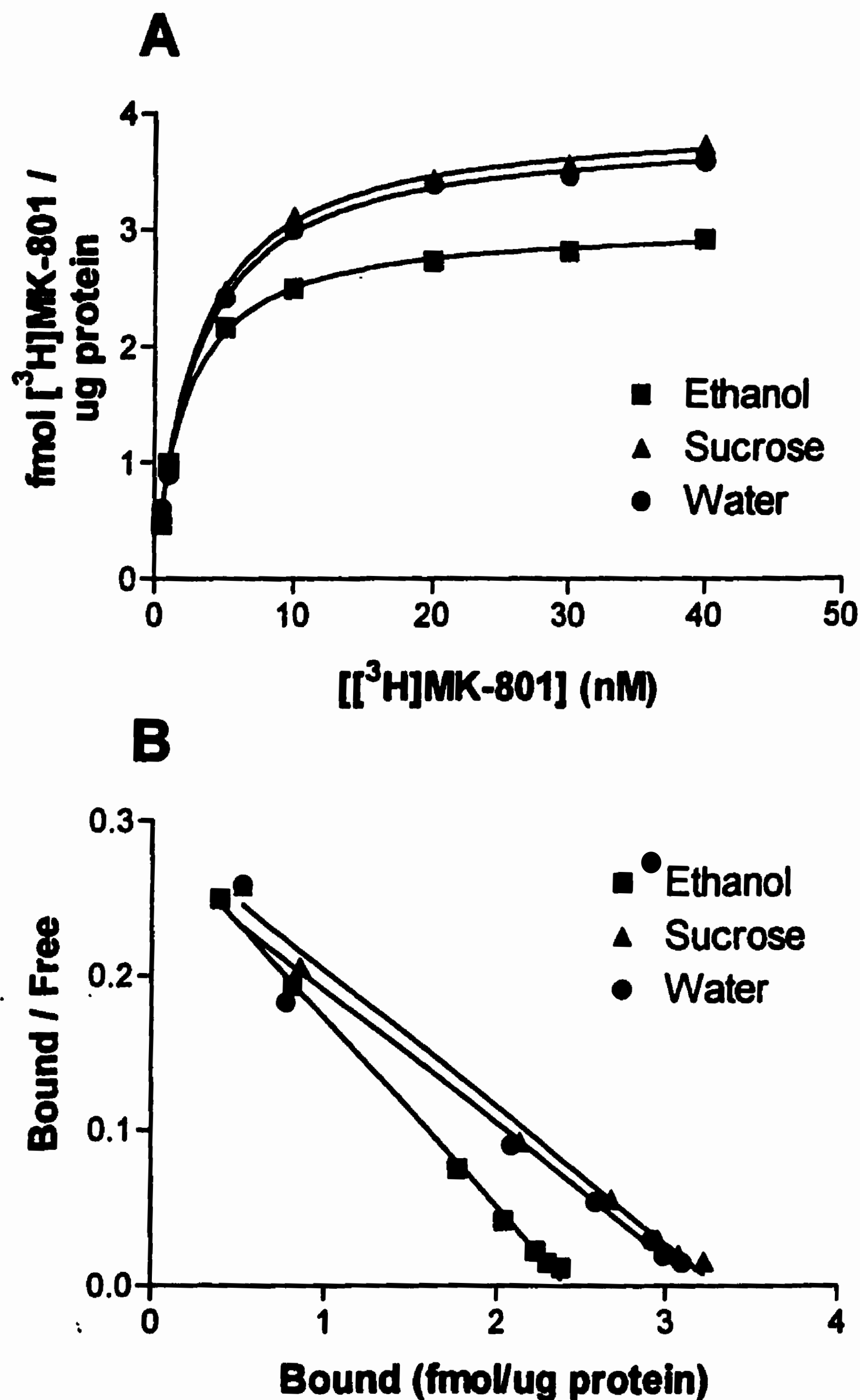



Figure 2. Representative saturation isotherm (A) and Scatchard plot (B) of [$^3$H]MK-801 specific binding in the cerebral cortical membrane preparation of individual PD 60 offspring of pregnant guinea pigs that received chronic daily oral administration of 4 g ethanol / kg maternal body weight, isocaloric-sucrose / pair feeding or water.

