MODERATE ETHANOL CONSUMPTION BY THE PREGNANT GUINEA PIG INCREASES ETHANOL PREFERENCE IN OFFSPRING

By

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Abstract

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Ethanol teratogenicity involving the developing brain is the leading preventable cause of mental deficiency in the Western world. Chronic prenatal ethanol exposure (CPEE) may be a risk factor for ethanol abuse and altered responsiveness to nicotine in postnatal life. Previous studies in our laboratory have utilized maternal oral administration of a high-dose (4 g ethanol/kg maternal body weight/day) ethanol regimen that induces structural and functional deficits in the fetus and postnatal offspring. The objective of this thesis was to test the hypotheses that moderate CPEE produces in postnatal offspring: (i) structural and functional teratogenic effects; (ii) increased ethanol preference; (iii) altered responsiveness to acute nicotine; and (iv) increased nicotinic acetylcholine receptors (nAChR) in forebrain structures, namely the hippocampus and frontal cortex. Pregnant Dunkin-Hartley-strain guinea pigs were given 24-h access to aqueous ethanol solution (5%, v/v) sweetened with sucralose (1g/L) or aqueous sucralose solution (1g/L) throughout gestation. Spontaneous locomotor activity in an open field was measured in offspring on postnatal day (PD) 10. Beginning around PD 40, ethanol preference in the offspring was determined using a two-bottle-choice paradigm. Each animal was given 2-h daily access to aqueous ethanol solution (0 - 3%, v/v) and water over 33 days of testing. Subsequently, hippocampal and frontal cortical tissues were obtained for the measurement of nAChR population by radioligand binding. Moderate maternal consumption of the aqueous ethanol produced growth restriction in postnatal offspring of both sexes, and increased spontaneous locomotor activity in male offspring only. These postnatal outcomes are similar to the teratogenic effects produced by a high-dose, binge-type ethanol regimen. Compared with control, offspring from mothers that consumed ethanol throughout gestation exhibited greater preference for aqueous ethanol, and a decrease in the concentration of nAChRs in the frontal cortex, but not the hippocampus. These data demonstrate that, in the guinea pig, moderate maternal consumption of ethanol is a useful model for studying ethanol neurobehavioural teratogenicity; and chronic prenatal exposure to ethanol enhances ethanol preference in young adult offspring and altered expression of nAChRs in the frontal cortex.

CO-AUTHORSHIP

The research described in this thesis was conducted under the supervision of Dr. James N. Reynolds and Dr. James F. Brien by Kayla M. Shea. Matthew Jefkins and Rebecca Grunwald assisted with the experiments in Chapter 3.4 - 3.6. Elyse Michaud and Kayla Shea collaborated on experiments in Chapter 3.8 - 3.9.

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LIST OF ABBREVIATIONS AND SYMBOLS

5-HT	serotonin
ACh	acetylcholine
ADHD	attention-deficit hyperactivity disorder
ad libitum	at will
AEE-SI	adolescent ethanol exposure plus social isolation
ANOVA	analysis of variance
ARND	alcohol related neurodevelopment disorder
BEC	blood ethanol concentration
BGS	brain growth spurt
BSA	bovine serum albumin
BW	body weight
CNS	central nervous system
CORT	corticosterone
CPEE	chronic prenatal ethanol exposure
DA	dopamine
DID	drinking in the dark
dL	decilitre(s)
EDTA	ethylenediaminetetraacetic acid
et al.	et alia, and others
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorders
g	gram(s)
g	gravitational force
GD	gestational day
GH	group housed
h	hour(s)
HCl	hydrochloric acid
HPA	hypothalamic–pituitary–adrenal
HPG	hypothalamic-pituitary-gonadal
i.g.	intragastric
i.p.	intra peritoneal
kg	kilogram(s)
L	litre(s)
LTP	long term potentiation
mg	milligram(s)
mM	millimolar
mm	millimetre(s)
min	minute(s)
n	number of determinants
NA	nucleus accumbens
nAChR	nicotinic acetylcholine receptor(s)
nM	nanomolar
NP	non-alcohol preferring
P	alcohol preferring

p	statistical probability
PD	postnatal day
pH	percent hydrogen
pFAS	partial fetal alcohol syndrome
s	second(s)
s.c.	subcutaneous
SEM	standard error of the mean
SI	social isolation
VTA	ventral tegmental area
α	alpha
β	beta
^{o}C	degree Celsius
μL	microlitre(s)
μM	micromolar
/	per
[^{3}H]	tritium
v/v	volume per volume
w/v	weight per volume
±	plus or minus
%	percent
μ	micro
<	less than
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Chapter 1: General Introduction

Ethanol is a known teratogen that results in structural and functional defects in offspring exposed during gestation, via maternal consumption of alcoholic beverages, including neurobehavioural problems resulting from central nervous system (CNS) injury (Jones and Smith, 1973; Spohr et al., 1993). In the human, the multifaceted structural and functional manifestations of ethanol teratogenicity are referred to collectively as fetal alcohol spectrum disorders (FASD) (Koren et al., 2003). The most severe human manifestation is fetal alcohol syndrome (FAS), comprising growth restriction, craniofacial dysmorphology and persistent CNS dysfunction (Jones and Smith, 1973). The CNS injury can manifest as behavioural and cognitive deficits involving learning and memory, attention, executive function, visuospatial abilities and emotional management (Steinhausen et al., 1993; Steinhausen and Spohr, 1998). FASD has an incidence of 9-10 cases per 1000 live births (Koren et al., 2003; Lupton et al., 2004) and is the leading preventable cause of mental deficiency in the Western world (Abel and Sokol, 1987).

The guinea pig is a key mammalian animal species used to study ethanol neurobehavioural teratogenicity because it's prenatal brain development, including the brain growth spurt (Dobbing and Sands, 1979), and the pharmacokinetics of ethanol in the maternal-fetal unit are similar to the human situation (Clarke et al., 1988a). The guinea pig has been used extensively in our laboratory to study ethanol neurobehavioural teratogenicity, and the structural and functional deficits produced in offspring after high-dose (4g/kg maternal body weight) chronic prenatal ethanol exposure (CPEE) are well characterized (Abdollah et al., 1993; Catlin et al., 1993). Maternal volitional ethanol consumption instead of oral intubation of high-dose ethanol has been used recently in the mouse to study ethanol neurobehavioural teratogenicity because there is decreased risk of malnutrition, decreased maternal stress and increased maternal care with this paradigm while producing similar postnatal teratogenic outcomes (Allan et al., 2003). At the outset of this thesis research project, the teratogenic effects of moderate CPEE in guinea pig offspring were unknown.

CPEE is a risk factor for ethanol consumption in postnatal life in the human (Baer et al., 1998, 2003; Yates et al., 1998), rat and mouse (Chotro et al., 2007). The underlying mechanism(s) for CPEE-induced increased ethanol preference in offspring is unknown, but may involve nicotinic cholinergic receptors (nAChRs) in the CNS. These receptors are located throughout the CNS, and are important in learning and memory along with the stimulating and reinforcing effects of ethanol (Larsson and Engel, 2004). In the human, tobacco smoking and ethanol drinking are directly related; over 80 percent of alcoholics smoke tobacco (Grant et al., 2004). CPEE alters the postnatal response to nicotine, including altered nicotine-induced locomotor activity (Nagahara and Handa, 1999b). Despite the known CPEE-induced alterations in postnatal response to nicotine, the effect of CPEE on nAChRs had not been previously studied before this research.

In the rat, adolescent ethanol exposure (AEE) and social isolation (SI) appear to be risk factors for increased volitional ethanol consumption (Spear, 2002; Siciliano and Smith, 2001; Wolffgramm and Heyne, 1991). The effects of SI and AEE on volitional ethanol consumption in the guinea pig were unknown before this thesis research.

The main goal of this thesis research was to test the hypothesis that moderate maternal intake of ethanol by the pregnant guinea pig induces in the offspring: neurobehavioural teratogenicity, increased ethanol preference mediated by CNS nAChRs, increased nAChRs in the CNS and altered response to acute nicotine. Furthermore, a separate study was conducted to determine if SI and AEE plus SI increases ethanol preference in the adult female guinea pig.

Chapter 2: Literature Review

2.1. Fetal Alcohol Spectrum Disorders

Ethanol is a teratogen: CPEE via maternal consumption of alcoholic beverages during pregnancy is known to produce structural and functional defects in the offspring. The link between ethanol and negative birth outcomes is referred to throughout history (reviewed in Calhoun and Warren, 2007). In fact, Aristotle famously stated that ‘‘Foolish, drunken and harebrained women most often bring forth children like unto themselves, morose and languid’’ (Krous, 1981). William Sullivan in 1899 concluded that alcohol had a direct toxic effect on the embryo (Golden, 2005) in an epidemiological study of 600 children of female alcoholic prisoners. Despite this observation, it was not until 1968 that ethanol developmental toxicity was reported in the medical literature (Lemoine et al., 1968). In this article, physical and behavioural problems were documented in over 100 children of women who drank heavily during pregnancy (Lemoine et al., 1968). It was not until the early 1970s that fetal alcohol syndrome (FAS) was introduced as a clinical term for children affected by ethanol teratogenicity. The diagnostic criteria established for FAS were a triad of principal features including CNS dysfunction, growth deficiency, and craniofacial dysmorphology (Jones et al., 1973; Jones and Smith, 1973). The craniofacial dysmorphology (Figure 2.1) includes thin upper lip, short palpebral fissures, epicanthal folds, micrognathia and flattened midface (Sampson et al., 1997). CNS dysmorphology and dysfunction constitute the most debilitating manifestations of FAS,

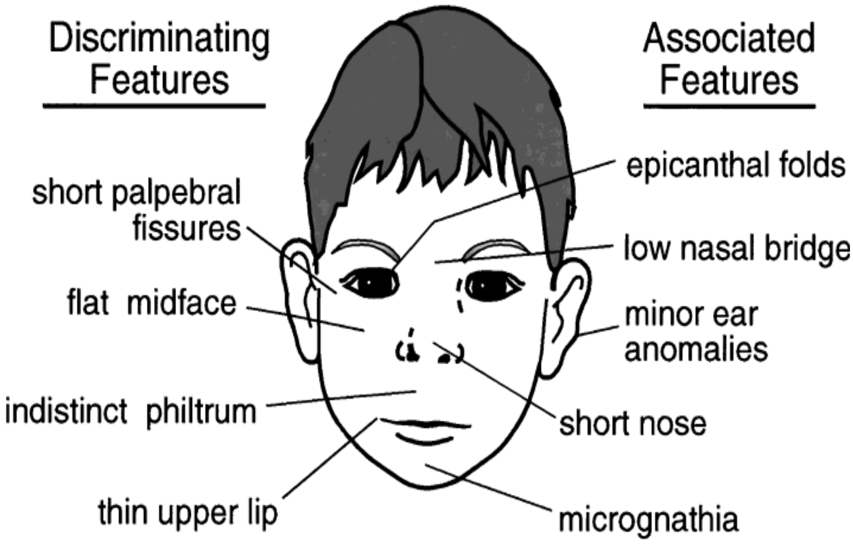


Figure 2.1. Craniofacial dysmorphology of the FAS phenotype in the young child (Modified Sampson et al., 1997).

as these persist throughout postnatal life (Spohr et al., 1993) and can manifest as intellectual, neurological and behavioural abnormalities (Steinhausen et al., 1993; Steinhausen and Spohr, 1998).

More recently, the term fetal alcohol spectrum disorders (FASD) was introduced as an umbrella term to include all children affected by prenatal ethanol exposure, including people with some, but not all, of the principle diagnostic features of FAS (Koren et al., 2003; Lupton et al., 2004). These children may not exhibit the craniofacial dysmorphology or growth restriction, but still have the neurobehavioural problems resulting from CNS injury. Other diagnostic terms in the FASD spectrum include partial FAS (pFAS) and alcohol related neurodevelopment disorder (ARND) (Abel, 1984). One of the brain regions that is a target of ethanol teratogenicity is the hippocampus (Butters et al., 2000). CPEE, via maternal consumption of ethanol, can alter hippocampal synaptic plasticity including long term potentiation (LTP) and can adversely affect hippocampal-dependent learning, as well as cause selective loss of hippocampal CA1 pyramidal cells (Berman and Hannigan, 2000; McGoey et al., 2003; Mattson and Riley, 1998).

Ethanol neurobehavioural teratogenicity is the leading cause of preventable mental deficiency in the Western world (Abel and Sokol, 1987). It is estimated that the incidence of FASD is between 9-10 cases per 1000 live births (Koren et al., 2003; Lupton et al., 2004). In certain regions of Canada, especially among First Nations' communities, the incidence of FASD ranges from 100-200 per 1000 live births (Chudley et al., 2005). The cost of FASD annually to Canada is over 340 million dollars (Stade et al., 2009). In 2002 in the United States, medical care, special education, residential care, and lost productivity associated with FAS was estimated to cost two million dollars per diagnosis

(Lupton et al., 2004). It is clear therefore that FASD is a major public-health problem that needs to be addressed comprehensively. These clinical and epidemiological data demonstrate the severity and magnitude of this public-health problem in countries where ethanol is a major lifestyle factor and the leading cause of drug-induced mental deficiency (Abel and Sokol, 1987).

