EFFECTS OF PRENATAL EXPOSURE TO NICOTINE ON WORKING MEMORY, ACTIVITY, SENSORY GATING, AND DOPAMINE RECEPTOR BINDING IN ADOLESCENT AND ADULT MALE AND FEMALE RATS

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Abstract

Title of Thesis: Effects of Prenatal Exposure to Nicotine on Working Memory, Activity, Sensory Gating, and Dopamine Receptor Binding in Adolescent and Adult Male and Female Rats

Matthew Alan Rahman, Doctor of Philosophy, 1999

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Exposure to nicotine *in utero* results in a wide array of physical, behavioral, and cognitive effects on the offspring. Some of these effects are evident at birth and some do not appear until later in life. In the present experiment, pregnant female Sprague-Dawley rats were administered nicotine (6 or 12 mg/kg/day) or saline via osmotic minipump chronically throughout pregnancy and the offspring were examined during adolescence and adulthood for changes in body weight, radial-arm and water maze performance, passive avoidance performance, acoustic startle reactivity, pre-pulse inhibition, locomotor activity, and dopamine D1 receptor binding in the medial prefrontal cortex. Effects of prenatal nicotine exposure depended on nicotine dosage, the sex and

age of the subject. Specifically, females and males that had been exposed to the highest dose of nicotine *in utero* weighed less than the other offspring throughout the experiment. These females also made more mistakes on the radial-arm maze and were less active during adolescent testing than the other females. When tested in adulthood, the females that had been exposed to 6 mg/kg/day nicotine showed enhanced pre-pulse inhibition (an index of sensory gating). Adolescent males that had been exposed to 6 mg/kg/day nicotine took longer to complete the water maze task, but were more active than the other adolescent males. When tested as adults, males that had been exposed to either dosage of nicotine performed more poorly than non-drug-exposed males on the radial-arm maze (they made fewer correct choices before an error was made and took longer to complete the task) and showed impaired pre-pulse inhibition. However, these same males showed enhanced passive avoidance learning. Dopamine D1 receptors of either sex were not significantly affected by prenatal exposure to nicotine. In summary, prenatal exposure to nicotine resulted in physically smaller offspring, impaired spatial memory performance, and mixed results on non-spatial memory, pre-pulse inhibition, and locomotor activity without affecting the D1 receptor system in the medial prefrontal cortex. Implications of these findings with regard to previous work in animals and relevance to human smoking during pregnancy are discussed.

Effects of Prenatal Exposure to Nicotine on

Working Memory, Activity, Sensory Gating, and

Dopamine Receptor Binding in

Adolescent and Adult

Male and Female Rats

by

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Introduction

Cigarette smoking during pregnancy results in pathophysiologic consequences in children, including spontaneous abortion, fetal resorption, reduced birth weight, and weakened physical condition (CDC, 1994a; CDC, 1995; Levin et al., 1998). In addition to these hazards, epidemiologic investigations have suggested that children of smoking mothers have decreased learning and cognitive abilities as well as increased hyperactivity and conduct disorders (Levin et al., 1998). Because these human studies are correlational causality of tobacco or any of its key constituents (most importantly, nicotine) cannot be determined. Animal models have proven to be valuable in the investigation of causal contributions of nicotine to behavioral and cognitive effects (e.g., Grunberg et al., 1991; Slotkin, 1992). Animal studies (mostly using rats and mice) have reported that prenatal exposure to nicotine results in offspring that show: 1) decreased learning abilities (Genedani et al., 1983; Martin et al., 1971); 2) decreased cognitive (*e.g.*, memory performance) abilities (e.g., Levin, 1993a); 3) increased activity (Richardson et al., 1994); and 4) altered brain development including, alterations in gene expression (Slotkin et al., 1997), receptor densities (Fung et al., 1989; Slotkin et al., 1987a), and general alterations in overall brain development (Lichtensteiger et al., 1988). Unfortunately, these experiments have several methodologic limitations: 1) routes of administration have included several techniques that are not appropriate models of exposure to nicotine or tobacco (e.g., nicotine in drinking water, oral gavage of smokeless tobacco extract); 2) dosages of nicotine via