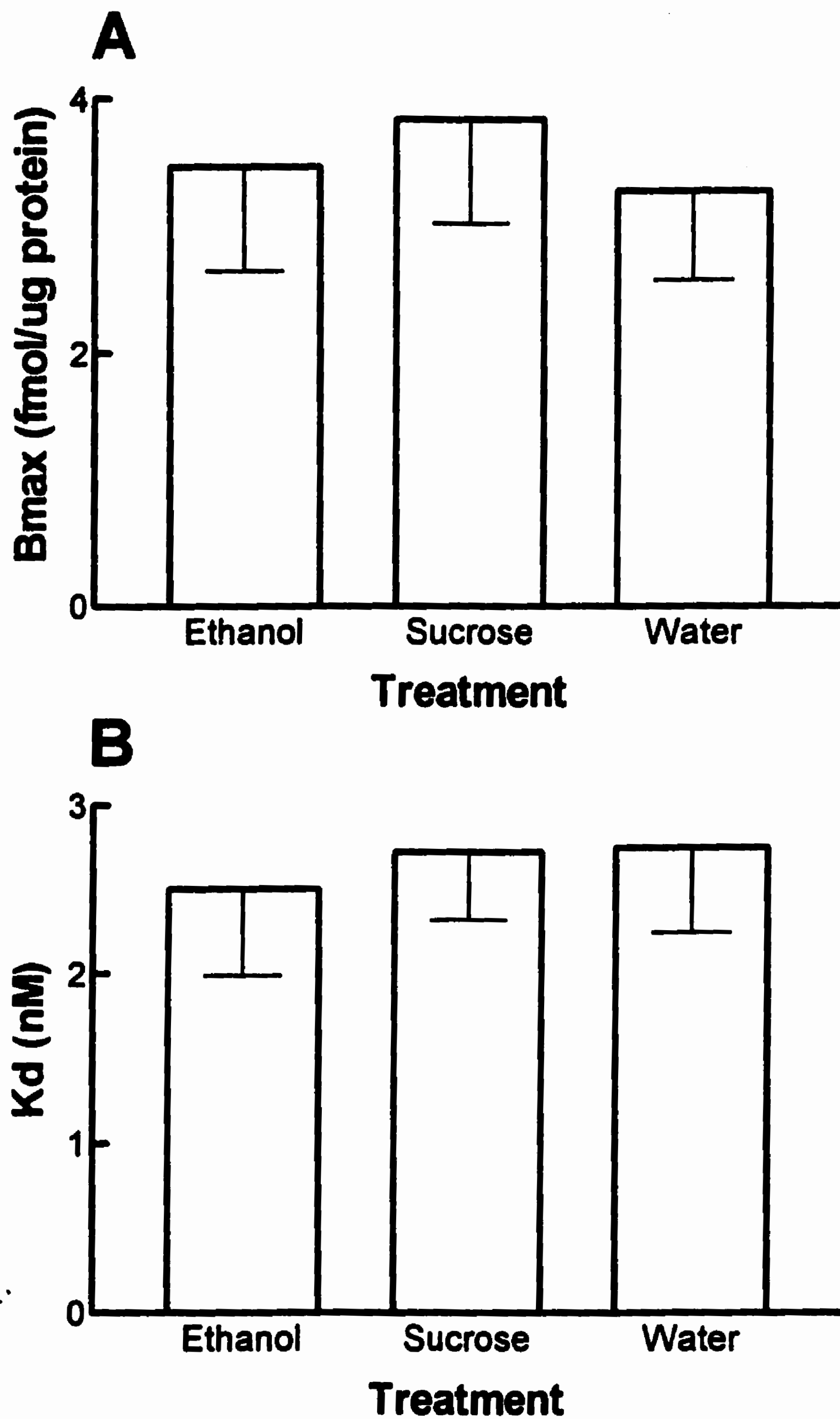
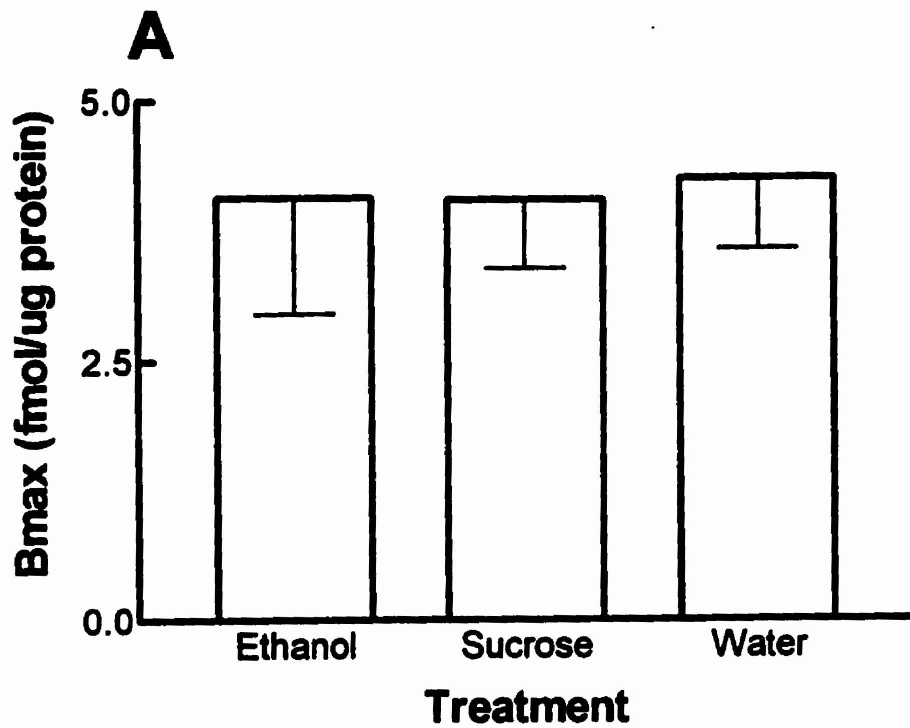



Figure 3. Bmax (A) and Kd (B) for [$^3$H]MK-801 binding to cerebral cortical NMDA receptors in the PD 10 offspring of pregnant guinea pigs that received chronic daily oral administration of 4 g ethanol / kg maternal body weight (n=6 offspring from 3 litters), isocaloric-sucrose / pair feeding (n=6 offspring from 3 litters) or water (n=6 offspring from 3 litters).