2.2. Animal Model of Ethanol Neurobehavioural Teratogenicity

The guinea pig is a suitable mammalian species to use in studies of ethanol neurobehavioural teratogenicity because the pharmacokinetics of ethanol in the maternal-placental-fetal unit of the guinea pig are similar to those of the human, in that maternal hepatic biotransformation of ethanol plays a major role (Clarke et al., 1986, 1988a; Martensson, 1984). The timing of the brain growth spurt (BGS) is advantageous in the guinea pig. The BGS comprises a period of rapid brain growth, including proliferation and migration of neural cells, axonal elongation, dendritic arborization and synaptogenesis, and the BGS is sensitive to the neurotoxic effects of ethanol (Dobbing and Sands, 1979). The BGS has different ontogenic timing in the human and other mammalian species used to study ethanol neurobehavioural teratogenicity. In the human, the BGS is perinatal; in the guinea pig it occurs during the last third of gestation; and in the rat or mouse the BGS occurs in early postnatal life up to postnatal day (PD) 10 (Figure 1.2) (Dobbing and Sands, 1979). In the rat or mouse, only the direct (neonatal target site) effect of ethanol on the BGS can be studied, whereas in the guinea pig, the direct (fetal brain site) and indirect (maternal and placental target sites) effects of maternal ethanol administration on fetal brain development, including the BGS, and the

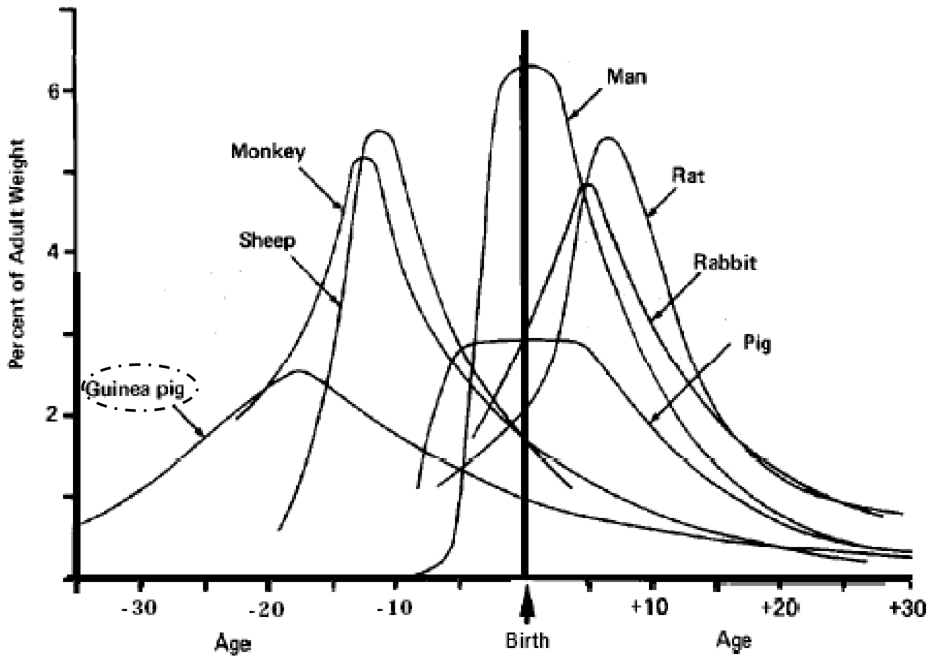


Figure 2.2: Brain growth spurt of various mammalian species. The brain growth spurt is presented as a first-order velocity curve for brain weight gain as a percentage of adult brain weight with respect to age. The unit of time on the abscissa is day for the guinea pig, 5 days for sheep, 4 days for monkey, months for human, days for rats, weeks for pig and 2 days for rabbit. (Modified from Dobbing and Sands, 1979).

consequent neurobehavioural outcome throughout postnatal life can be studied, which is more relevant to the human situation.

The guinea pig model of ethanol neurobehavioural teratogenicity is well established and commonly used in our laboratory. In this model, timed pregnant animals are administered orally 4g ethanol/kg maternal body weight (BW)/day by intubation into the oral cavity, in two equally divided doses given two hours apart. The apparent peak maternal blood ethanol concentration (BEC) at one hour after the second divided dose is about 250 mg/dL (Catlin et al., 1993; Nash et al., 2007). In this model, CPEE neonates have a smaller birth weight compared with controls (isocaloric-sucrose/pair-fed (nutritional) control and water-treated (handling) control) which reflects the growth restriction seen in the human (Abdollah et al., 1993). CPEE male and female offspring are hyperactive when tested in the open field (Bailey et al., 1999, 2001; Abdollah et al., 1993). This hyperactivity is ethanol dose-dependent with higher maternal BEC associated with greater increases in spontaneous locomotor activity. (Catlin et al., 1993). Hyperactivity is reliably observed in other mammalian animal models of ethanol teratogenicity (Gilbertson and Barron et al., 2005).

2.3. Moderate Ethanol Exposure to Study Ethanol Neurobehavioural Teratogenicity

Animal studies of ethanol neurobehavioural teratogenicity typically involve pregnant animals consuming an ethanol-containing liquid diet (Chernoff, 1977; Middaugh et al., 1988; Zhou et al., 2003), or receiving ethanol via intragastric (i.g.) intubation (Boehm et al., 1997; Gilliam et al., 1989). Some animal models of ethanol neurobehavioual teratogenicity involve maternal volitional ethanol consumption instead of oral intubation of high dose ethanol or ingestion of ethanol-containing liquid diet (Allan et al., 2003). There are several reasons for this shift in animal models. In liquid diet models, the maternal BEC is typically not high (83 mg/dL) in pregnant animals (Sutherland et al., 1997) but there is a risk of malnutrition during gestation (Allan et al., 2003). In studies using i.g. administration of high-dose ethanol there is maternal stress of varying magnitude, which can confound fetal and postnatal outcomes (Slone and Redei, 2002; Ward and Wainwright, 1989a). In the behavioural assessment of offspring, the quality of maternal care can influence the measured outcomes. Pregnant animals that consumed high-dose ethanol exhibit diminished maternal care of their offspring when they still receive ethanol or when ethanol is removed (due to withdrawal effects) following parturition (Allan et al., 2003). Cross-fostering of the offspring is often used to decrease the difference in maternal care between treatment and control groups but can confound behavioural testing (Allan et al., 2003; Ward and Wainwright, 1989b).

A model of ethanol neurobehavioural teratogenicity using maternal volitional consumption was established in the mouse using sweetened ethanol solution in a free-choice paradigm (Allan et al., 2003). The animals had 22-hour free access to 0.66 % (w/v) aqueous saccharin solution with or without 10% (v/v) ethanol. The animals consumed up to 14g ethanol/kg maternal BW/day in their home cage. This model is beneficial because the animals drink ethanol voluntarily throughout pregnancy, provide appropriate maternal care of their offspring and have a lower mortality rate. The offspring with CPEE still display the same neurobehavioural effects observed in previous mouse models, including increased exploration of novel objects and decreased contextual fear (Allan et al., 2003).

This model has been developed in the rat and expanded further in the mouse such that there currently are several models available for study (reviewed in Green and Grahame, 2008). For example, some research studies involve drinking in the dark (DID) models, in which the pregnant dams have restricted access to ethanol during the dark phase, when nocturnal animals are active, to increase the dose of ethanol consumed in a short time period (binge-like exposure) (Boehm et al., 2008; Crabbe et al., 2009; Rhodes et al., 2005, 2007)

Recent studies in the pregnant guinea pig used a drinking paradigm, in which animals were given free access to 4% (v/v) aqueous ethanol solution as their sole source of fluid throughout the third-trimester-equivalent period (Gauthier et al., 2005, 2009; Ping et al., 2007). The maternal and fetal serum ethanol concentrations were 50 ± 0.01 mg/dL (Gauthier et al., 2005), which is below the legal limit of 80 mg ethanol/ 100 ml blood for intoxication for driving a motor vehicle in Canada. Pregnancy outcomes including birth weight were not reported; hence it is unclear whether structural and functional manifestations of ethanol neurobehavioual teratogenicity demonstrated in our laboratory for maternal administration of high-dose ethanol, were produced in this model of moderate maternal ethanol consumption.

2.4. Prenatal Ethanol Exposure as a Risk Factor for Postnatal Ethanol Abuse

2.4.1. Clinical Evidence

The interaction between environmental influences and genetic factors as predictors of substance-abuse disorders has been studied using a variety of approaches. Family, twin, adoption and molecular biology studies all indicate that there is a strong genetic component in the development of drug abuse and drug dependence (Camí and Farré, 2003). Another component that may play an important role in the development of substance-abuse disorders is prenatal exposure to ethanol and/or other drugs (Spear and Molina, 2005). In humans, it is difficult to determine the relative roles of genetic and environmental factors in the etiology of substance abuse. Until recently, prenatal drug exposure has not been considered as a causal factor. It is well established that ethanol consumption early in adolescence is associated with abuse of ethanol and other drugs later in life (Spear, 2002). Taking this concept further, Spear and Molina (2005) have postulated that the use of ethanol in preadolescence or adolescence is a consequence of still earlier exposure to the pharmacological effects of ethanol, most notably during prenatal life. In the first comprehensive study Baer et al. (1998) assessed the relative importance of prenatal ethanol exposure and family history of alcoholism for predicting ethanol use. Prenatal ethanol exposure was found to be a significant predictor of drinking patterns, including the average number of drinks consumed per occasion, age of first intoxication, ethanol dependence, and negative consequences of ethanol use at 14 and 21 years of age (Baer et al., 1998, 2003). Similarly, Yates et al. (1998), in a study of 197 adoptees, found that prenatal ethanol exposure is a risk factor for abuse of ethanol, nicotine and other drugs by offspring, and that the impact of prenatal ethanol exposure on the development of drug abuse disorders is independent of genetic and other environmental factors. The studies by Yates et al. (1998) and Baer et al. (1998, 2003) support the notion that moderate-to-high ethanol consumption by the mother during pregnancy is as reliable or more reliable for predicting ethanol intake by offspring compared with a family history of alcoholism.

2.4.2. Evidence from Animal Studies

Increased self-administration of ethanol by adult offspring after prenatal ethanol exposure was first reported in animal studies using rats (Bond and DiGusto, 1976). Subsequent studies in the rat and mouse confirmed this finding (Pepino et al., 1999; Randall et al., 1983), demonstrated no change (Abel and York, 1979; McGivern et al., 1984) or only a limited increase (Hilakivi, 1986; Hilakivi et al., 1987; Reyes et al., 1985) in ethanol preference in adult offspring after prenatal ethanol exposure (reviewed in Chotro et al., 2007). These discrepancies are likely the result of differing experimental methods used in the studies. Variables that are likely to affect ethanol preference after prenatal ethanol exposure are sex of the offspring, age at testing, conditions of testing and, most importantly, the timing and magnitude of ethanol exposure during development (Chotro et al., 2007). Animal studies of the role of prenatal ethanol exposure in ethanol preference in postnatal life have used maternal high-dose ethanol consumption during gestation, ethanol consumption throughout gestation and lactation, or binge-like ethanol consumption during pregnancy (reviewed by Chotro et al., 2007). There are relatively few published studies in which the pregnant animal consumed a low-to-moderate dose of ethanol throughout the day, and adult offspring were tested for ethanol preference (reviewed in Chotro et al., 2007). In one study in the rat, pregnant dams consumed 5% (w/v) aqueous ethanol solution throughout gestation and lactation, and the offspring exhibited an increase in ethanol preference when tested with 5% (w/v) aqueous ethanol at 28 days of age (Buckalew, 1979). In another rat study, when the pregnant dams consumed 4% (w/v) aqueous ethanol as the sole liquid source throughout gestation, there was no increased preference by offspring for 8% (w/v) ethanol, but there was an ethanol preference in the offspring if the dams continued to consume ethanol solution until weaning (Honey and Galef, 2003).

The limited number of studies in the pregnant rat with moderate ethanol exposure only throughout gestation involved binge-like ethanol consumption with ethanol administration via i.g. intubation (Chotro and Aries, 2003; Aries and Chotro 2005a,b). The maternal BEC in these studies was not reported, but would likely be much higher than the 50 mg/dL ethanol concentration in the pregnant guinea pigs that consumed 4% (v/v) aqueous ethanol solution (Gauthier et al., 2005).

2.4.3. Potential Mechanisms of Increased Ethanol Preference

The underlying mechanism of increased ethanol preference in offspring induced by exposure to ethanol has not been completely elucidated (Abate et al., 2008). Spear and Molina (2005) postulate that increased ethanol preference after CPEE could be a result of familiarity with it as a consequence of prenatal ethanol exposure or due to the positive reinforcing effects of ethanol learned through conditioning. Indeed, prenatal ethanol exposure induces fetal learning about ethanol through classical conditioning involving the chemosensory or pharmacological properties of ethanol (reviewed in Chorto and Arias, 2006; Youngentob and Glendinning, 2009). Rats can acquire sensory cues and can have memory of prenatal experience with ethanol when exposed during the last few days of fetal life, as seen by increased response to ethanol flavour (Chotro and Spear, 1997). Infant rats process the chemosensory effects of ethanol during the course of intoxication induced by i.g. administration of ethanol in doses that range between 1.5 and 3.0 g/kg BW (Molina and Chotro, 1989; Molina et al., 1989). Rats exposed to ethanol via maternal administration during prenatal life (1 or 2 g/kg maternal BW) manifest an increased intake of ethanol and increased appetitive responses to the taste of ethanol when tested on PD 14-15 (Arias and Chotro, 2005). This may be, at least in part, mediated through the endogenous opioid system, as rats given prenatal exposure to ethanol and naloxone together do not have an increased ethanol preference or increased appetitive response to the taste of ethanol (Chotro and Arias, 2006). Another study in the rat demonstrated that increased ethanol preference can be induced by making ethanol smell and taste better (Youngentob and Glendinning, 2009). In this study, prenatal ethanol exposure was associated with increased acceptability of ethanol taste and smell in adult offspring, although this study was unable to determine if there was a positive (increased preference) or negative (decreased aversion) reinforcement of the olfactory acceptance of the ethanol by the offspring.

The human neonate displays an apparent memory of, and enhanced responsiveness to, ethanol odour (Faas et al., 2000). Offspring of mothers reporting moderate ethanol intake during pregnancy (mothers who consumed alcohol at least once a week, reaching a level of ingestion equivalent to 22.1 ± 2.4 g of ethanol per occasion) during pregnancy displayed increased reactivity (head and facial movements) to ethanol, but not lemon, odour when it was presented during the first 1-2 or 7-14 days after birth (Faas et al., 2000). Along with increased reaction to the ethanol odour, infants born to moderate drinkers exhibited higher hedonic reactions towards ethanol as measured by smiling, suckling, and licking. There were no differences in the positive responses or aversive gestures (gaping and nose and eye wrinkling) promoted by lemon (Faas et al., 2000). None of the women in this study had binge-like drinking episodes or chronic patterns of ethanol consumption based on their ethanol intake patterns. There was no difference in the birth-related morphologic and neurobehavioral parameters in the offspring from mothers that consumed ethanol as compared to control offspring.

2.5. Ethanol and Nicotine

2.5.1. Nicotinic Acetylcholine Receptors in the Central Nervous System

CNS nAChRs are ligand-gated ion channels (Figure 2.3) that belong to the superfamily of neurotransmitter receptors including $GABA_A$, glycine and serotonin (5-HT_3) receptors (Gotti and Clementi, 2004). Neuronal nAChRs consist of heteromeric or homomeric combinations of α (α2- α10) and β (β2- β4) subunits, and these receptors have different functional properties based on their subunit composition (Gotti et al., 2007). The dominant nAChR isoform consists of α and β subunits usually in an $(\alpha4)_2(\beta2)_3$ stoichiometry (Picciotto et al., 2000). Over 90% of CNS nAChRs contain α4 and β2 subunits (Romanelli et al., 2007) and these receptors are involved in memory, attention and information processing (Picciotto et al., 2000; Court et al., 1997; Court and Clementi, 1995). Ethanol can act on nAChRs by binding to and stabilizing them in the open state or by stimulating burst frequency by increasing ion-channel opening rates (Wu et al., 1994; Liu et al., 1994). Ethanol also increases the response to nicotine by $\alpha_2\beta_2$, $\alpha_3\beta_2$ and $\alpha_4\beta_2$ nAChRs (Davis and de Fiebre, 2006).