osmotic minipump (an appropriate model for nicotine exposure) have been lower than dosages that mimic nicotine exposure that human fetuses experience (*e.g.*, 0.1, 0.2, 0.5, 1.0 and up to 6.0 mg/kg/day as opposed to 6.0 - 12.0 mg/kg/day) (Winders et al., 1998); and 3) cognitive, behavioral, and neural effects have not been examined within the same offspring. The purpose of this dissertation research was to fill these gaps by examining effects of prenatal exposure to appropriate dosages of nicotine, during adolescence and adulthood, on cognitive performance, behavior and activity, sensory gating, and dopamine receptor binding in relevant brain areas, within the same male and female offspring.

This dissertation begins by discussing background information on the effects of cigarette smoking during pregnancy in humans: the prevalence, the effects on children, and costs to society. Next follows a discussion of what has been discovered using prenatal nicotine administration in animals, including effects on working memory with particular attention to animal models of memory performance. The Introduction ends with a discussion of the physical chemistry of dopamine and nicotine and their associated receptor systems. The Methods section delineates exactly how the experiment was performed and analyzed. The Results section presents the findings of this experiment. These findings reveal that: nicotine administration during pregnancy results in altered cognitive and behavioral abilities; these changes are complex and are revealed at different times in the life span; these changes are dependent upon the sex of the offspring; and altered cognitive and behavioral abilities are not associated with changes in neurochemical receptors in the specific area of the brain examined.

Cigarette Smoking During Pregnancy in Humans

Prevalence

Tobacco use remains the leading preventable cause of death in the United States, causing more than 400,000 deaths each year and resulting in more than $50 billion in direct medical costs (CDC, 1994b). More than 48 million Americans adults smoke (61 million including those under age 18), 22.7 million of them adult women of child-bearing age (CDC, 1995; USDHHS, 1996). Cigarette smoking by pregnant females has serious negative behavioral, cognitive, and health effects in offspring. Whereas the incidence of smoking in the general population has decreased 25% in recent years (CDC, 1994a; 1965-1994, Mactutus, 1989), smoking among females has increased (CDC, 1995; USDHHS, 1996). Smoking among females of child-bearing age has been reported to be as high as 25-33% in recent years (Levin et al., 1998; Levin et al., 1996b; Slotkin, 1998; USDHHS, 1988; USDHHS, 1996). Among females of lower socio-economic status (SES) the prevalence of smoking is even higher (Bardy et al., 1994). Although pregnant woman commonly report decreases in or cessation of smoking behavior during pregnancy (Fricker et al., 1985; Stewart et al., 1985; Streissguth et al., 1983), biochemical markers of cigarette smoking measured in maternal and fetal blood indicate high prevalence of smoking by the mothers. For example, Pley and colleagues (1991) reported significant discrepancies between self-report data and carboxy-hemoglobin levels. Bardy and colleagues (1993) found that 38% of women who had reported being non-smokers during pregnancy had cotinine (the primary metabolite of nicotine) in

their blood at levels similar to those women who reported that they continued smoking.

Effects on Children

Cigarette smoking during pregnancy can result in: increased infant mortality and decreased birth weight (Aronson et al., 1993; Brooke et al., 1989; Eliopoulos et al., 1996; Morrison et al., 1993; Picone et al., 1982); reduced APGAR scores (Drage et al., 1966a; Drage et al., 1966b); learning impairment (Butler et al., 1973; Dunn et al., 1977; Hardy et al., 1972); cognitive deficits (Dunn et al., 1977; Rantakallio, 1983); attentional and conduct deficits including attention deficit disorder (ADD) and attention deficit hyperactivity disorder (ADHD) (Denson et al., 1975; Naeye et al., 1984; Streissguth, 1984); poor impulse control (Fergusson et al., 1993; Kristjansson et al., 1989); impaired attention and orientation (Landesman-Dwyer et al., 1979; Picone et al., 1982; Streissguth et al., 1983); poor vigilance performance and decreased reaction time performance (Kristjansson et al., 1989); and hyperactivity without attentional deficits (Denson et al., 1975).