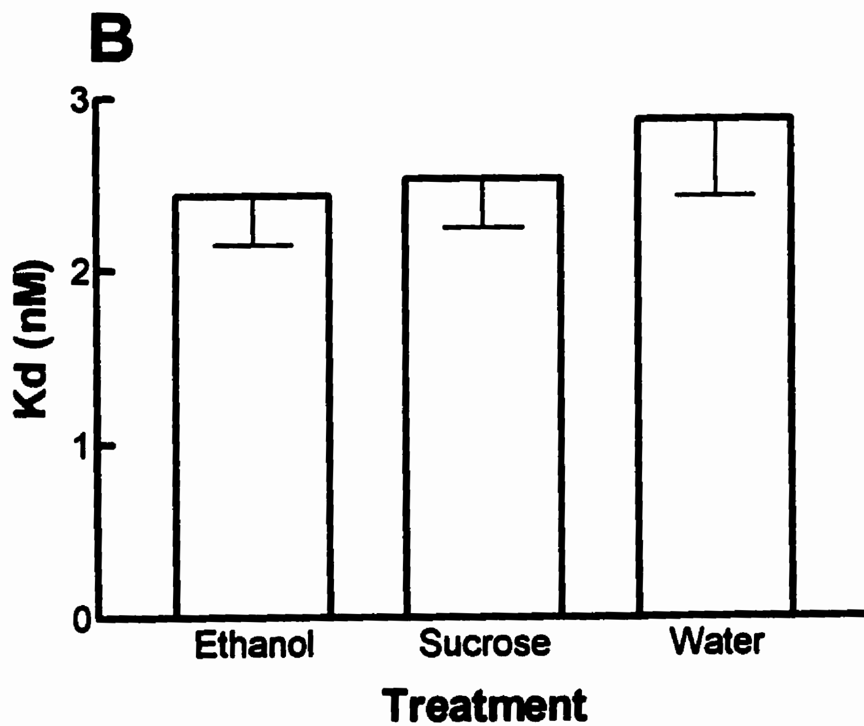




Figure 4. Bmax (A) and Kd (B) for [$^{3}$H]MK-801 binding to cerebral cortical NMDA receptors in the PD 20 offspring of pregnant guinea pigs that received chronic daily oral administration of 4 g ethanol / kg maternal body weight (n=6 offspring from 2 litters), isocaloric-sucrose / pair feeding (n=6 offspring from 2 litters) or water (n=6 offspring from 2 litters).