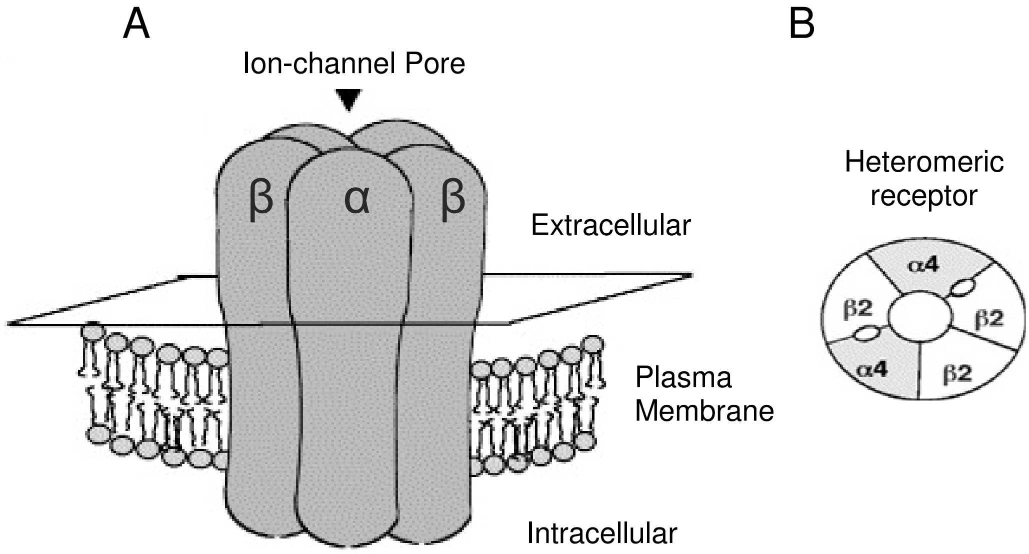


Figure 2.3. Structure and cellular localization of nAChRs in the CNS. (A) Pentameric arrangement of an assembled nAChR showing the ion-channel pore. (B) Subunit arrangement in the heteromeric α4β2 nAChR isoform. (Modified from Gotti and Clementi, 2004).

2.5.2. Pharmacological Interactions between Ethanol and Nicotine

Tobacco smoking and ethanol drinking are highly correlated in the human population. Recent estimates suggest that 80-90% of alcoholics smoke, and that 70% of alcoholics are heavy smokers, whereas only 10% of the general population is classified as heavy smokers (Grant et al., 2004; DiFranza and Guerrera, 1990). Smokers consume twice as much ethanol as non-smokers (Carmody et al., 1985). Administration of either ethanol or nicotine, a major psychoactive component in tobacco, activates the mesolimbic dopaminergic system, leading to increased dopamine (DA) release in limbic structures such as the nucleus accumbens (NA) (Larsson and Engel, 2004). Chronic nicotine exposure leads to an upregulation of nAChRs, through stabilization of the receptor with slow desensitization and increased sensitivity to acetylcholine (Vallejo et al., 2005), which is modulated by ethanol (Dohrman and Reiter, 2003). Mecamylamine, a noncompetitive and nonselective CNS-acting nAChR antagonist (Shytle et al., 2002), decreases: (i) ethanol-induced locomotor activity stimulation in mice; (ii) ethanol-induced DA release in mice and rats; (iii) ethanol self-administration in a high ethanol-preferring line (P) of rats ; (iv) ethanol self-administration in P rats when infused directly into the ventral tegmental area (VTA), which contains the neuronal cell bodies of the mesolimbic dopaminergic system (Larsson and Engel, 2004). In mice, mecamylamine reduces ethanol consumption in high-ethanol consuming animals in a dose-dependant manner, while leaving sucrose consumption intact (Hendrickson et al., 2009). Mecamylamine also attenuates the stimulant and euphoric affects of ethanol in human social drinkers, and decreases the self-reported desire to consume ethanol (Chi and de Wit, 2003).

2.5.3. Prenatal Ethanol Exposure and Postnatal Response to Nicotine

In the rat, prenatal and neonatal exposure to ethanol alters the behavioural response to nicotine in postnatal life, manifesting as decreased nicotine-induced stimulation of spontaneous activity (Nagahara and Handa, 1999b) and attenuation of nicotine-induced memory enhancement (Nagahara and Handa, 1999a). When neonatal rats are given ethanol, there is an increased nicotine-induced analgesia and place-conditioning (Rogers et al., 2004). Rat offspring exposed to ethanol, nicotine or ethanol plus nicotine throughout gestation and lactation have differential responses to nicotine in postnatal life (Matta and Elberger, 2007). Rat offspring with prenatal and neonatal exposure to ethanol plus nicotine had (i) faster initiation of acquisition of nicotine self-administration, (ii) greater percentage of animals acquiring nicotine self-administration, and (iii) higher nicotine intake compared with saline self-administration (Matta and Elberger, 2007). Rats that received prenatal and neonatal nicotine alone or ethanol alone did not differ from controls in acquisition of nicotine self-administration.

The timing of developmental drug exposure is important in regard to the long-term changes in response to nicotine (Gilbertson and Barron, 2005). Nicotine-induced hyperactivity occurs in rats if the animals have been exposed to ethanol or ethanol plus nicotine in the first week of post-natal life. In contrast, if the exposure to ethanol, or ethanol plus nicotine is delayed to the second postnatal week, hypoactivity occurs in response to nicotine (Gilbertson and Barron, 2005).

It has been proposed that, if similar effects occur in the human, then prenatal ethanol exposure may increase the risk for the rewarding effects and dependence liability of nicotine later in postnatal life (Rogers et al., 2004). It seems reasonable, therefore, to speculate that CPEE may change the nature of the interaction between ethanol and CNS nAChR function in postnatal life, thereby increasing ethanol preference and ethanol self-administration. Both increases and decreases in the behavioural response to nicotine have been reported as a consequence of prenatal and/or perinatal exposure to ethanol, and thus it is difficult to predict in which direction the population of nAChRs might be altered by CPEE. However, changes in the response to nicotine and increased self-administration in CPEE offspring suggest the possibility that there may be an increase in the number of CNS nAChRs. Despite extensive evidence that demonstrate that prenatal ethanol exposure alters response to nicotine in postnatal life, the effects of CPEE on CNS nAChR expression and function have not been studied.

2.6. Factors Associated with Increased Consumption of Ethanol

2.6.1. Social Isolation Increases Ethanol Consumption

Social isolation has been shown to increase ethanol self-administration in rats by as much as 30% (Wolffgramm and Heyne, 1991), which is associated with irreversible changes in dopamine D1 receptor-mediated neurotransmission (Wolffgramm and Heyne, 1995). In a study of ethanol self-administration in P and NP rats, social isolation selectively increased volitional ethanol intake in P rats (Ehlers et al., 2007), indicating that a significant interaction occurs between the genetic liability to excessive ethanol consumption and specific environmental experiences, to promote ethanol drinking behaviour. In a recent study in Long-Evans rats, ethanol preference after social isolation was examined using operant self-administration to distinguish between the effects of isolation rearing on ethanol seeking- and drinking-related behaviors (McCool and

Chappell, 2009). During operant self-administration, isolation housing increased the response rate and ethanol consumption selectively, without increasing consumption of or response rate to sucrose (McCool and Chappell, 2009). These data indicate that social isolation leads to increased ethanol consumption specifically, and not to a general increase in consummatory behaviour.

2.6.2. Adolescent Ethanol Exposure Increases Ethanol Preference

Early onset ethanol drinking is related to the use of alcohol in adulthood both in men and women (Pitkänen et al., 2005). When ethanol consumption is initiated before the age of 16, the risk of heavy drinking in adulthood is greatly increased. Importantly, emotional behaviour and school success at eight years of age did not predict the age of onset of drinking.

In animal studies, adolescent ethanol consumption is associated with ethanol abuse in adulthood (Spear, 2002; Siciliano and Smith, 2001), especially in male rats (Siciliano and Smith, 2001). The mechanism for this is unknown. Adolescence is a critical period during which excessive ethanol exposure can act on the emerging personality and, perhaps, on the rapidly developing adolescent cerebral cortex. Adolescent substance abuse is related to long-lasting brain injury, altered cognitive capabilities, electrophysiologic changes and/or hormonal effects that alter sexual maturation (Smith, 2003).

2.7. Research Rationale, Hypothesis and Objectives

The guinea pig is a good animal model to study ethanol neurobehavioural teratogenicity. Previous studies conducted in the guinea pig using a daily dose of 4 g ethanol /kg maternal BW, administered throughout gestation, reliably produces structural and functional deficits. Animal models in other species using more moderate prenatal ethanol exposure generally produce similar defects as compared to high ethanol exposure, with less maternal stress, less nutritional deficits and better maternal care.

Prenatal ethanol exposure is a risk factor for increased ethanol preference and altered responsiveness to nicotine in postnatal life, which may be related to changes in the nAChRs. Social isolation and adolescent ethanol exposure are also known to increase ethanol preference.

This thesis tested the following hypotheses:

1. Moderate maternal ethanol intake is sufficient to produce ethanol neurobehavioural teratogenicity.
2. CPEE, via moderate maternal ethanol intake, results in increased preference for and self-administration of ethanol in a two-bottle-choice paradigm in offspring.
3. CPEE, via moderate maternal ethanol intake, increases the number of nAChRs in the hippocampus and prefrontal cortex in offspring.
4. CPEE, via moderate maternal ethanol intake, leads to altered response to nicotine in postnatal life in offspring.
4. Social isolation and adolescent ethanol exposure increase ethanol preference in the adult female guinea pig.

These hypotheses were tested in the guinea pig according to the following research objectives, which were to:

1. Develop a model of ethanol neurobehavioural teratogenicity using moderate maternal ethanol consumption.

2. Characterize physical and behavioural ethanol teratogenicity in offspring including decreased birth weight and increased spontaneous locomotor activity.
3. Determine volitional ethanol consumption in young adult CPEE offspring and the effect of mecamylamine, a CNS nAChR antagonist, on this behaviour.
4. Determine CPEE-induced changes in the nAChR population in the prefrontal cortex and hippocampus.
5. Determine the effects of social isolation and adolescent ethanol exposure on volitional ethanol consumption.

Chapter 3: Methods

3.1. Experimental Animals and Reagents

Female nulliparous Dunkin-Hartley-strain guinea pigs (500-800 g; Charles River Canada, St. Constant, QC) were mated with males of the same strain using an established protocol (Elvidge, 1972). Gestational day (GD) 0 was defined as the last day of full vaginal-membrane opening, where term is around GD 68. Vaginal-membrane status, body weight and general health were monitored daily throughout gestation. Animals were housed in separate cages with a 12-h/12-h light/dark cycle with lights on starting at 0800 h, at an ambient temperature of 23^{o}C. Offspring were weighed and monitored daily for general health and at PD 15-17 were weaned from their mothers and separated by sex. All animals were cared for according to the principles and guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the Queen's University Animal Care Committee.

Reagent grade tris-hydrochloric acid (HCl), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), mecamylamine and (-) – nicotine hydrogen tartrate salt were purchased from Sigma Chemical Co Oakville, ON. Sucrose and SX18-4 Scintiliverse scintillation cocktail were purchased from Fisher Scientific (Unionville, ON). - [^{3}H]- nicotine (specific activity, 75 Ci/mmol) was purchased at Perkin-Elmer, Boston, MA. Anhydrous Ethyl Alcohol was purchased from Commercial Alcohols, Brampton, ON. Sucralose was from McNeil Consumer Healthcare, Guelph, ON. Xylazine hydrochloride (Rompun) was from Bayer Healthcare, Animal Health Division, Toronto ON, and Ketamine Hydrochloride was from Bioniche Animal Health Canada Inc, Belleville, ON (purchased from McGill University Animal Research Center, QC).

3.2. Chronic Treatment Regimens

Prior to mating, female guinea pigs were given 24-h/day access to either tap water sweetened with 1g/L sucralose (n=10) or an aqueous ethanol solution containing 1 g/L sucralose (n=10). To acclimatize the animals to consumption of ethanol, the concentration of ethanol in the aqueous sweetened solution was increased in a stepwise manner every three days starting at 0% (v/v) on day one and ending at the final treatment solution concentration of 5% (v/v) on day 30. During this time, food was available *ad libitum.* After the female animals had stabilized their consumption of the 5% (v/v) ethanol solution, they were mated, during which time they had access to both water and their treatment solution. The females were separated from the males on GD 1-2, and thereafter, they had *ad libitum* access to food and their treatment solution. After parturition, the ethanol and control solutions were diluted to 0.5 g/L sucralose with or without 2.5% (v/v) ethanol. On PD 2, the solutions were again diluted to 0.25 g/L sucralose with or without 1.25% (v/v) ethanol. On PD 3, the solutions were replaced with tap water.

3.3. Spontaneous Locomotor Activity

Spontaneous locomotor activity was measured in the offspring using an open-field apparatus (480 mm x 480 mm x 100 mm), equipped with two banks of infrared beams to measure movement in the horizontal and vertical planes, and a software acquisition system (TSE Actimot, Scientific Products and Equipment , Concord Ontario). The apparatus was housed in a quiet room, and testing was carried out between 0900 h and 1500 h in dimmed lighting. Animals were tested at PD 10 for a period of 30 min. The measured variables were time moving and distance traveled in the open field during 5-min intervals in the test session.

3.4. Ethanol Preference

Thirty-three offspring, eight males and eight females with CPEE and eight male and nine female control animals were chosen for ethanol-preference testing. Starting on PD 40, animals were tested for ethanol preference using a modified two-bottle-choice paradigm (Wang et al., 2003). In the home cage, food and water were removed for two h. Thereafter, each animal was weighed and transferred into a testing cage. Food and two drinking solutions were introduced and left for two h with *ad libitum* access. One of the drinking bottles contained water and the other an unsweetened aqueous ethanol solution. The concentration of ethanol was raised gradually, starting at 0% on testing days 1-3 and increased by 0.3% every three days, for a total of 30 days of testing, until a final ethanol solution of 3% (v/v). The side of the cage on which each bottle was placed was switched daily to avoid a side bias. The volumes of the two drinking solutions and the amount of food consumed were recorded daily. Ethanol preference was determined by calculating the total dose of ethanol consumed with the density of ethanol being 0.789 g/mL. Each animal was returned to its home cage at the end of the 2-h hour ethanol-preference-testing session.