These effects can be long lasting and may be permanent. Denson and colleagues (1975) reported that mothers of hyperkinetic elementary-school-aged children smoked more than mothers of nonhyperkinetic age-matched control or dyslexic children. In a study examining 452 four-year-old children in Seattle, Streissguth and colleagues (1984) reported impaired attention and orientation in children whose mothers reported that they smoked during pregnancy. Fried and colleagues (1989) reported that three-year-old children of smoking mothers had

impaired verbal memory and impaired general cognitive performance on the McCarthy Scale of Children's Abilities. Later, Fried and colleagues (1992) identified deficits in cognitive and receptive language abilities in 136 five-year-old and 137 six-year-old children of smoking mothers. Additionally, in a follow-up study of 127 six-year-old children, Fried (1992) reported significant deficits in verbal memory, increased impulsive behavior which lead to deficits on response inhibition tasks and increased errors of commission on sustained vigilance tasks. Hyperactivity and impaired visual and auditory vigilance performance (increased errors of commission and impulsive responding) has also been reported in children 4-7 years old (Kristjansson et al., 1989) and in auditory processing in 110 children 6-11 years old (McCartney et al., 1994). Dunn and colleagues (1976; 1977) reported effects of maternal smoking on children aged 6½ on both physical development, stature, and intellectual development (1977). Additionally, maternal-smoking-related hyperactivity has been significantly associated with increased conduct disorders in 1265 children followed from birth to age 15 in New Zealand (Fergusson et al., 1993) and in 177 boys aged 7-12 in a 6-year longitudinal study conducted in Chicago (Wakschlag et al., 1997). In another long-term study, Fogelman and Manor (1988) reported that while maternal-smoking-related deficits in height were found in boys but not girls by age 16, deficits in overall intellectual achievement were still found in both sexes at age 23. Perhaps even more disturbing, in an Atlanta study examining 221 children diagnosed with idiopathic mental retardation and 400 control children, Drews and colleagues (1996) reported that children whose mothers smoked at

least one pack a day during pregnancy had more than a 75% increase in the occurrence of idiopathic mental retardation, even when controlling for potential confounds such as maternal age at delivery, birth weight, race, maternal education, economic status, parity, and alcohol use. Drews (1996) argues that maternal smoking may be a preventable cause of mental retardation. In addition, children (particularly adolescent girls) of smoking mothers show evidence of greater risk of subsequent drug use including smoking later in life (Kandel et al., 1994).

Costs to Society

It appears obvious that smoking during pregnancy incurs a high societal cost. In a recent meta-analysis of the literature on smoking-related birth defects, Di Franza and Lew (1995) reported that smoking during pregnancy accounts for an estimated 19,000 - 141,000 incidents of tobacco-induced miscarriages; 32,000 - 61,000 incidents of low birth weight; 14,000 - 16,000 smoking-related admissions to the neonatal intensive care unit (NICU); 1,900 - 4,800 postnatal deaths due to perinatal complications; and 1,900 - 4,800 cases of Sudden Infant Death Syndrome (SIDS). These incidents of fetal death and increased health care costs, longer hospital stays and greater life support requirements for both infant and mother have been estimated to cost more than $60 million per year (Aronson et al., 1993). These figures may underrepresent the total contribution of smoking during pregnancy because actual biochemical markers of maternal smoking (maternal and fetal cord blood levels of cotinine, carboxyhemoglobin, and thiocyanate (Mercelina-Roumans et al., 1995; Mercelina-Roumans et al.,