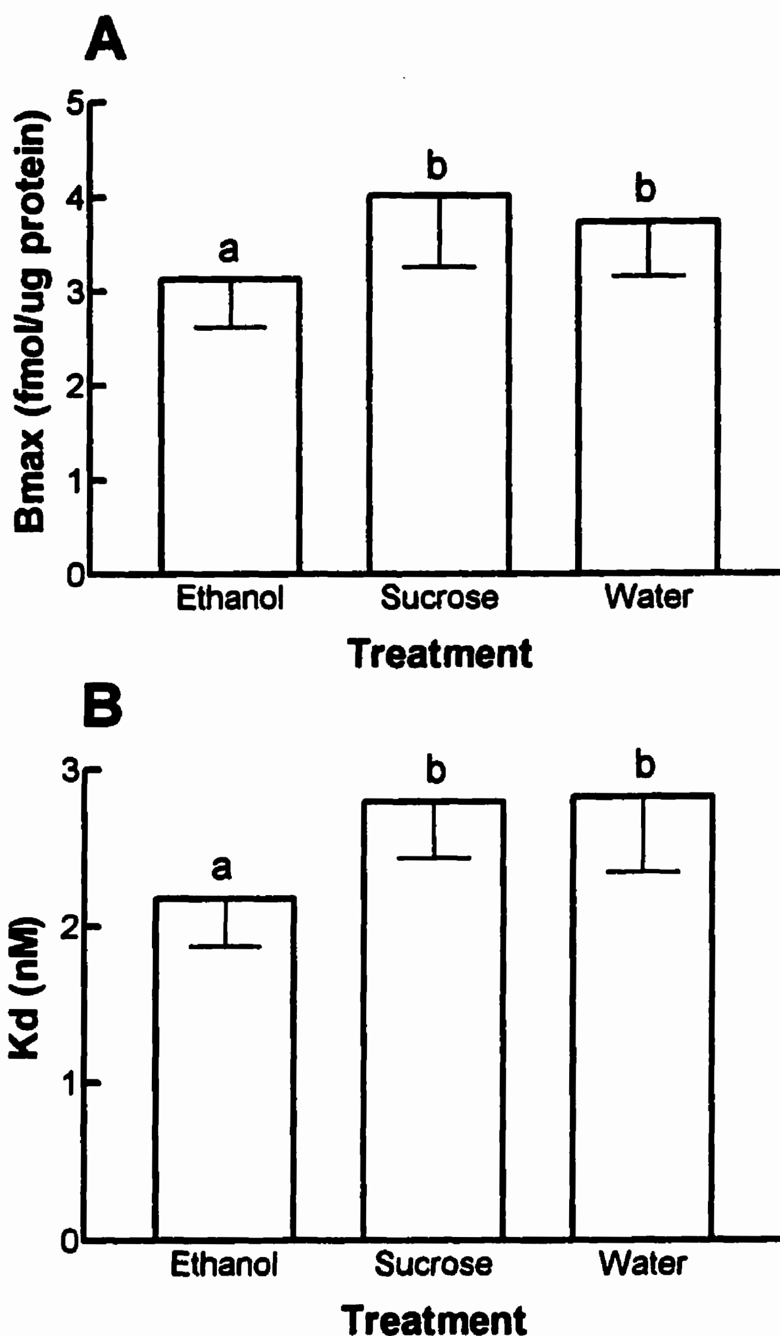



Figure 5. Bmax (A) and Kd (B) for [$^3$H]MK-801 binding to cerebral cortical NMDA receptors in the PD 60 offspring of pregnant guinea pigs that received chronic daily oral administration of 4 g ethanol / kg maternal body weight (n=6 offspring from 3 litters), isocaloric-sucrose / pair feeding (n=11 offspring from 3 litters) or water (n=8 offspring from 2 litters). There were statistically significant decreases in the Bmax and Kd values for the PD 60 offspring of the ethanol treatment group compared with the sucrose and water treatment groups ($F_{2,22}$=3.686 and $F_{2,22}$=5.941, respectively). Group means with different letters are statistically different ($p \leq 0.05$) from each other.

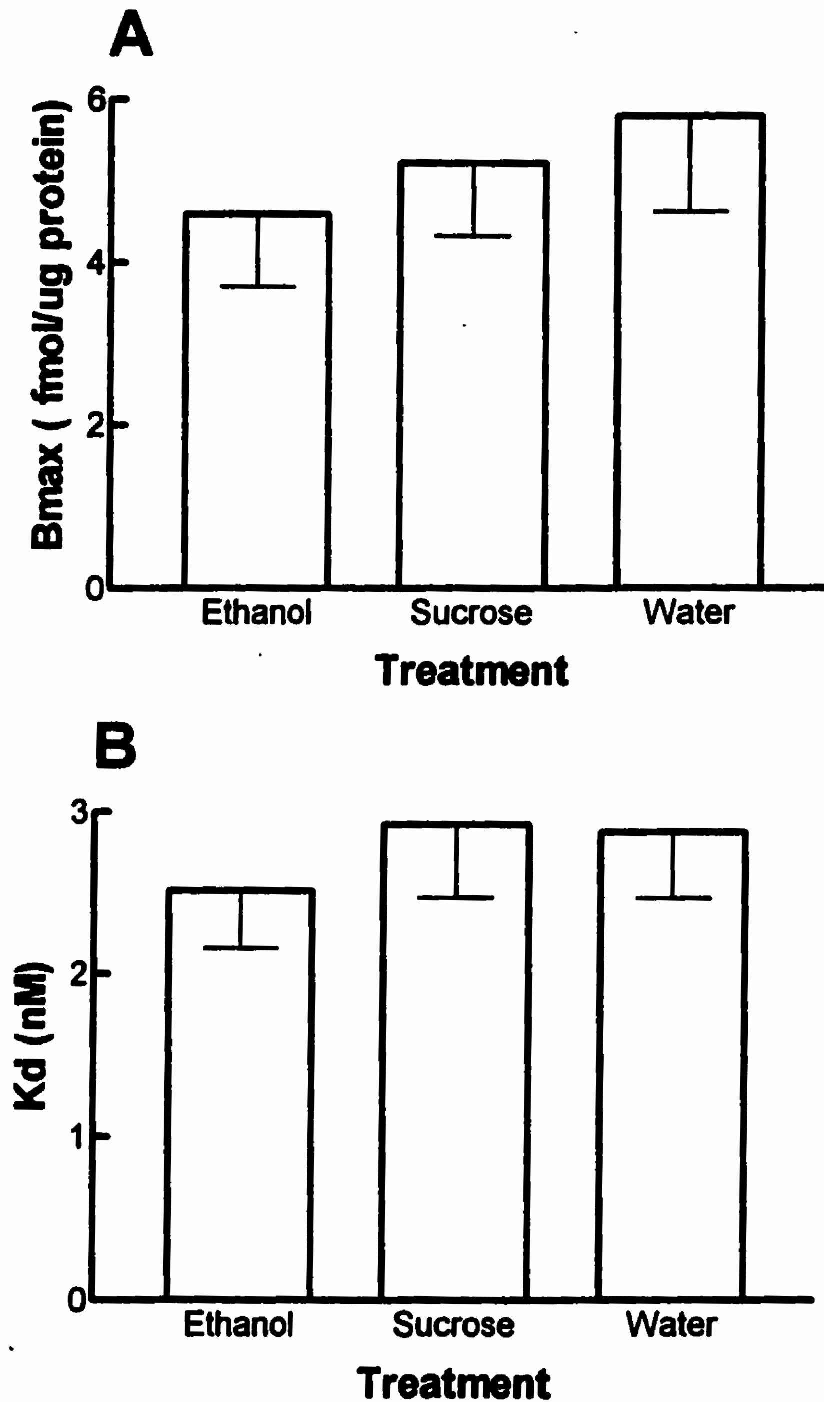



Figure 6. Bmax (A) and Kd (B) for [$^3$H]MK-801 binding to hippocampal NMDA receptors in the PD 60 offspring of pregnant guinea pigs that received chronic daily oral administration of 4 g ethanol / kg maternal body weight (n=6 offspring from 3 litters), isocaloric-sucrose / pair feeding (n=11 offspring from 3 litters) or water (n=8 offspring from 2 litters).