3.5. Mecamylamine Administration and Ethanol Preference

The role of nAChRs in ethanol preference was tested in the guinea pig offspring after the two-bottle-choice experiment described above was completed around PD 70. Each animal received intraperitoneal (i.p.) injection of either 1 mg mecamylamine/kg BW, a CNS-acting, noncompetitive nAChR antagonist or saline vehicle (control), and was then tested for ethanol preference each day, using the two-bottle-choice experiment as described previously with an aqueous ethanol solution of 3% (v/v). Each animal received daily injection of saline for three days followed by daily injection of mecamylamine for three days.

3.6. Acute Nicotine Challenge and Spontaneous Locomotor Activity

Seventeen offspring, four male and four female guinea pigs with CPEE, and four male and five female animals from control litters, were tested with acute nicotine challenge. Spontaneous locomotor activity in the open field after acute nicotine challenge was used to determine the effect of CPEE on nicotine response and was performed as described in Chapter 3.3. Starting at about PD 40, individual animals were removed from their home cage, transported to a holding room, and then received subcutaneous (s.c.) injection of nicotine followed by measurement of spontaneous locomotor activity for 30 min using the method described above. Each animal received 0, 0.17, 0.35 or 0.7 mg nicotine/ kg BW. This procedure was repeated with the same dose of nicotine for three days, after which the animal was given a 48-h no-treatment period (drug elimination/ washout). Thereafter, spontaneous locomotor activity in response to acute nicotine challenge was repeated using a different dose of nicotine, until each animal in the study received all four doses (three days for each nicotine dose with a 48-h no-treatment period between each of the four different nicotine doses). The initial nicotine dose and the order of administration of the subsequent doses were randomly assigned. At the end of each testing day, the animal was transported back to its home cage.

3.7. [^{3}H]-Nicotine Receptor Binding

At about PD 100, the individual guinea pigs were euthanized, by s.c. injection of ketamine (50 mg/kg BW) and xylazine (5 mg/kg BW) followed by decapitation. The brain was rapidly excised, and the frontal cortex and hippocampus were isolated, weighed and flash-frozen in liquid nitrogen. The samples were stored at -80 ^{o}C until analyzed. The tissue was thawed on ice and homogenized in 10 volumes of buffer containing 10 mM Tris HCl, 0.3 M sucrose and 2 mM EDTA (pH 7.4). The homogenate was centrifuged at 3000 x *g* for 10 min at 4 ^{o}C. The supernatant was collected and centrifuged at 13000 x *g* for 20 min at 4 ^{o}C. The pellet was resuspended in the sucrose buffer and centrifuged at 13000 x *g* for 20 min at 4°C. After this wash step, the pellet was resuspended in assay buffer containing 50 mM Tris HCl (pH 8.0). The protein concentration was determined by the Bradford assay (1976) with BSA as the standard.

Binding assays were performed as described by Hellstrom-Lindahl et al., (1999) with modification. Briefly, 250-300 μg of membrane protein were incubated with 5 nM (-)-[^{3}H]-nicotine (specific activity, 75 Ci/mmol; Perkin-Elmer, Boston, MA) in a final volume of 0.5 ml at 4°C for 60 min. Non-specific binding was determined in the presence of 1 mM (-) nicotine (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). The reaction was terminated by vacuum filtration through pre-wetted Whatman GFB glass fibre filters, followed by three washes of the retentate on the filter with three 5-ml aliquots of ice-cold assay buffer. The filter was transferred to a vial containing 5 ml of SX18-4 ScintiVerse scintillation cocktail (Fisher Scientific, Unionville, ON) and the radioactivity was quantified by liquid scintillation spectroscopy using a Beckman LS 6500 scintillation counter. The assay was performed in triplicate for each sample.

3.8. Social Isolation, Adolescent Ethanol Exposure and Ethanol Preference

Female guinea pigs (300-600 g) were divided into three groups of eight animals each: (i) group-housed (control, GH), (ii) singly-housed (social isolation, SI), or (iii) adolescent ethanol exposure plus social isolation (AEE-SI). Female guinea pigs were selected because these animals were going to be used in another breeding study with volitional consumption of ethanol during pregnancy. The GH animals (400-600 g) were housed in pairs, whereas the SI animals (400-600 g) were housed individually. The AEE-SI animals (300- 400 g) received ethanol, via oral intubation of 1g ethanol/kg BW for two days in two equally divided doses, two hours apart, followed by 2 g ethanol/kg BW in two equally divided doses, two hours apart, for three days. Around PD 40, ethanol-preference testing was conducted using the two-bottle-choice experiments as described in Chapter 3.4, except that the food and water deprivation was four h.

3.9. Mecamylamine Administration and Ethanol Preference

The role of nAChRs in ethanol preference was tested in individual guinea pigs after the two-bottle choice experiments described in Chapter 3.8 were completed. Each animal received i.p. injection of either 0.5 mg mecamylamine/kg BW or saline vehicle (control), and was then tested for ethanol preference using the two-bottle-choice method as described above with an aqueous ethanol solution of 3% (v/v). Each animal received daily injection of saline for three days followed by daily injection of mecamylamine for three days.

3.10. Statistical Analysis

The data are presented as group means ± standard error of the mean (SEM) with the exception of the pregnancy outcome parameters, maternal death and spontaneous abortion, which are reported as the number of occurrences. Pregnancy outcome, birth weight and nicotine receptor binding data were analyzed by Student's *t*-test for unpaired data. Spontaneous locomotor activity at PD 10 and after acute nicotine challenge, and ethanol preference before and after mecamylamine administration were analyzed by two-way analysis of variance (ANOVA) followed by post-hoc *t*-test analysis with Bonferroni correction for multiple comparisons. All statistical analysis was performed using Prism 5 (GraphPad Software Inc., San Diego, CA). Two groups of data were considered to be statistically different when $p < 0.05$. Male and female offspring from each litter were studied in the experiments, and sex was included as a factor in the statistical analysis. If there was no statistically significant sex effect, the data for male and female animals in each experimental group were combined.

Chapter 4: Results

4.1. Animal Model of Ethanol Neurobehavioual Teratogenicity

4.1.1. Pregnancy Outcomes

The average maternal ethanol consumption was 2.2 g/ maternal kg BW, with a range 1.9 g/kg BW to 2.85 g/kg BW. The pregnancy outcome data are presented in Table 4.1. There was no difference in number of pups or the distribution of male and female offspring between the two treatment groups. In the ethanol treatment group, there was one spontaneous abortion and four instances of perinatal death, whereas in the control group, there was one maternal death and two instances of perinatal death. Maternal food consumption (Figure 4.1) was higher in the control group ($t_{18} = 2.9$, $p < 0.01$), and post-hoc analysis revealed that this occurred entirely in the first trimester of pregnancy ($t_{18} = 4.3$, $p < 0.001$, Figure 4.1). There was an effect of chronic maternal volitional ethanol consumption to decrease the length of gestation ($t_{18} = 2.9$, $p < 0.01$, Figure 4.2) and to decrease offspring body weight at PD 1($t_{70} = 6.2$, $p < 0.001$, Figure 4.3).

4.1.2. Spontaneous Locomotor Activity

Spontaneous locomotor activity was measured in PD 10 offspring (Figure 4.4). Two-way ANOVA revealed a significant interaction between treatment group (ethanol versus control) and sex ($F(1, 46) = 4.1$, $p < 0.05$). Post-hoc analysis demonstrated that the interaction was due to increased spontaneous locomotor activity (time moving (t_{46} =2.5, $p < 0.05$) and distance traveled ($t_{46} = 2.4$, $p < 0.05$)) selectively in the male offspring of pregnant guinea pigs that consumed 5% (v/v) aqueous ethanol solution throughout gestation. Spontaneous locomotor activity of female offspring was not different between the two treatment groups. Interestingly, control male offspring exhibited less spontaneous locomotor activity (time moving (t_{22} =3.0, $p < 0.01$) and distance traveled (t_{22} =3.0, $p < 0.01$) compared with control female offspring, and chronic maternal consumption of 5% (v/v) ethanol solution eliminated this sex-dependent difference.

4.2. Postnatal Ethanol Preference and Role of nAChRs

4.2.1. Ethanol Preference

In the two-bottle-choice experiment, offspring exposed to ethanol via chronic maternal ethanol consumption had higher ethanol preference, as demonstrated by the dose of ethanol consumed across the different concentrations of aqueous ethanol solution, starting on PD 40 and ending on PD 70 (Figure 4.5). Two-way ANOVA revealed main effects of treatment group (ethanol versus control) (F (1, 270) = 7.9, $p < 0.01$) and concentration of aqueous ethanol solution (F (8, 270) = 13.8, $p < 0.001$). For the dose of ethanol consumed, post-hoc analysis demonstrated that there was increased ethanol preference in the offspring of the maternal ethanol treatment group for the aqueous ethanol solutions with higher ethanol concentration, specifically 2.3, 2.6 and 3.0% (v/v) (t_{29} =2.6, $p < 0.05$; $t_{29} = 2.2$, $p < 0.05$; and $t_{29} = 2.3$, $p < 0.05$; respectively). There was also a significant main effect of sex on the total fluid consumption in the two-bottle-choice experiments (F (3, 261) = 9.4, p < 0.0001, Figure 4.6). Post-hoc analysis demonstrated that in both ethanol and control groups, male offspring consumed more fluid than female offspring (t_{16} = 7.0, p < 0.001; t_{16} = 2.9, p < 0.05, respectively). Ethanol females also consumed less fluid than control females (t_{16} = 4.4, p < 0.001), but total fluid consumption by male offspring of the two treatment groups was not different.

4.2.2. Role of nAChRs in Ethanol Drinking Behaviour

Mecamylamine, administered by i.p injection at a dose of 1mg/kg BW, decreased consumption of food (Figure 4.7). Two-way AVOVA revealed a significant main effect of mecamylamine (F (1, 58) = 73, p < 0.0001), and an interaction between treatment group (ethanol versus control) and mecamylamine administration (mecamylamine versus saline) (F (3, 58) = 4.1, p < 0.05), on food consumption. Two-way ANOVA also revealed a significant interaction between treatment group (ethanol versus control) and mecamylamine administration (mecamylamine versus saline) (F (3, 58) = 4. 181, p < 0.01) on water consumption. Post-hoc analysis revealed that the interaction was due to a selective decrease in total fluid intake in the control female offspring (mecamylamine versus saline, t = 3.7, p < 0.01). As mecamylamine produced a general decrease in food and fluid intake, the effects of this dose of drug on ethanol preference could not be determined.

4.3. Spontaneous Locomotor Activity Response to Acute Nicotine Challenge

The guinea pig offspring were tested for spontaneous locomotor activity after acute s.c. administration of nicotine to determine if CPEE altered this behavioural response. There was no difference between CPEE and control offspring in the spontaneous locomotor activity elicited in response to 0, 0.17, 0.35 or 0.7 mg nicotine/kg BW (Figure 4.8), as measured by total distance traveled (F (3, 60) = 1.25, p = 0.27) and time moving (F (3, 60) = 1.95, p = 0.17). There was no difference in the spontaneous-locomoter-activity response of the guinea pig to nicotine or saline (control) administration throughout the experiment (Figure 4.9). Whether saline was administered as the first or last dose, the subsequent spontaneous locomotor activity (distance) was not different (t_7 = 0.199, p = 0.8). The total distance traveled after administration of the highest dose of nicotine (0.7 mg/kg BW) also was not different if the animal received it as the first dose or the last dose (t_6 = 0.068, p = 0.074). This was the same for time moving in the open field (data not shown).

4.4. nAChR Concentration in the Frontal Cortex and Hippocampus

The concentration of nAChRs in the frontal cortex and hippocampus of the offspring at PD 100 was determined by a radioligand binding assay using [^{3}H] (-) - nicotine. Chronic maternal ethanol consumption decreased the concentration of nAChRs selectively in the frontal cortex (t_{30} = 2.13, p < 0.05), but not the hippocampus (Figure 4.10), and there was no sex effect. There was no correlation between the concentration of nAChRs in the frontal cortex and the dose of ethanol consumed, expressed as g/kg BW in the two-bottle-choice paradigm (Figure 4.11).

4.5. Social Isolation, Adolescent Ethanol Exposure and Ethanol Preference

Female guinea pigs were studied to determine the role of SI and combined AEE-SI on ethanol preference. Two-way ANOVA for the independent variables of housing condition (SI versus GH versus AEE-SI) and concentration of aqueous ethanol solution revealed significant main effects of housing (F (2, 180) = 9.9, p < 0.001) and concentration of ethanol solution (F (2, 180) = 8.8, p < 0.001) on ethanol preference. Post-hoc analysis demonstrated that GH animals consumed less ethanol than SI animals (t_{16} = 3.5, p < 0.01). AEE plus SI animals consumed less ethanol compared with SI guinea pigs (SI versus AEE-SI, t_{16} = 3.0 , p <0.05) (Fig. 4.12).

4.6. Mecamylamine Administration and Ethanol Preference

The female guinea pigs in the SI and GH groups were studied for ethanol preference after i.p. administration of 0.5 mg mecamylamine/kg BW, to determine the role of nAChRs in ethanol preference (Figure 4.13). There was a differential effect of mecamylamine in the two groups, with the drug decreasing ethanol preference in the SI group (t_{14} = 2.3, p < 0.05), and having no effect on ethanol preference in GH animals (t_{14} = 0.5, p = 0.6).

Table 4.1: Pregnancy outcome data.

Pregnancy outcome variable	Treatment	
	Ethanol (10)	Control (10)
Litter size	3.8 ± 0.3	3.4 ± 0.2
Male littermates (%)	54 ± 11	41 ± 8
Female littermates (%)	46 ± 11	59 ± 8
Perinatal death	4	2
Maternal death	0	1
Spontaneous abortion	1	0

The number of pregnant guinea pigs is reported in parentheses. Perinatal death, maternal death and spontaneous abortion are reported as the number of occurrences. The remaining data are presented as group mean ± SEM. (*), $p < 0.05$, unpaired t-test.