1996) indicate that pregnant women are smoking more than they self-report (Pley et al., 1991). Even when potential confounding variables (such as low socioeconomic status, poor nutrition and perinatal care, and increased risk-taking behavior) are accounted for, smoking during pregnancy still has a major effect on health and behavior of offspring (Brooke et al., 1989; Fried et al., 1992; Levin et al., 1998; Morrison et al., 1993; Slotkin, 1998). Additionally, a high proportion of smokers tend to be poly-substance abusers. These other substances may have effects by themselves and in combination with nicotine (Cutler et al., 1996; Evenden et al., 1993; McGivern et al., 1996; Sobrian et al., 1995). Therefore, in addition to epidemiologic studies of the effects of cigarette smoking in humans, animal models are important to gauge and elucidate effects of maternal tobacco smoking (specifically) on offspring in the absence of potentially confounding covariables mentioned above.

Animal Models of Cigarette Smoking

Nicotine = Cigarette Smoking?

Despite the overwhelming epidemiologic evidence regarding the health risks of exposure to cigarette smoke *in utero*, it is critical to conduct controlled experiments to determine causality. Animal models offer a valuable tool to conduct such experiments, but the adequacy of an animal model for human smoking behavior must be addressed. Smoke derived from smoking tobacco cigarettes is an extremely complex mixture of compounds. Cigarette smoke aerosol is a lightly charged, highly concentrated matrix of submicron particles contained in a gas with each particle being a multicompositional collection of

compounds arising from distillation, pyrolysis, and combustion of tobacco which is contained in a matrix of atmospheric gases (nitrogen, oxygen, carbon dioxide, carbon monoxide, hydrogen, argon, and methane). Inside a burning cigarette, a large variety of chemical and physical processes are occurring in an oxygen-deficient, hydrogen-rich environment, with temperatures as high as 950°C. There are two major regions inside the burning zone of the cigarette where products are released: a heat producing combustion zone, and a pyrolysis/distillation zone just downstream of the combustion zone. The vast majority of organic smoke products are formed in the pyrolysis/distillation zone (Dube et al., 1982). Approximately 4,000 distinct compounds have been identified in tobacco smoke (*see Tables 1 and 2*). One or many of these compounds may have adverse effects on mother and fetus.

Studies examining exposure of animals to cigarette smoke (Bertolini et al., 1982) and infusions of smokeless tobacco have produced mixed results with some of these studies finding effects in animals that are similar to those found in humans and some finding few or no effects (Paulson et al., 1993). These equivocal findings may result from differences in animal dosing, efficacy of administration, and clearance rates across species and strains. Nicotine, the principle addictive pharmacologic agent in tobacco also has been identified as the principle behavioral teratogenic agent (Riley et al., 1987; Skalko, 1989; Vorhees, 1989) through numerous reports (e.g., Benowitz, 1986; Levin et al., 1998; Mactutus, 1989; Murrin et al., 1987; Navarro et al., 1989a; Slotkin, 1992; USDHHS, 1988). While other components of cigarette smoke may have

deleterious effects on the developing fetus (e.g., HCN, carbon monoxide) the above reports clearly indicate that nicotine, by itself, is sufficient and necessary to cause a spectrum of effects similar to those found in offspring of human smokers. These findings are particularly important because of the increasing use of alternative forms of nicotine administration as therapy for smoking cessation, especially if given to a pregnant woman who wishes to quit smoking. Nicotine, when administered to pregnant rats, results in a spectrum of effects that mimic those effects found in offspring of human smoking mothers. These effects include growth retardation (Cutler et al., 1996; Paulson et al., 1994; Paulson et al., 1993; Peters et al., 1982; Slotkin et al., 1987b; Sobrian et al., 1995), increased likelihood of hyperactivity (Richardson et al., 1994; Tizabi et al., 1997), memory deficits as indexed by performance in various mazes such as the Morris water maze, radial-arm maze, or open field maze (Cutler et al., 1996; Levin et al., 1996b; Martin, 1986; Paulson et al., 1993; Peters et al., 1982; Sobrian et al., 1995; Sorenson et al., 1991), impaired avoidance learning (Bertolini et al., 1982; Paulson et al., 1993; Peters et al., 1982; Sobrian et al., 1995), and impaired operant conditioning (Martin et al., 1971; Martin et al., 1982).