3.6 Effect of Chronic Prenatal Ethanol Exposure on NMDA Receptor Activation by Glutamate in the Cerebral Cortex

The ability of glutamate to potentiate [$^3$H]MK-801 binding to NMDA receptors was examined in the adult (PD 60) cerebral cortex. Because MK-801 binds at an intrachannel site, NMDA receptor activation controls MK-801 binding. Increasing glutamate concentrations (1nM to 100μM) were added to a constant concentration of glycine (50nM) and [$^3$H]MK-801 (5nM). Chronic prenatal ethanol exposure did not alter the maximal potentiation of [$^3$H]MK-801 binding by glutamate or NMDA receptor affinity for glutamate (Figure 7).

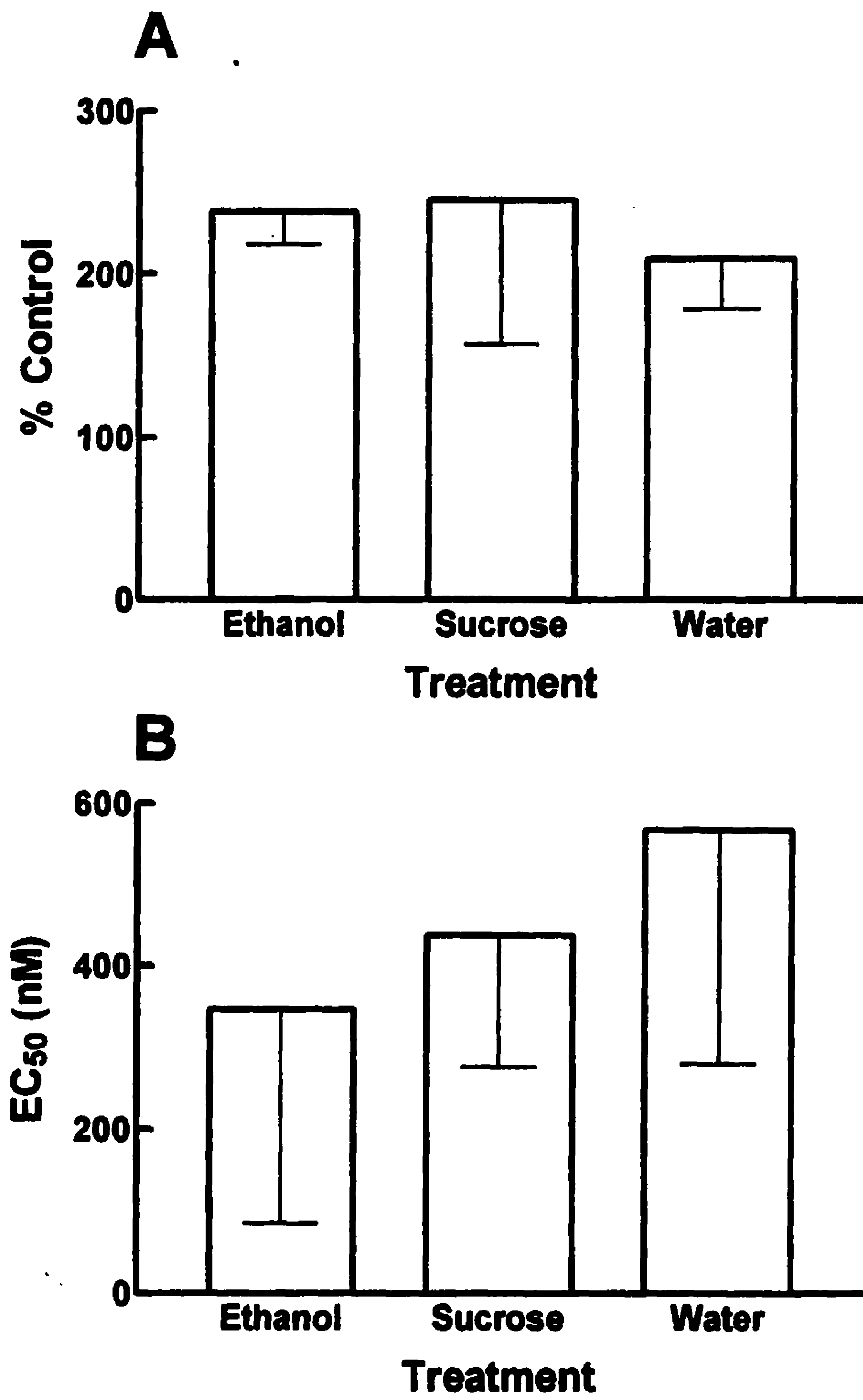



Figure 7. Emax (A) and $EC_{50}$ (B) for glutamate stimulated [$^3$H]MK-801 binding to cerebral cortical NMDA receptors in PD 60 offspring of pregnant guinea pigs that received chronic daily oral administration of 4 g ethanol / kg maternal body weight (n=6 offspring from 3 litters), isocaloric-sucrose / pair feeding (n=11 offspring from 3 litters) or water (n=8 offspring from 2 litters).