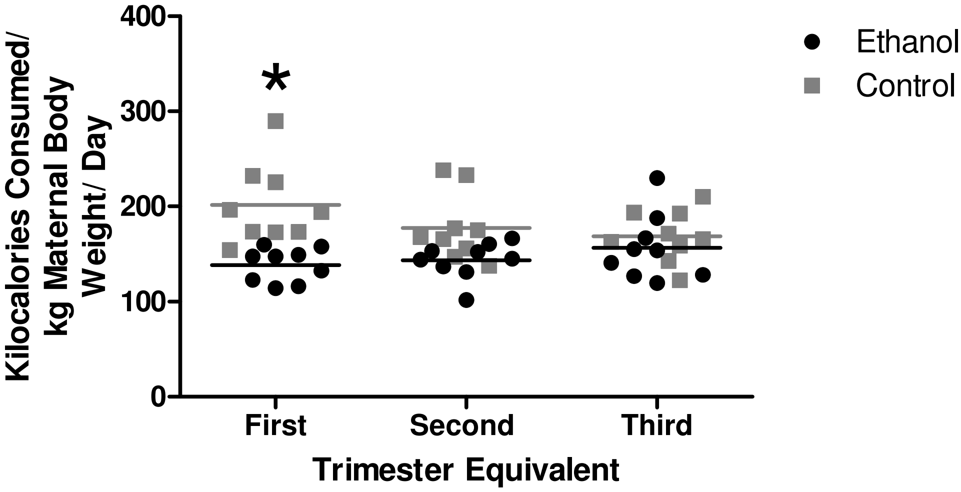


Figure 4.1: Maternal caloric intake in each of the three trimester equivalents. There was a significant decrease in caloric consumption during gestation, (n= 9 animals per group), (*) $p < 0.01$. Post-hoc analysis revealed that this decrease occurred in the first-trimester equivalent of pregnancy.

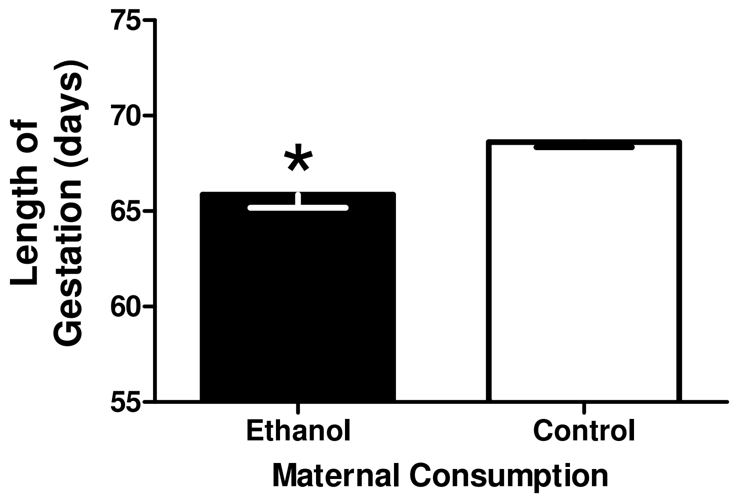


Figure 4.2: The effect of moderate ethanol intake on the length of gestation. There was an effect of chronic maternal ethanol consumption to decrease the length of gestation, (n= 10 animals per group, *$p < 0.01$).

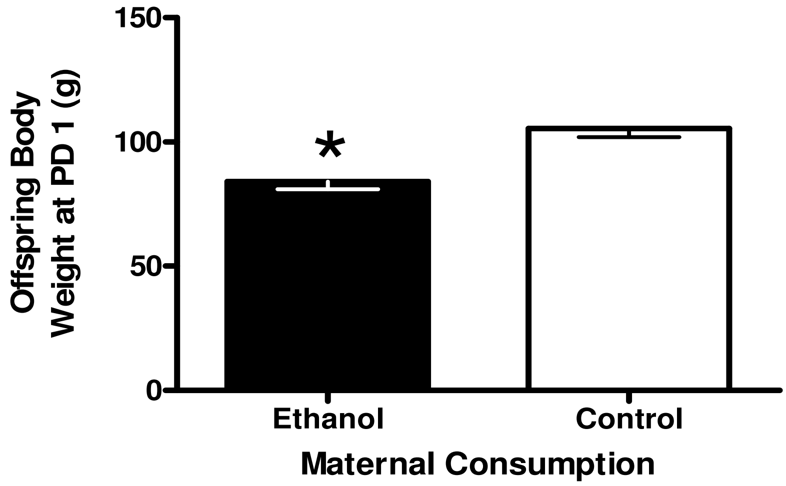


Figure 4.3: The effect of moderate ethanol intake on offspring body weight at PD 1. There was an effect of chronic maternal ethanol consumption to decrease the body weight of offspring (n = 29 ethanol, 28 control, *$p < 0.001$).

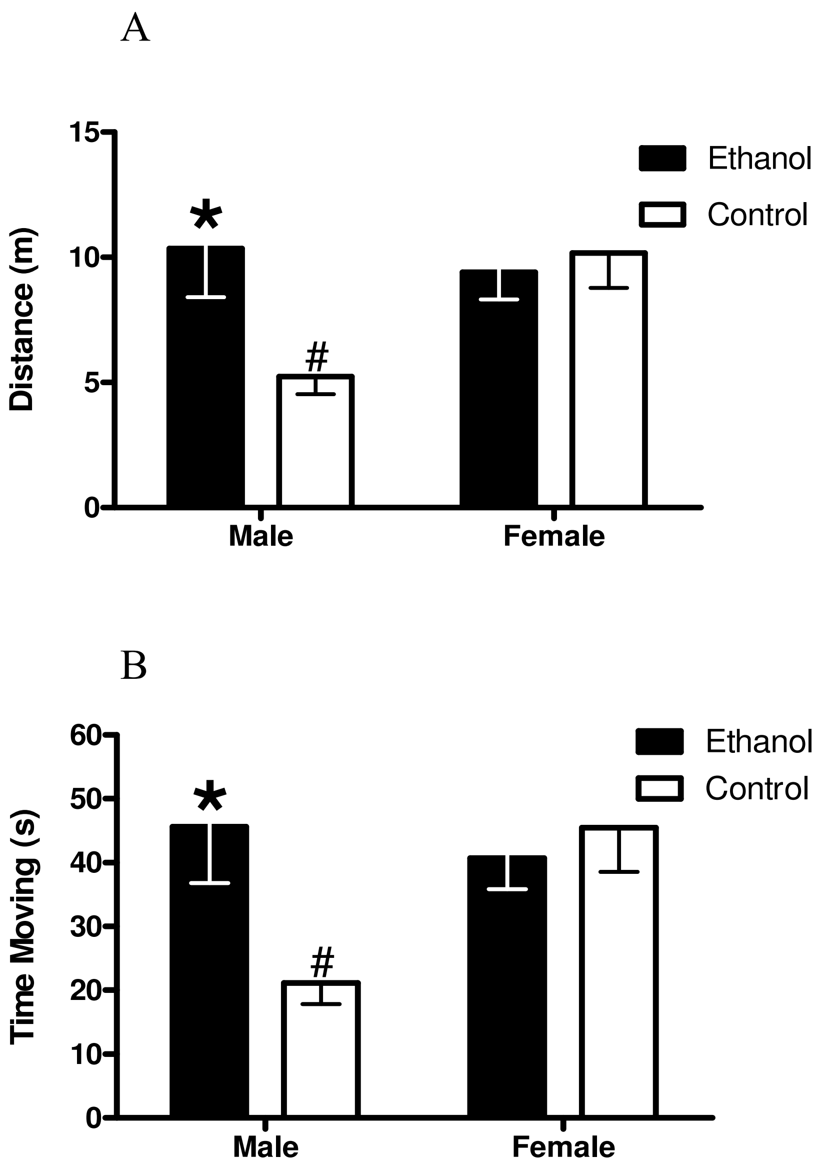


Figure 4.4: Spontaneous locomotor activity of PD 10 male and female offspring of the ethanol and control groups.
A) Distance traveled in the open field. (*), $p < 0.05$ for ethanol compared with control. (#), $p < 0.01$ for male control offspring compared with female control offspring.
B) Time moving in the open field. (*), $p < 0.05$ for ethanol compared with control. (#), $p < 0.01$ for male control offspring compared with female control offspring.

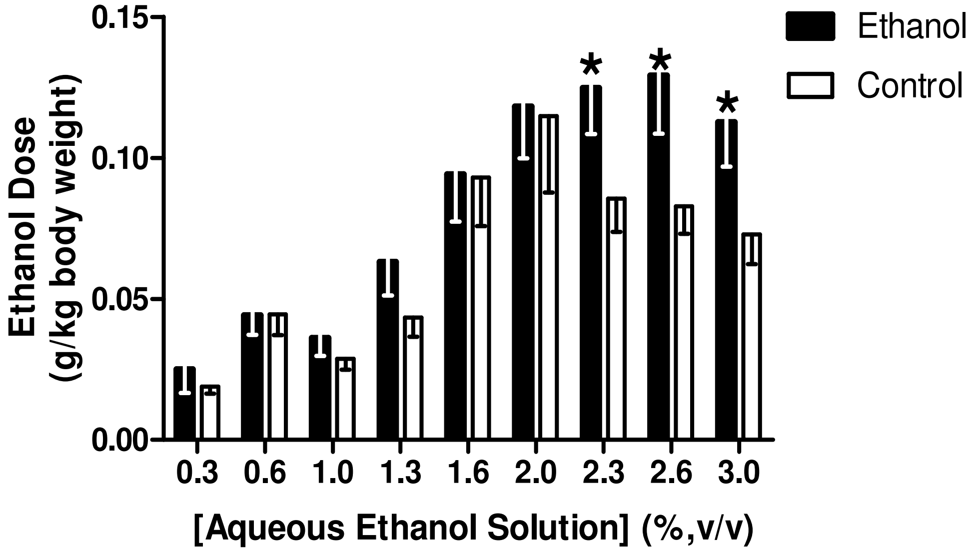


Figure 4.5: Dose of ethanol consumed by offspring in a limited-access, two-bottle-choice paradigm, starting on PD 40 and ending on PD 70. Offspring of the chronic maternal ethanol intake group demonstrated increased ethanol preference as measured by the dose of ethanol self-administered, (*), $p < 0.05$ for ethanol compared with control.

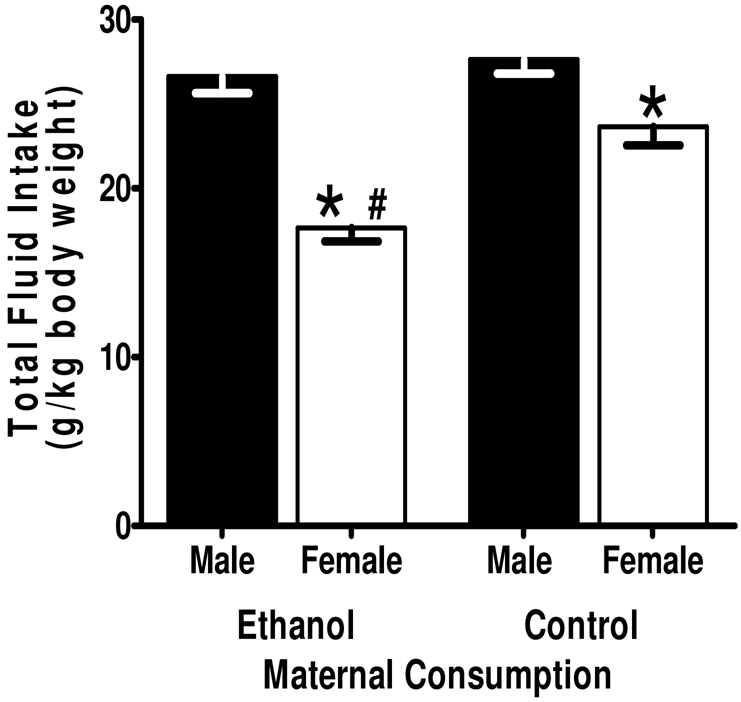
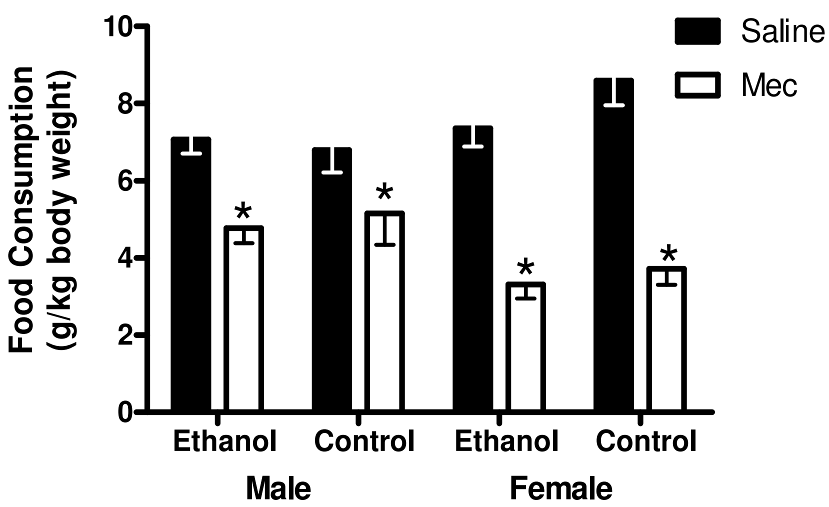


Figure 4.6: Total fluid consumption in a limited-access, two-bottle-choice paradigm, starting on PD 40 and ending on PD 70 for male and female offspring. Female offspring in each treatment group consumed less fluid than male offspring. Female offspring of the chronic maternal ethanol intake group consumed less total fluid than female and male offspring of the control group. (*), $p < 0.05$ for female offspring of ethanol or control group compared with male offspring of the respective group. (#), $p < 0.001$ for female offspring of ethanol group compared with female and male offspring of control group.