Routes of Administration

The next important consideration in evaluating animal models of cigarette smoking is route of administration to the animal subjects. Several routes of administration have been employed in previous studies. These routes include: inhalation of cigarette smoke; oral gavage of smokeless tobacco extract; addition

of nicotine to drinking water; injection of nicotine, either via single or multiple daily injections; or continuous infusion, usually by osmotic minipump or by implanted cannulae. Each of these routes of administration carries with it important methodologic considerations (Murrin et al., 1987). Normally in humans smoked tobacco is self administered many times per day. This form of administration results in transient peak boli of nicotine throughout the course of the day overlaying a steadily increasing level of nicotine in the blood (Benowitz, 1986; Benowitz et al., 1993; Benowitz et al., 1994). In addition to nicotine, tobacco smoke contains many other potentially pharmacologically-active compounds, such as carbon monoxide and cyanide (Mactutus, 1989). These compounds, in particular, as w ell as nicotine cause transient hypoxia and ischemia through interference with oxygen delivery (increased levels of carboxyhemoglobin) and uterine and placental vasoconstriction (Martin et al., 1971). Injected nicotine replicates this bolus type of administration. However, many investigators inject very large doses of nicotine only once or twice per day. This large bolus of nicotine results in peak plasma concentrations that far exceed any found in human smokers (Benowitz et al., 1994; Levin et al., 1993b; Mercelina-Roumans et al., 1996) even when different rates of metabolism and clearance in animals are taken into account. Additionally, such high concentrations of nicotine result in increased placental vasculature constriction resulting in hypoxic injury not seen with lower or sustained nicotine concentrations (Benowitz, 1986; Dow et al., 1975; Levin et al., 1993b; Muneoka et al., 1997; Navarro et al., 1988; Slotkin et al., 1987; Slotkin et al., 1987a;

Slotkin et al., 1987b; Werler et al., 1985). In pilot studies undertaken by the author some rats injected daily with nicotine (single daily subcutaneous injection of 1.0 mg/kg nicotine in a volume of 1.0 ml/kg) went into transient respiratory crisis. Some of these animals required supportive intervention while others spontaneously recovered after several minutes. Studies examining effects of experimentally-induced hypoxia and effects of injected nicotine have found behavioral and biochemical similarities (Martin et al., 1971; Slotkin, 1992). Finally, the stress of repeated handling and injecting pregnant rats has been shown to have additional effects with behavioral and biochemical sequelae (Bertolini et al., 1982; Peters, 1986a; Peters, 1986b).

Addition of nicotine to drinking water avoids these bolus injection problems and is simple and noninvasive. However, when this method is used, rats become hypodipsic and hypophagic (Murrin et al., 1987). This reduction in feeding and drinking behavior and resultant poor nutrition has obvious deleterious effects on mother and offspring and may confound interpretation of subsequent behavioral or cognitive outcomes. Additionally, oral nicotine bioavailability is poor. Approximately 45% of nicotine is absorbed in this manner and much of that is metabolized through first-pass metabolism by the liver (Benowitz et al., 1993).