# 4. DISCUSSION

## 4.1 Discussion of Thesis Research

The objective of this thesis research was to determine, in the guinea pig, the effects of chronic prenatal ethanol exposure, via maternal ethanol administration throughout gestation, on the NMDA receptor population in the cerebral cortex during postnatal life. Furthermore, it was important to elucidate if there was a relationship between altered NMDA receptor population, hyperactive behaviour, and brain growth restriction during postnatal life including adulthood. Chronic oral administration of 4 g ethanol / kg maternal body weight / day produced behavioural (hyperactivity) and morphological (decreased brain and cerebral cortical weights) changes that diminished during postnatal life, and neurochemical (decreased NMDA receptor $B_{max}$ and $K_d$ for [$^3$H]MK-801) changes that occurred in adult offspring. The adult offspring of the isocaloric-sucrose / pair-fed treatment group were not different from the progeny of the water treatment control group with respect to locomotor activity, body, brain and cerebral cortical weights, and NMDA receptor number and affinity. These results demonstrate that the neurobehavioural teratogenic effects in the offspring of the ethanol treatment group are primarily the result of chronic prenatal ethanol exposure and not fetal undernutrition or a maternal oral-intubation-induced stress response.

In the present study, the offspring of the chronic ethanol treatment group exhibited increased horizontal and vertical spontaneous locomotor activity at all three postnatal ages (PD 10, 20 and 60). As the prenatal-ethanol-exposed offspring matured, they were less hyperactive. Previous studies conducted in the guinea pig have demonstrated that chronic prenatal ethanol exposure increased locomotor activity up to and including

adulthood (Abdollah 93). Hyperactivity is a reliable index of ethanol CNS teratogenicity because it is one of the most common manifestations of CNS dysfunction resulting from chronic prenatal ethanol exposure and occurs in all mammalian species studied to date (Driscoll et al., 1990).

Chronic prenatal ethanol exposure produced microencephaly in the offspring, as demonstrated by the decreased brain and cerebral cortical weights at each of the three postnatal ages studied compared with the isocaloric-sucrose / pair-fed and water treatment control groups. The offspring of both the ethanol and isocaloric-sucrose/pair-fed treatment groups had lower birth weight than the offspring of the water group. This decrease in body weight of the offspring in the ethanol and isocaloric-sucrose / pair-fed treatment groups indicates that fetal undernutrition is primarily responsible for this growth restriction. At PD 10, there was no difference in the offspring body weight among the three treatment groups. At PD 20, the body weight of the offspring for the isocaloric-sucrose / pair-fed treatment group was transiently decreased compared with the ethanol and water treatment groups; however, by PD 60 (adulthood), there was again no difference in body weight among the three treatment groups. Chronic prenatal ethanol exposure decreased brain and cerebral cortical weights throughout postnatal life that persisted into adulthood, compared with the isocaloric-sucrose and water treatment groups. The isocaloric-sucrose / pair-feeding treatment had a suppressant effect on body, brain and cerebral cortical weights at PD 20 only compared with the water treatment group; the effect on brain and cerebral cortical weights was of smaller magnitude than the effect of chronic prenatal ethanol exposure. The nature and mechanism of this transient effect of isocaloric-sucrose / pair-feeding treatment on body weight at PD 20 remain to be determined. The brain weight data demonstrate that this maternal ethanol regimen

produced microencephaly that persisted into adulthood, which is a common manifestation of the CNS dysmorphology of ethanol teratogenicity (Clarren and Smith, 1978b, Driscoll et al., 1990).

Several studies have been conducted using various mammalian species (e.g. rat and guinea pig) and treatment protocols in order to determine if chronic prenatal ethanol exposure alters NMDA receptors in various brain regions, including the cerebral cortex (Chiu et al., 1999), hippocampus (Savage et al., 1991, Abdollah and Brien, 1995) and combined cerebral cortical-hippocampal preparation (Diaz-Granados et al., 1997). The results of these studies indicate that the effects of chronic prenatal ethanol exposure on the NMDA receptor population are dependent on the ethanol dosage regimen, the time of ethanol exposure during gestation, as well as the brain region and developmental age that are examined. Previous research in our laboratory has demonstrated that chronic prenatal ethanol exposure, using the maternal ethanol regimen utilized in the present study, increases the number of NMDA receptors and decreases their affinity for [$^3$H]MK-801 in the cerebral cortex of the near-term fetal guinea pig (Chiu et al., 1999). In the present study, chronic prenatal ethanol exposure had different effects on NMDA receptor number and affinity in the cerebral cortex during postnatal life that depended on the age of the offspring, ranging from no effect on $B_{max}$ and $K_d$ at PD 10 and PD 20 to decreased number of NMDA receptors and increased affinity for [$^3$H]MK-801 at PD 60. This decrease in NMDA receptor number in the cerebral cortex of the adult guinea pig offspring resulting from chronic maternal ethanol administration is similar to the NMDA receptor binding data for the PD 45 rat hippocampus (Savage et al., 1991) and the PD 20-23 rat combined cerebral cortical-hippocampal membrane preparation (Diaz-Granados et al., 1997) following chronic prenatal ethanol exposure.