A



B

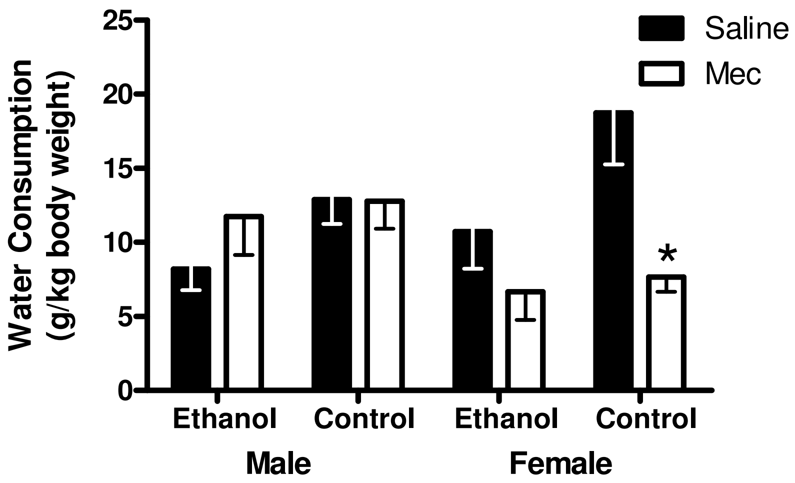
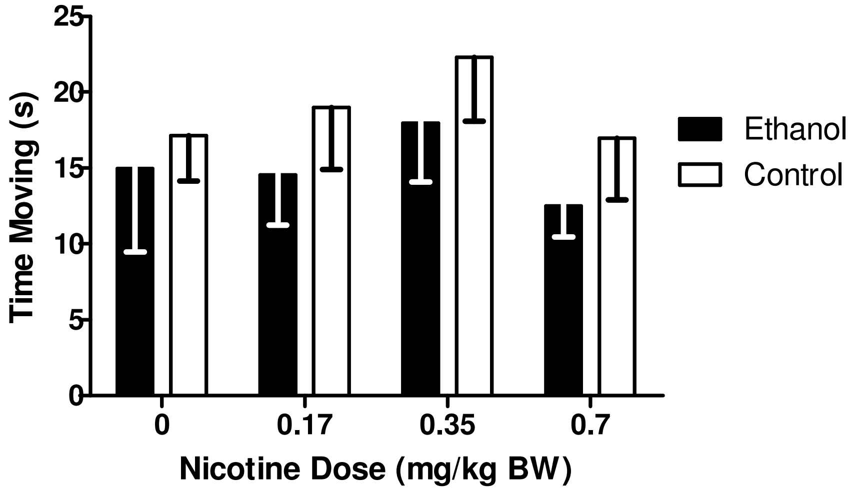


Figure 4.7: Food and water consumption in the two-bottle-choice experiment after i.g. administration of 1mg/kg BW mecamylamine, starting on PD 71 and ending on PD 76 for male and female offspring of the chronic maternal ethanol and control groups.
A) Food consumption. Mecamylamine decreased food consumption in both male and female offspring of the ethanol and control groups, (*), $p < 0.05$ for saline compared with mecamylamine.
B) Total water intake. Mecamylamine decreased water consumption in female control animals, (*), $p < 0.05$ for saline compared with mecamylamine.

A



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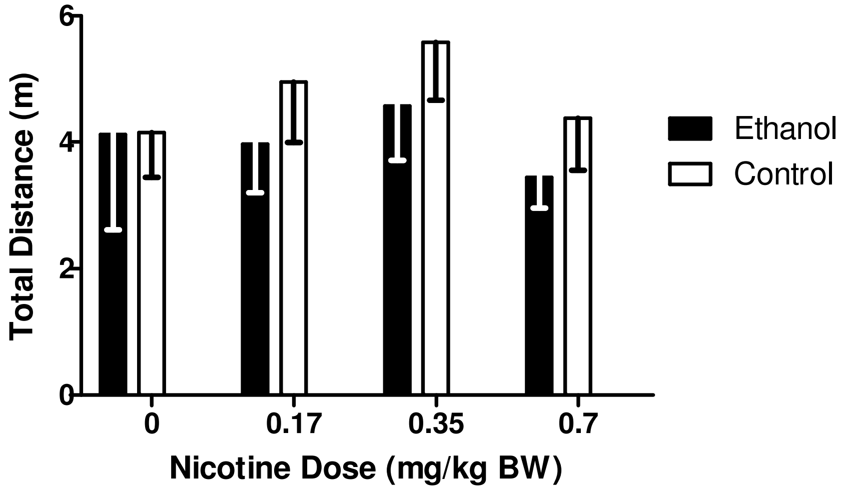

Figure 4.8: Spontaneous locomotor activity in response to acute s.c. administration of different nicotine doses to offspring of the ethanol and control groups.
A) Time moving in the open field after each dose of nicotine. Chronic maternal ethanol consumption did not alter the responsive to nicotine.
B) Distance traveled in the open field after each dose of nicotine. Chronic maternal ethanol consumption did not alter the response to nicotine.

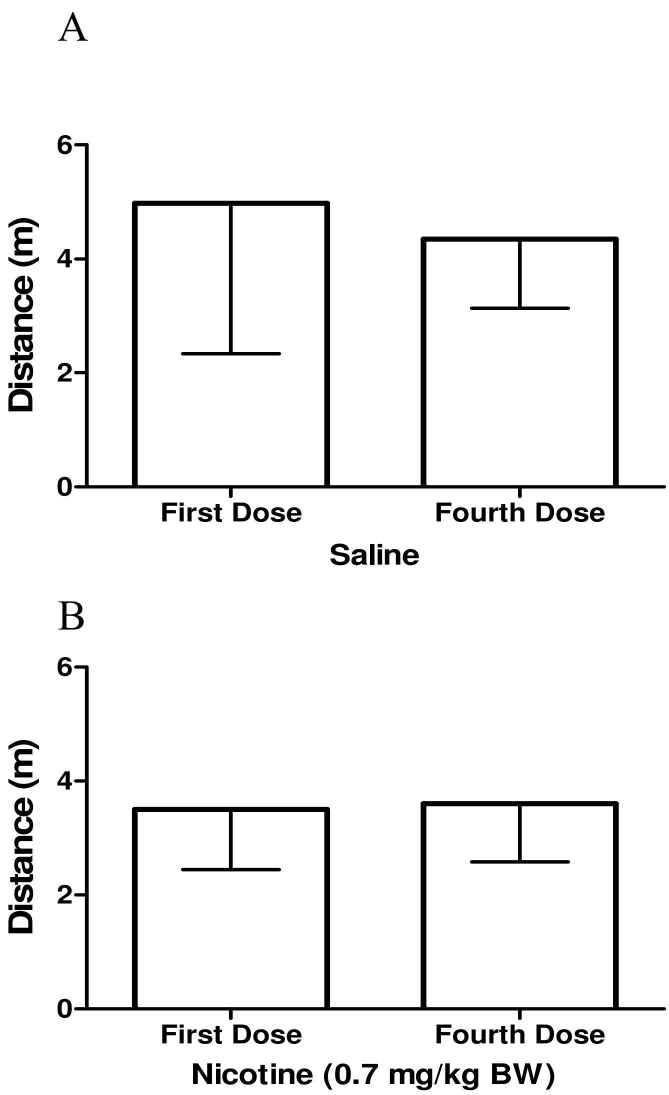

Figure 4.9: Distance traveled in the open field in response to s.c. injection of saline or 0.7 mg nicotine/kg BW after the first or last dose in the paradigm.
A) Distance traveled in the open field in response to saline as the first or fourth dose for control and ethanol offspring (combined). The animals did not change in their response to saline throughout the paradigm.
B) Distance traveled in the open field in response to 0.7 mg nicotine/kg BW as the first or fourth dose for control and ethanol offspring (combined). The animals did not change in their response to nicotine throughout the testing.

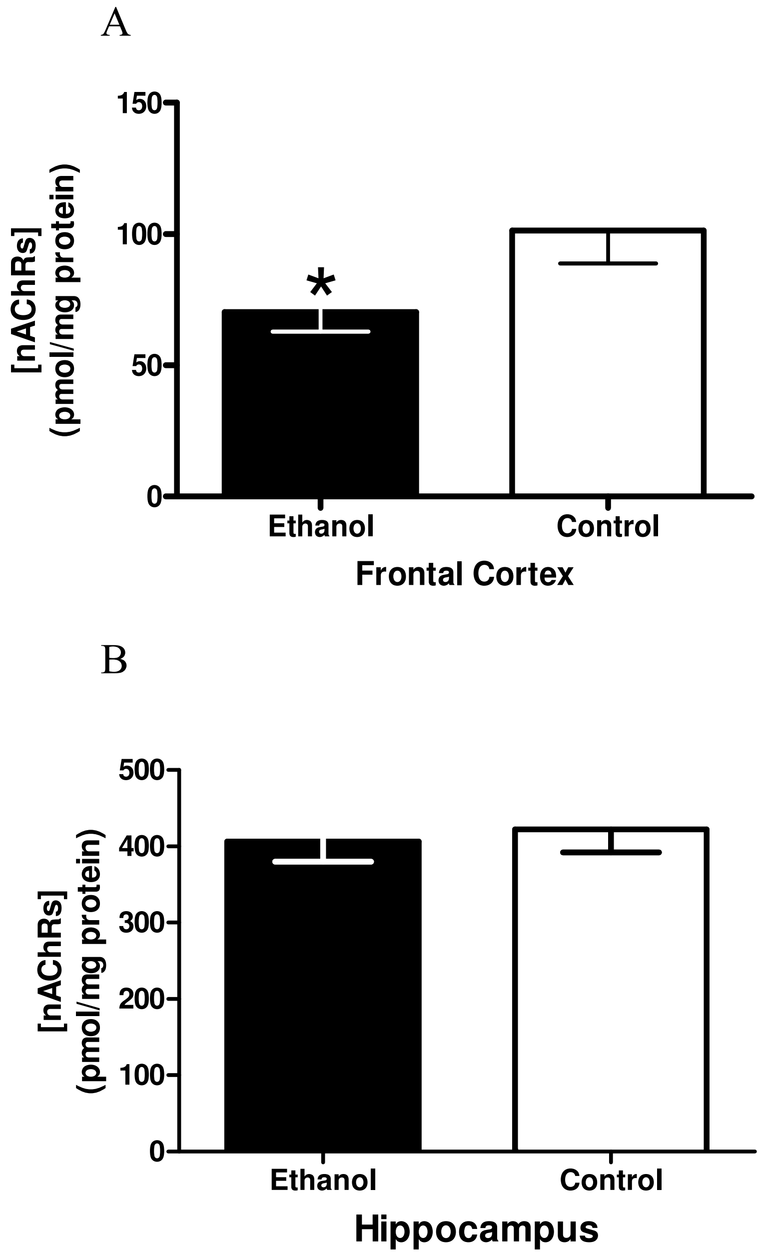

Figure 4.10: Concentration of nAChRs in the frontal cortex and hippocampus in PD 100 offspring of the chronic maternal ethanol and control groups.
A) Concentration of nAChRs in the frontal cortex. Chronic maternal ethanol intake decreased the concentration of nAChRs, (*), $p < 0.05$.
B) Concentration of nAChRs in the hippocampus. Chronic maternal ethanol intake did not alter the concentration of nAChRs.

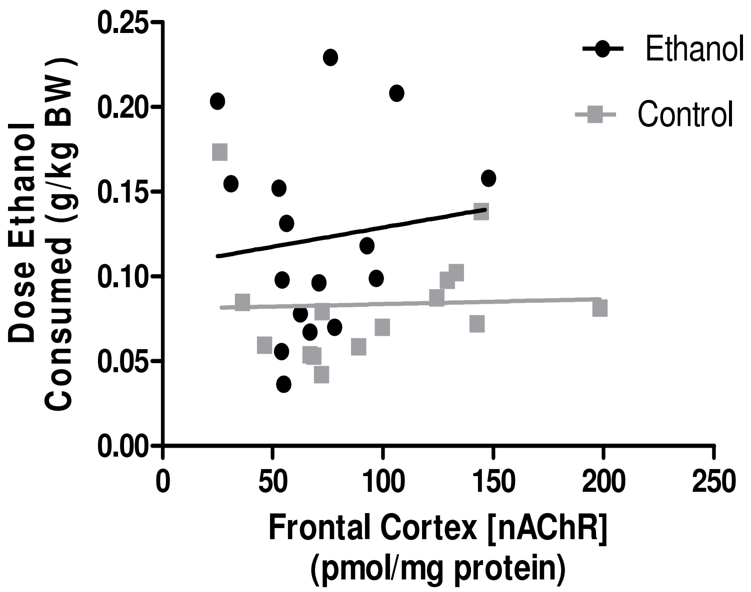

Figure 4.11: Plot of the dose of 3% (v/v) aqueous ethanol consumed in the two-bottle choice experiment and concentration of nAChRs in the frontal cortex in PD 100 offspring of the chronic maternal ethanol and control groups. There was no correlation between the two variables.

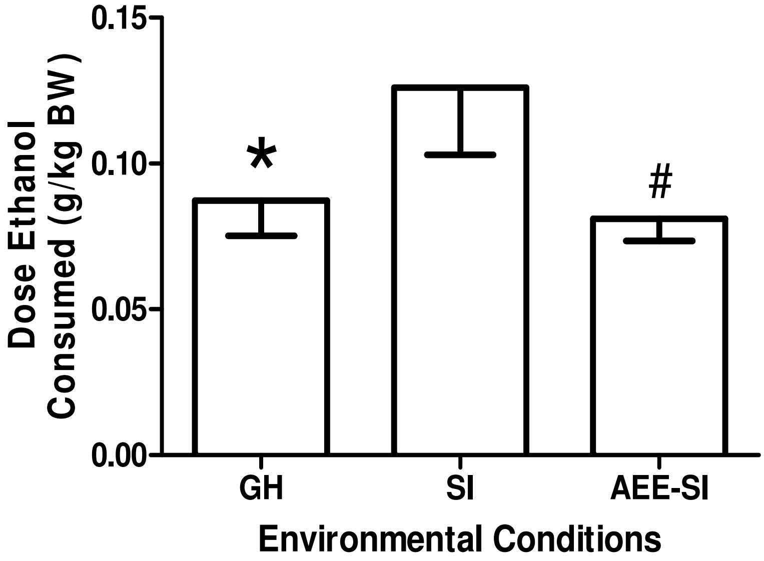

Figure 4.12: Ethanol preference of female guinea pigs in different housing conditions and adolescent ethanol exposure.
Social isolations (SI) increased ethanol preference. Adolescent ethanol exposure plus social isolation (AEE-SI) animals did not have increased ethanol preference compared with group housed (GH) animals. (*), $p < 0.01$ for GH versus SI, (#), $p < 0.05$ for AEE-SI versus SI.

A

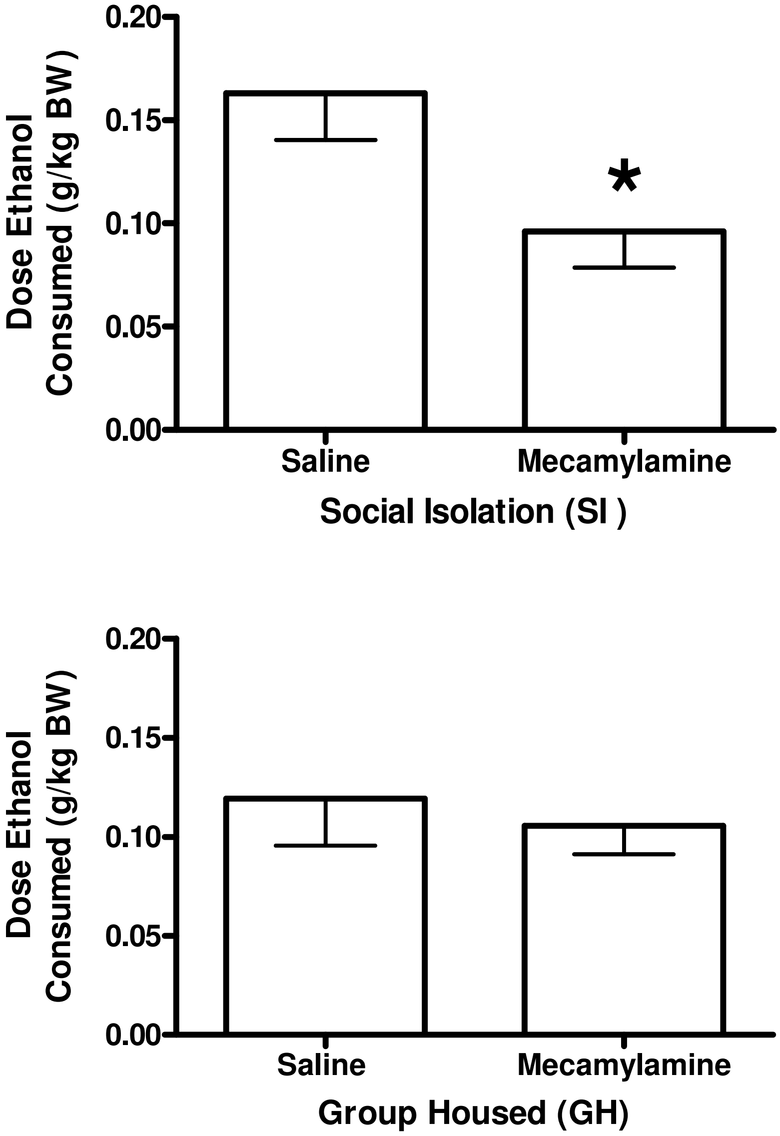

Figure 4.13: The effect of i.p. administration of 0.5 mg mecamylamine/kg body weight on the dose of 3% (v/v) aqueous ethanol consumed in a two-bottle-choice experiment in social isolation (SI) and group housed (GH) animals.
A) Mecamylamine decreased ethanol preference in the SI animals (*****), $p < 0.05$.
B) Mecamylamine did not alter ethanol preference in the GH animals.

Chapter 5: Discussion

5.1. General Discussion

This study determined in the guinea pig, whether (i) moderate CPEE in the guinea pig, via maternal consumption of an aqueous ethanol solution, increases ethanol preference, alters nicotine responsiveness in young adult offspring and changes the expression of nAChRs in the hippocampus and frontal cortex of adult offspring, and (ii) SI and AEE plus SI increases ethanol preference in the adult female. Moderate CPEE decreased length of gestation and birth weight and induced a sex-dependent increase in spontaneous locomotor activity in young male (PD 10) offspring. Moreover, guinea pig offspring of mothers that consumed moderate amounts of ethanol daily throughout gestation exhibited increased ethanol preference in a two-bottle-choice paradigm and a selectively decreased nAChR expression in the frontal cortex. CPEE did not alter the offspring's locomotor-activity response to acute nicotine administration. In the adult female, SI, but not AEE plus SI, increased ethanol preference, which was sensitive to the effects of mecamylamine, a CNS-active nAChR antagonist. These thesis-research findings are discussed in relation to published literature regarding prenatal ethanol exposure as a risk factor for adult ethanol abuse, the postulated role of CNS nAChRs in ethanol drinking behaviour and the etiology of substance-abuse disorders.

CPEE offspring in this study were born earlier and had lower birth weight than control offspring. This growth restriction was not solely the result of the gestational age of the animals. In a previous study, control near-term fetal guinea pig offspring at GD 63 (term, about GD 68), were found to have a greater body weight than the CPEE offspring born around GD 66 in this thesis research, indicating that the difference in neonatal birth weight between control and CPEE offspring in the current study was due to ethanol and not gestational age. The CPEE offspring in this study were born on GD 66 (range, GD 63-68), compared to control offspring that were born on GD 68 (range, GD 67-69). The decreased gestational length in this study is consistent with clinical data which indicate that women who consume alcohol during pregnancy are at risk for preterm delivery (Albertsen et al., 2004; Shiono et al., 1986). This is true even in pregnant women who consume lower doses of ethanol. In a study of over 2700 live births, women who consumed less than the equivalent of two alcoholic beverages per week in the late stages of pregnancy were at increased risk for preterm delivery (Lundsberg et al., 1997).

In both CPEE and control offspring, females had greater spontaneous locomotor activity in the open field. In the rat, females have frequently been found to exhibit greater spontaneous locomotor activity in the open field as compared with male littermates (Hyde and Jerussi, 1983; Hendley et al., 1985; Prus et al., 2008). There are several factors that may contribute to this sex difference. In the rat, there are differences in the acetylcholine (ACh) profile, with females exhibiting greater ACh release and more CNS cholinergic neurons as compared with the male rat (Takase et al., 2007) which is related to the greater spontaneous locomotor activity in females. The open field is stressful for rodents (Bond and Di Giusto, 1977; Gabriel et al., 2006), and in the rat, there is a sex-dependent difference in the release of stress hormones in the open field, which may be related to sex-dependent increased spontaneous locomotor activity. Corticosterone (CORT) is the major adrenal corticosteroid secreted in the rat, and the amount of CORT released by the adrenal gland in the open field (and in response to other stressors) is greater in females compared with males (Brett et al., 1983). CPEE female offspring consistently demonstrate an enhanced response to stressors such as intermittent footshock, acute restraint, forced swimming, noise and shaking, as compared to CPEE male offspring (reviewed in Weinberg et al., 2008). Furthermore, the greater amount of CORT release in females may be due to stress-induced estrogen acting on the hypothalamic–pituitary–adrenal (HPA) axis (Burgess and Handa, 1992) and/or inhibition of the HPA response to stress by increased androgen receptor stimulation in males (Handa et al., 1993).

In the current study there was a treatment effect, in which CPEE male offspring had increased spontaneous locomotor activity in the open field compared with control male offspring, indicating that the male guinea pig may be more susceptible to the neurobehavioural teratogenic effects of ethanol. Rats with prenatal ethanol exposure have sex-dependent altered behaviour in aversive or stressful situations (Gabriel et al., 2006). In the rat, CPEE differentially affects behavioural and hormonal responses of males and females in the elevated plus maze, a stressful behavioural task, indicating that the mechanism(s) underlying these responses are sex-dependent (Osborn et al., 1998). In this study, CPEE-induced hyperactivity in the guinea pig is an ethanol-dose dependent phenomenon (Catlin et al., 1993), in which the magnitude of hyperactivity in the offspring is dependent on the chronic maternal ethanol dosing regimen administered during pregnancy.

It is proposed that the use of ethanol in adolescence may be, at least partially, a consequence of ethanol exposure and its pharmacological effects in prenatal life (Spear and Molina, 2005). In the current study, pregnant guinea pigs consumed a 5% (v/v) aqueous ethanol solution daily throughout gestation, and the offspring exhibited increased ethanol preference when tested in a two-bottle-choice paradigm. In previous rat and mouse studies of the effect of CPEE on postnatal ethanol preference, CPEE occurred via maternal high-dose ethanol consumption during gestation, ethanol consumption throughout gestation and lactation, or binge-like ethanol consumption during pregnancy (reviewed in Chotro et al., 2007). In the limited number of studies involving relatively moderate ethanol exposure (1-2 g/kg maternal body weight) during gestation, ethanol was administered by gastric intubation (Chotro and Aries, 2003; Aries and Chotro 2005a,b) and was, therefore, binge-like exposure with assumed higher BEC compared to what is seen when ethanol is consumed throughout the day. The differing methods used in investigating the role of prenatal ethanol exposure in postnatal ethanol preference make it challenging to compare the findings of this thesis research to other studies. The results of this thesis indicate that CPEE does not need to involve maternal administration of high-dose ethanol during pregnancy and lactation, or in a binge-like regimen, in the guinea pig to produce long-lasting changes in postnatal ethanol preference.

The data of this thesis support clinical data which indicate that prenatal ethanol exposure in the human leads to increased response to ethanol in offspring and that prenatal ethanol exposure is a significant risk factor for ethanol abuse throughout postnatal life (Baer et al., 1998, 2003). Although genetic background is an important risk factor for alcohol-abuse problems, moderate-to-high-dose ethanol ingestion during gestation is as reliable or more reliable for predicting ethanol-drinking behaviour in adolescent and adult offspring compared with a family history of alcoholism (Yates et al., 1998). The exact mechanism(s) of this behavioural alteration remains to be elucidated, although several possible explanations have been suggested.

The role of nAChRs in ethanol preference of the CPEE and control offspring could not be determined in this thesis because mecamylamine at a dose of 1 mg/kg body weight decreased general volitional consumption of both food and water during the two-bottle-choice testing. The dose of this nAChR antagonist was given based on the literature (Le et al., 2000), although it appears that the guinea pig is more sensitive to the effects of mecamylamine. In the human, high dose mecamylamine (30–90 mg/day) has parasympathetic-blocking activity, producing constipation, urinary retention, dryness of the mouth and skin, dilation of the pupils, and loss of visual accommodation in some patients (Shytle et al., 2002). In the study of the female guinea pig described in Chapter 3.9, a dose of 0.5 mg mecamylamine/kg body weight was used, which appeared to be better tolerated and selectively decreased ethanol consumption, but not food or water intake (data not shown). In future studies, this dose of mecamylamine could be used in CPEE offspring to test for the role of nAChRs in ethanol preference.

Varenicline, a selective $\alpha 4\beta 2$ nAChR partial agonist is currently being used for smoking cessation (Coe et al., 2005). Varenicline is effective in smoking cessation by stimulating nAChR-mediated activity, thereby leading to 32%-45% decrease in DA release in the reward center that is produced by smoking a cigarette (Coe et al., 2005; Hays et al., 2008). Furthermore, varenicline competitively inhibits nicotine from interacting with the nAChR, via its antagonist activity (Coe et al., 2005; Rollema et al., 2007a, b). Since ethanol reward is partially mediated through the dopaminergic pathway in the reward center via action at the $\alpha 4\beta 2$ nAChR, it was hypothesized that varenicline would be useful in decreasing ethanol consumption. In the rat, varenicline decreases ethanol-seeking behaviour and consumption (Steensland et al., 2007). In a double-blind, placebo-controlled investigation of the effects of varenicline (2 mg/day vs. placebo) on ethanol self-administration in heavy-alcohol-drinking smokers, the drug decreased ethanol consumption, self-reported desire for alcohol and the positive reinforcing effects of ethanol (McKee et al., 2009). It is important to note that varenicline is associated with altered consciousness and visual disturbances, and in the United States, pilots and air traffic controllers are not permitted to take this drug (Hays et al., 2008), yet it has the potential to be used in the future as a research tool to further elucidate the role of nAChRs in increased ethanol preference in CPEE offspring.

There are other mechanism(s) that may have a role in increased ethanol preference in postnatal life after CPEE. The first is that CPEE induces fetal learning about ethanol through classical conditioning, involving the smell and taste of ethanol (reviewed in Chorto and Arias, 2006; Youngentob and Glendinning, 2009). The human neonate is more responsive to ethanol odour when exposed to ethanol via maternal ethanol consumption during pregnancy (Faas et al., 2000). Rat offspring appear to have memory of prenatal ethanol exposure (Chorto and Spear, 1997) and have an increased appetitive response to the taste of ethanol in infancy (Arias and Chotro, 2005b). Rats have an increased acceptability of ethanol taste and smell after CPEE even when tested in adulthood (Youngentob and Glendinning, 2009). To determine if the guinea pig retains memory of the chemosensory attributes of ethanol from prenatal experience with the drug into postnatal life, CPEE and control offspring could be presented with aqueous ethanol, quinine hydrochloride (bitter) and sucrose (sweet) tastes in a two-bottle choice paradigm with each solution paired with water (Youngenob and Glendinning, 2009). If the CPEE guinea pigs had an increased intake of ethanol and quinine hydrochloride but not sucrose as compared to controls, it would indicate that CPEE increased acceptability of the ethanol which could be attributed directly to the attenuated aversion to ethanol's quinine-like taste quality.

An alternate explanation is that increased ethanol preference in postnatal life in CPEE offspring is a result of the positive reinforcing properties of ethanol, specifically from a shift in the dose-dependent ethanol-induced DA release after CPEE (Blanchard et al., 1993). The mesolimbic DA system, including areas of the ventral tegmental area (VTA) and NA, are stimulated by ethanol (Yoshimoto et al., 1992). In ethanol preferring (P) rats, there is an underlying decrease in DA systems in certain limbic structures, including the medial prefrontal cortex, as compared to ethanol non-preferring (NP) rats (Samson et al., 1992), indicating a link between altered neurochemistry and a predisposition for increased ethanol consumption. CPEE is known to alter the development of the dopaminergic system in the striatum and frontal cortex in mice and rats, with a decrease in DA, DA uptake sites and activity of the DA neurons in midbrain structures of postnatal CPEE offspring (Druse et al., 1990; Shen et al., 1999). Finally, CPEE alters the DA release to ethanol in postnatal life in the adult rat (Blanchard et al., 1993). In control rat offspring, DA release is increased in the NA and striatum in response to 0.5 g ethanol/kg body weight, but not 1.0 g ethanol/kg body weight. In contrast, CPEE offspring do not exhibit an ethanol-induced DA release at 0.5 g ethanol/kg, although male CPEE offspring do show enhanced DA released in response to 1.0 g ethanol/kg BW in the NA and striatum. In CPEE female offspring, there is a decrease in DA release in the striatum and no change in the NA in response to both doses of ethanol. CPEE and control offspring were subsequently tested for ethanol preference and there was no difference between the two groups. In the absence of an altered behavioural outcome of increased ethanol preference, there were underlying neurological changes in the NA and striatum. The release of DA in response to administered ethanol in these brain regions could be tested in CPEE guinea pig offspring, using microdialysis (Blanchard et al., 1993). This would be of particular interest, as there is an increased ethanol preference in these animals and the correlation between any physical biochemical changes in the brain and ethanol consumption could be analysed. Alternately, the role of the D1 and D2 receptor in the increased ethanol preference induced by CPEE in the guinea pig could be examined by manipulation of D1 and/or D2 receptors (Moody et al., 1993). Specifically, the D1 antagonist SCH23390 and the D2 antagonist sulpiride can be administered alone or in conjunction to determine their effect on ethanol consumption in the offspring. The effect of the D1 agonist SK&F38393 and D2 agonist quinpirole on ethanol preference in the offspring could also be tested.