Binding characteristics of the ligand, glutamate, were examined to determine whether chronic prenatal ethanol exposure alters its ability to open the NMDA receptor ion channel. [$^3$H]MK-801 binding was used to measure NMDA receptor activation because it binds to an intrachannel site that is exposed only when the NMDA receptor is in the activated state. The three treatment regimens did not alter the maximal NMDA receptor activation produced by the addition of glutamate or the NMDA receptor affinity for glutamate. These results indicate that chronic prenatal ethanol exposure causes an increase in NMDA receptor affinity for [$^3$H]MK-801, but does not alter receptor affinity for glutamate, in the adult cerebral cortex. This finding can be explained by the fact that different NR2 subunits have quite different affinities for MK-801, whereas NR2 subunit affinity for glutamate is fairly constant (Laurie and Seeburg, 1994). The fact that there was no change in NMDA receptor affinity for glutamate has important ramifications when coupled with the fact that there was a decrease in NMDA receptor number in the adult cerebral cortex: equivalent release of glutamate will cause less post-synaptic response in offspring exposed to ethanol prenatally compared with the offspring of the isocaloric-sucrose / pair-fed and water treatment groups. Based on these findings, it appears that cerebral cortical post-synaptic neurons will be less sensitive to the presence of glutamate in the synaptic cleft for offspring that received chronic prenatal ethanol exposure.

A second NMDA receptor activation assay was attempted to determine if chronic prenatal ethanol exposure caused any change in NMDA receptor sensitivity to the essential co-agonist, glycine. Several experimental conditions were used but ultimately, consistent results were not attained. It is believed that these experiments were unsuccessful due to contamination of the solutions by glycine of unknown source.

Solutions prepared with deionized water (Nanopure system) or deionized ultra-filtered bottled water caused activation of NMDA receptors without the addition of glycine.

There are several possible mechanisms that could explain the decreased NMDA receptor number in the adult offspring that were chronically exposed to ethanol throughout gestation. Decreased number of neurons expressing NMDA receptors, increased turnover of NMDA receptors, decreased transcription of NMDA receptor DNA or decreased mRNA translation (Kumari and Ticku, 1998) could produce a decrease in the number of NMDA receptors. Studies in the fetal and adult rat have shown that prenatal ethanol exposure can alter selectively the number of neurons in the somatosensory cortex (Miller, 1997) and in other cerebral cortical regions including the principal sensory nucleus (Miller, 1995), ventricular zone and subventricular zone (Miller, 1996a). It is noteworthy that there was a trend toward decreased number of NMDA receptors in the hippocampus of the adult guinea pig resulting from chronic prenatal ethanol exposure (19% decrease for ethanol treatment compared with water control treatment; $0.05<p<0.10$). In this regard, a study in our laboratory has demonstrated that chronic maternal administration of 4 g ethanol / kg maternal weight / day produces a 25% decrease in the number of CA1 pyramidal neurons in the hippocampus of the adult guinea pig (Abdollah et al., 1993). This morphologic effect of chronic prenatal ethanol exposure is temporally related to the trend of decreased number of hippocampal NMDA receptors and the hyperactivity of the adult offspring observed in a previous study (Abdollah et al., 1993, Chiu et al., 1999).

Chronic maternal administration of ethanol increased the affinity of NMDA receptors for [$^3$H]MK-801, but did not alter the affinity for glutamate in the cerebral

cortex of the adult guinea pig. NMDA receptors are heteromeric ligand-gated ion channels consisting of NR1 subunits together with NR2 subunits (Monyer et al., 1994). Investigation using recombinant NMDA receptors has demonstrated that affinity for MK-801 is closely associated with the subunit composition of the receptor, whereas glutamate affinity is relatively independent of the subunit composition (Laurie and Seeburg, 1994). The cerebral cortex contains a heterogeneous population of NMDA receptor isoforms composed of different subunit subtype combinations. The $K_d$ for [$^3$H]MK-801 binding, therefore, reflects the average $K_d$ value for the NMDA receptor population. The increased affinity for [$^3$H]MK-801 resulting from chronic prenatal ethanol exposure could be due to loss of NMDA receptor isoforms with relatively lower affinity for [$^3$H]MK-801 from the population of cerebral cortical NMDA receptors, an alteration in the NMDA receptor subunit composition (Follessa and Tieku, 1995) and / or post-translational modifications such as receptor phosphorylation. Future experiments should focus on elucidating the ontogeny of cerebral cortical NMDA receptor subunit expression during prenatal and postnatal life, and the effects of chronic prenatal ethanol exposure on NR1, NR2A, NR2B, NR2C and NR2D subunit subtype expression. This may provide insight into the differential effects of prenatal ethanol exposure on prenatal versus postnatal NMDA receptor expression in the guinea pig cerebral cortex.

There was no a temporal correlation between spontaneous locomotor activity and changes in the cerebral cortical NMDA receptor population at PD 60. The lack of a relationship between locomotor activity and the cerebral cortical NMDA receptor population indicates that locomotor activity is not solely influenced by the cerebral cortical NMDA receptor population.

In conclusion, this study has provided important new knowledge about the effects of chronic prenatal ethanol exposure on behaviour, morphology and the cerebral cortical NMDA receptor population of the postnatal guinea pig. By examining three postnatal ages (PD 10, 20 and 60), developmental profiles of the effects of chronic prenatal ethanol exposure on cerebral cortical NMDA receptor number and affinity, cerebral cortical weight and exploratory locomotor activity during postnatal life have been elucidated. The mechanism by which chronic prenatal ethanol exposure decreases the number of cerebral cortical NMDA receptors and increases their affinity for [$^3$H]MK-801 in the adult offspring and the relationship of this neurochemical effect to the transient hyperactivity and cerebral cortical growth restriction remains to be determined.