A third alternate mechanism to explain increased ethanol preference after CPEE is that increased fear and stress in CPEE offspring promotes ethanol consumption. Self-administration of ethanol in the rat is increased in response to various stressors including electric shock, immobilization and psychological or emotional stress (reviewed in Pohorecky, 1990). Ethanol consumption is sometimes delayed until the stressor is extinguished, as with the electric food-shock. In non-human primates stress, via maternal-deprivation, SI or maternal deprivation plus SI, resulted in increased volitional ethanol consumption, which was positively correlated with plasma cortisol and corticotropin concentrations (Higley et al., 1991). Stress is also associated with increased ethanol consumption in the human, although this is more difficult to ascertain due to the complex interaction of many factors, including mood and personality of the subject, drinking environment, cultural influence, experience with ethanol, sex, and type of alcoholic beverage (reviewed by Pohorecky, 1991).

In the human, high maternal ethanol consumption during pregnancy is related to increased basal cortisol concentrations in the blood of infants, as well as increased cortisol after a stressful event (Jacobson et al., 1999). Prenatal ethanol exposure is also related to increased stress reactivity, measured by greater negative affect and elevated heart rate, in infants at 5-7 months of age (Haley et al., 2006). The HPA axis is one of the main regulators of the stress response (Weinberg et al., 2008) and fetal injury resulting in changes of the HPA axis is associated with increased cortisol in adults (Phillips et al., 2000).

In rodents and non-human primates (reviewed by Weinberg et al., 2008) CPEE offspring are hyper-responsive to a variety of stressors in postnatal life, which is associated with alterations in the HPA axis. The specific changes in the axis as a result of CPEE are complex, with alterations at the level of the hypothalamus, pituitary gland, adrenal gland and hippocampus, which are often sex-dependent. There are also alterations in interactions between the HPA and hypothalamic-pituitary-gonadal (HPG) axis. CPEE leads to long-lasting disregulation in the HPA axis, with underlying increased HPA tone throughout postnatal life and also increased HPA activity in response to stress (Weinberg et al., 2008). The ontogeny of the HPA axis in the guinea pig is well-understood with extensive prenatal development (Matthews, 1998). In our laboratory, we have previously demonstrated that in the guinea pig, CPEE acts as a maternal and fetal stressor, causing activation of the HPA axis in the near-term fetus and offspring (Iqbal et al., 2005; Iqbal et al., 2006b). Cortisol is the primary glucocorticoid hormone in the guinea pig (Matthews, 1998) and CPEE offspring manifest increased saliva cortisol concentration after exposure to swim stress (Iqbal et al., 2006b).

The interaction between HPA axis disregulation in guinea pig offspring following CPEE and increased ethanol consumption could be investigated in the future by measuring saliva cortisol concentrations in the offspring. The basal cortisol concentrations as well as the cortisol levels after a stressful event and/or during the two-bottle choice paradigm could be measured, to determine if there is a relationship between cortisol levels and ethanol preference. Any positive correlations would indicate that alterations in the HPA axis are implicated in increased ethanol preference and suggest a mechanism for increased risk for ethanol abuse following CPEE.

CPEE altered the expression of nAChRs, with a selective receptor loss in the frontal cortex of the adult guinea pig. These data suggest that the nAChR system in the frontal cortex and hippocampus of the guinea pig has differential sensitivity to the effects of CPEE. CNS nAChRs are ligand-gated ion channels that are important in memory, attention and information processing (Picciotto et al., 2000; Court and Clementi, 1995). With increased cognitive demands, there is recruitment of the cholinergic system in different brain regions, including the frontal cortex and hippocampus, and impaired function is likely to impact negatively tasks that require high attention (Dumas et al., 2008). In addition, nAChRs are implicated in ethanol self-administration (Larsson and Engel, 2004). CPEE offspring have altered behavioural responsiveness to nicotine in postnatal life (Nagahara and Handa 1999a,b; Rogers et al., 2004), which indicates that brain nAChRs are potential targets of ethanol neurobehavioural teratogenicity.

Alterations in the brain nicotinic cholinergic system have been implicated in other neurobehavioural disorders, namely, attention-deficit hyperactivity disorder (ADHD) and depression (Beane and Marrocco, 2004; Fowler et al., 2008). These two disorders are of interest because of their high comorbidity associated with FASD. ADHD is characterized by hyperactivity, impulsivity and/or inattention and distractibility that are persistent and hinder academic or occupational functioning (Fletcher et al., 1991; Lahey et al., 1994). There is high comorbidity of ADHD in individuals with FASD and a possible link exists between these two developmental disabilities (O'Malley and Nanson, 2002). There is a higher rate of depression in people with FASD than in the general population (Barr et al., 2006; Famy et al., 1998). nAChRs may regulate, at least in part, HPA function, which in turn plays an important role in the etiology of depression (Fowler et al., 2008). Interestingly, the clinical literature suggests that individuals with ADHD or depression are more likely to smoke tobacco, possibly as a means of self-medication (Pomerleau et al., 1995; Breslau, 1995; Rabenstein et al., 2006).

Acute nicotine treatment decreases spontaneous locomotor activity, (Stolerman, 1990) whereas chronic nicotine treatment results in behavioural sensitization (Clarke and Kumar, 1983; Clarke et al., 1988b) and increased spontaneous locomotor activity (Stolerman, 1990; Prus et al., 2008). Nicotine administration increases DA release in the NA (Sziraki et al., 2001), and increased spontaneous locomotor activity after nicotine exposure is mediated partially through mesolimbic dopaminergic pathway activation (Clarke et al., 1988b; Prus et al., 2008). Increased response to nicotine after chronic exposure also may be the result of desensitization and subsequent up-regulation of nAChRs (Bertrand et al., 1990; Marks et al., 1983; Mochizuki et al., 1998; Vann et al., 2006; Zhang et al., 2000). Post-mortem analysis of tobacco abusers revealed that nAChRs are upregulated in a dose-dependent manner in the hippocampus and thalamus (Breese et al., 1997). In this thesis research, nicotine did not alter spontaneous locomotor activity in the open field at all doses of the drug. There was no sensitization to the nicotine, as seen by the 0.7 mg nicotine/kg BW dose eliciting the same magnitude decrease in spontaneous locomotor activity when the drug was administered as the first or last dose. The 48-h washout period after every three days of nicotine testing in the current study should have been sufficient for complete elimination of nicotine from the body. There was no difference in the behavioural response to nicotine in the CPEE and control offspring. Future studies should focus on the known changes that occur to the nAChRs after chronic nicotine exposure and determine whether CPEE induces in the offspring different behavioural response to chronic nicotine exposure and if these changes are related to altered nAChRs in the brain.

SI in the female guinea pig led to increased ethanol preference in a two-bottle-choice paradigm as compared with ethanol consumption in GH guinea pigs. This is consistent with the literature, which indicates that long-term SI in the rhesus monkey (Kraemer and McKinney, 1985) and the rat leads to increased ethanol consumption (McCool and Chappell, 2009; Wolffgramm and Heyne, 1991), which may be mediated by the CNS dopaminergic system (Wolffgramm and Heyne, 1995). The guinea pig, like other animal species, is vulnerable to environmental factors that increase ethanol consumption. It is currently unknown if there is an interaction between increased ethanol preference induced by CPEE and these environmental factors. People with FASD may have a predisposition for increased risk for ethanol abuse throughout life but also may be exposed to one or more environmental factors that are associated with increases ethanol abuse, including alcohol availability, parental attitudes, inconsistent discipline, physical abuse and parental neglect (Enoch, 2006). The data in this thesis research indicate that SI in the female guinea pig can be used as an environmental promoter of volitional ethanol intake and can be used in future studies to investigate the potential interaction between CPEE-induced ethanol preference and environmental factors.

AEE coupled with SI did not increase ethanol consumption in the female guinea pig. Forced administration of ethanol during the adolescent period actually reversed the effects of SI such that ethanol intake in the two-bottle choice paradigm was not different from that in GH guinea pigs. This decline in ethanol consumption may be related to the sex of the guinea pigs tested. In the rat, males exposed to ethanol as adolescents increase their ethanol consumption as adults, whereas ethanol-exposed females decrease their consumption slightly (Siciliano and Smith, 2001). The decline in ethanol consumption in this thesis research may have also been due to the paradigm used. In the rat, increased ethanol self-administration after AEE only occurs when the animal had free choice between water and aqueous ethanol solutions in adolescence. Long-term forced administration of ethanol only leads to physical dependence but not ethanol-seeking behaviour in the rat indicating that forced ethanol administration may lead to negative association with ethanol thereby decreasing volitional ethanol consumption (Wolffgramm and Heyne, 1995).

Ethanol preference by the SI guinea pigs in the current study was suppressed by mecamylamine, indicating that ethanol preference in these animals was, at least partially, dependent on the nicotinic cholinergic system in the brain. Interestingly, mecamylamine did not decrease ethanol consumption in GH or AEE-SI animals. The reason for this may be that ethanol consumption in these animals was not mediated through the CNS nAChR system or was the result of the demonstrated difference in the baseline intake of aqueous ethanol solution among the two groups.

5.2. Future Research Directions

Based on the results of this thesis research, future studies should focus on the following key areas.

1) In this thesis, the role of CNS nAChRs in ethanol preference and ethanol self-administration could not be determined, due to the apparent high sensitivity of the guinea pig to the 1mg mecamylamine/kg BW dose that was used. Future investigations are needed to study this receptor system in the guinea pig using a lower dose of mecamylamine. Experiments described in Chapter 3.9 were completed using a mecamylamine dose of 0.5 mg/kg BW, which selectively decreased volitional ethanol consumption in the female guinea pig, without disrupting food and water consumption. Future studies of the role of CNS nAChRs in ethanol self-administration after CPEE could therefore use this lower dose of mecamylamine.
2) Since the time this thesis research was initiated, varenicline, an $\alpha4\beta2$ nAChR partial agonist has become available for treatment of nicotine dependence (Hays et al., 2008). Future studies should include this more selective agent, alone and in combination with mecamylamine, to probe the role of the $\alpha4\beta2$ nAChR receptor in ethanol preference in postnatal life following CPEE. This nAChR isoform is

important to the molecular mechanism of the reinforcing effects of ethanol (Steensland et al., 2007) and currently it is unknown whether CPEE alters the function or population of this receptor subtype.

3) The frontal cortex and hippocampus were the brain areas selected for study in this thesis because they are targets of the neurobehavioural teratogenic effects of ethanol. As there is selective disruption of nAChRs in the brain following CPEE, more research is needed to elucidate the brain localization of this change. Of particular interest are the areas of the brain involved in drug addiction and reward, especially the VTA and NA. These brain areas cannot be easily dissected for radioligand binding studies as conducted in this thesis research, but can be studied using alternate techniques. Currently, autoradiography is being developed in our laboratory and could be used in the future to probe multiple receptor systems, including nAChRs, in selected brain regions, including the VTA and NA.

4) One of the goals of this thesis was to study the postnatal locomotor activity response to acute nicotine administration, to order to determine whether CPEE, via moderate chronic maternal intake of an aqueous ethanol solution, alters this response. The results were inconclusive. Further studies are needed to answer this question, including investigating the locomotor activity response to chronic nicotine exposure, which would be more similar to the situation of a tobacco smoker. Another way to determine whether the response to nicotine is altered by CPEE would be to measure the nicotine self-administration in the offspring. Rats and mice will self-administer nicotine (Shoaib et al., 1997; Martin-Garcia et al., 2009; reviewed in O'Dell and Khroyan, 2009). Conditioned place preference has

also been used to study nicotine preference in the rat (Horan et al., 1997) and is another option for future investigations of the impact of CPEE on nicotine response in postnatal life. Any behavioural changes observed in these studies could then in turn be correlated with alterations in nAChRs in the CNS.

Chapter 6: Summary and Conclusions

This thesis tested the hypothesis that moderate maternal consumption of ethanol by the pregnant guinea pig produces in the offspring (i) ethanol neurobehavioural teratogenicity, (ii) increase in ethanol preference that is related to CNS nAChRs, (iii) altered response to nicotine, and (iv) increase of nAChRs in the hippocampus and prefrontal cortex. In a separate study, the impact of social isolation (SI) and adolescent ethanol exposure (AEE) coupled with SI to increase ethanol preference in the adult female guinea pig was tested.

CPEE, via moderate ethanol consumption throughout gestation, decreased length of gestation and birth mass of the offspring, and increased spontaneous locomotor activity in male offspring. Male and female offspring with CPEE manifested increased ethanol preference in a two-bottle-choice paradigm. The role of CNS nAChRs in this behaviour was not determined, although there was decreased nAChRs in the frontal cortex, but not the hippocampus. These data indicate that in the guinea pig, maternal administration of high-dose ethanol throughout gestation is not needed to produce neurobehavioural teratogenicity and that moderate-dose ethanol is a risk factor for increased ethanol preference in offspring. SI female guinea pigs had higher ethanol preference compared with the GH and AEE-SI female guinea pigs, indicating that SI is a risk factor for increased ethanol preference. This provides a useful model in which to study the interaction between CPEE and environmental factors in offspring ethanol preference. AEE involving forced administration of ethanol during adolescence appeared to counteract the effect of SI in the female guinea pig, as the impact of SI to increase ethanol self-administration was prevented. It is possible that forced administration of ethanol during adolescence is aversive for the animal, leading to decreased ethanol drinking later in life. Interestingly, mecamylamine, a CNS-active nAChR antagonist, decreased ethanol preference selectively in SI guinea pigs, indicating that increased ethanol preference as a consequence of this environmental manipulation involves activation of nAChR in the CNS.

Future studies in the guinea pig are needed to further understand the role of CNS nAChRs in the neurobehavioural outcomes in offspring following moderate CPEE, specifically increased ethanol preference. These studies could involve using mecamylamine and varenicline, a CNS nAChR partial agonist. These studies also could involve investigating other key areas of the brain involved in addiction and reward, namely the VTA and NA, to determine if the nAChR signalling pathway is perturbed and whether changes in nAChR function in these brain regions correlates with the aforementioned neurobehavioural outcomes of moderate CPEE.

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