### 4.2 Future Research Directions

#### 4.2.1 Effect of Chronic Prenatal Ethanol Exposure on NMDA Receptor Expression in the Adult Cerebellum

The cerebellum, along with the cerebral cortex and hippocampus, is a target site for ethanol CNS teratogenesis. Children who suffer from disrupted cerebellar function exhibit motor deficits that are similar to the motor dysfunction observed in patients with the FAS (Clarren and Smith, 1987). Exposure to ethanol during development produces several morphological alterations in the cerebellum, including Purkinje cell loss (Marcussen 94) and cerebellar dysgenesis (Roebuck et al., 1998). To date, there is little known about whether chronic prenatal ethanol exposure affects the cerebellar NMDA receptor population. Future studies examining the cerebellum could produce valuable information whether altered NDMA receptors play a role in the mechanism of the motor deficits that occur in individuals suffering from the FAS.

#### 4.2.2 Effect of Ethanol Exposure During Critical Periods of Development on NMDA Receptor Expression

An extremely important question, that has clinical implications, is whether or not there are critical periods of development during which the fetal brain is sensitive to ethanol's effects on NMDA receptors. The brain growth spurt appears to be at least one of the developmental periods during which ethanol exposure can alter NMDA receptors. In preliminary studies conducted in the guinea pig in our laboratory, it was discovered that exposure to ethanol from GD 45 to 62 (the time of the brain growth spurt) produced an increase in the number of cerebral cortical NMDA receptors in the near-term fetus similar

to that produced by ethanol exposure throughout gestation (Chiu et al., 1999). This treatment paradigm should be used to examine alterations in cerebral cortical NMDA receptors during postnatal life, as well as the effects on offspring behaviour and cerebral cortical morphology. Future experiments that examine the effects of prenatal ethanol exposure during selected periods of development, including the brain growth spurt, could provide important new information about gestational times when consumption of ethanol should be avoided.

#### 4.2.3 Effect of Chronic Prenatal Ethanol Exposure on NMDA Receptor NR1 and NR2 Subunit Expression

Studies conducted with the rat have discovered that brain regions have distinct NMDA receptor developmental profiles. To date, no such studies have been reported for the developing guinea pig. Future experiments could be conducted to elucidate the ontogeny of cerebral cortical NMDA receptor subunit expression during prenatal and postnatal life. The increased affinity for [$^3$H]MK-801 of the cerebral cortical NMDA receptors resulting from chronic prenatal ethanol exposure could be due to an alteration in the NMDA receptor subunit composition (Follessa and Tieku, 1995). In view of this possibility, it would be important to determine the effect of chronic prenatal ethanol exposure on NR1, NR2A, NR2B, NR2C and NR2D subunit expression. It is important to examine chronic prenatal ethanol-induced changes in NMDA receptor subunit composition because alterations in subunit expression can have profound implications on the ability of a ligand to bind to the receptor. This study could provide insight into the differential effects of prenatal ethanol exposure on fetal versus postnatal NMDA receptor expression in the guinea pig cerebral cortex.

Immunohistochemistry using subunit-specific antibodies provides a useful experimental technique by which both the distribution and subunit composition of the NMDA receptor population can be determined. Various slices of the cerebral cortex can be used to determine whether chronic prenatal ethanol exposure alters the localization of NMDA receptors in the cerebral cortex. These studies should employ NR1-specific antibodies since NR1 subunits are essential for functional NMDA receptors (Meguro et al., 1992). Distribution of NMDA receptor NR2 subunits in the cerebral cortex can be determined using NR2-subunit specific antibodies. This technique can be used to determine the amount of NMDA receptor individual subunit protein found at various locations in the cerebral cortex. Chronic prenatal ethanol exposure may alter the distribution of NMDA receptors in the cerebral cortex, which could influence the effects of decreased NMDA receptor number.

4.2.4 Effect of Chronic Prenatal Ethanol Exposure on Cerebral Cortical Neurons

Chronic prenatal ethanol exposure produced a persistent decrease in cerebral cortical weight as well as a decease in the number of cerebral cortical NMDA receptors in the adult offspring. Therefore, future investigation would be warranted to determine if the chronic ethanol regimen used in the present study produces loss of cerebral cortical neurons in the adult guinea pig offspring that is correlated with the decreased NMDA receptor number. Previous studies in the rat cerebral cortex have shown that chronic prenatal ethanol treatment can cause neuronal cell loss in specific cerebral cortical structures, such as the motor and sensory cortices, as well as a loss of astrocytes (Guerri, 1998). Chronic prenatal ethanol exposure has been shown to cause a 25% decrease in hippocampal CA1 pyramidal neurons in the adult guinea pig (Abdollah et al., 1993,

Kimura et al., 1996). Employing techniques already established in our laboratory, another future direction could be to determine whether there is cerebral cortical neuronal cell loss at PD 60 and if so, its localization.

### 4.2.5 Effects of Chronic Prenatal Ethanol Exposure on other Behavioural Parameters

To date, the test primarily used to measure behavioural alterations produced by chronic prenatal ethanol exposure in the guinea pig has focused on spontaneous locomotor activity in the open field. Ethanol exposure during brain development has been shown to produce a myriad of behavioural effects in other animal models. Another interesting future direction could be to develop new tests to measure whether chronic prenatal ethanol exposure causes other behavioural alterations, including altered social recognition (Kelly and Tran, 1997), learning deficits (Klintsova et al., 1998) and inhibitory avoidance (Lochry and Riley, 1980, Abel, 1982, Tan, 1990) in the guinea pig.

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