Constant, low level infusion of nicotine throughout gestation circumvents the shortcomings mentioned above. Grunberg and colleagues have pioneered the use of osmotic minipumps as a technique to chronically administer nicotine to rats and have continued to successfully use this technique to reveal new

behavioral and biological findings of effects of nicotine that have been predictive of effects later confirmed in human studies (e.g., Acri et al., 1995; Acri et al., 1994; Faraday et al., 1998a; Grunberg, 1982; Grunberg, 1985; Grunberg, 1992; Grunberg et al., 1984; Grunberg et al., 1987; Winders et al., 1990). Specifically, for nearly 20 years Grunberg and colleagues have used Alzet (Alza Corp., Palo Alto, CA) osmotic minipumps to administer nicotine or saline for various durations to rats. This method of drug administration avoids the repeated stress of daily injections and provides a slow, continuous infusion.

This method does not, however, precisely mimic the topography of nicotine administration via cigarette smoking in which multiple boli of nicotine are overlaid on top of steadily increasing "background" levels — low during the night and increasing during the course of the day (Benowitz et al., 1994). Overall, however, this method provides an ideal balance of factors and is considered an excellent animal model of human nicotine intake (Levin et al., 1993b; Murrin et al., 1987; Navarro et al., 1988; Slotkin et al., 1987a; Slotkin et al., 1987b). In addition, nicotine's chronic as opposed to acute effects may be particularly relevant to understand behaviors of heavy smokers who are likely to maintain nicotinic cholinergic receptors in a chronically desensitized state as a result of frequent and intensive nicotine self-administration (Benwell et al., 1995). In addition, this particular method of drug administration is similar to some of the new nicotine replacement therapies (e.g., the nicotine patch). This consideration may be particularly relevant because women who wish to quit smoking during pregnancy may be counseled by their physicians to begin nicotine replacement

therapy during pregnancy (Levin et al., 1998; Slotkin, 1998).

A range of dosages has been evaluated by Grunberg and colleagues (including 2.5, 5.0, 6.0, 9.0, and 12.0 mg/kg/day) and they have found that these dosages result in reliable behavioral effects that parallel effects in humans that smoke (e.g., Grunberg, 1982; Grunberg, 1992; Winders et al., 1990; Winders et al., 1998). Of these dosages, 6.0 and 12.0 mg/kg/day establish clear dose-response effects in adult rats on body weight and feeding, locomotion, acoustic startle response, and pre-pulse inhibition of the acoustic startle response (see sections below). In addition, blood samples have been assayed for nicotine and cotinine to establish the meaningfulness of these dosages (Winders et al., 1998). Pharmacokinetic studies suggest that 1-2 pack per day smokers (20-40 cigarettes a day) have venous plasma cotinine levels of about 50 ng/ml and arterial plasma levels of approximately 100-150 ng/ml (Benowitz, 1986; Benowitz et al., 1993; Benowitz et al., 1994). Investigations with rats have used trunk blood which contains mostly arterial blood. Published reports with male rats suggest that the 6 mg/kg/day minipump preparation yields plasma nicotine levels of approximately 100-150 ng/ml, similar to the average human smoker (Richardson et al., 1994; Winders et al., 1998). Additionally, exposure to the 12 mg/kg/day minipump preparation results in plasma nicotine levels that range from 300-450 ng/ml in male rats (Winders et al., 1998). This plasma level is higher than that found in the average smoker. However, a ratio of between 4:1 (for brain nicotine levels) and 8:1 (for peripheral nicotine levels) should be taken into consideration when comparing drug plasma levels in rat with those found in

humans because rats metabolize nicotine at a much higher rate than humans (Plowchalk et al., 1992). Finally, Mercelina-Roumans and colleagues (1995; 1996) have measured venous plasma cotinine levels in mothers who smoke and in their neonates' cord blood. They reported that cotinine easily crosses the placental barrier in a positive linear relationship to maternal smoking behavior and that there seemed to be a limit of at least ten cigarettes smoked per day to reach detectable levels in neonate cord blood. Additionally, Peacock and colleagues (1991), have suggested a threshold of 13 cigarettes a day to be a minimum threshold to detect effects of cigarettes smoking on birth weight. Therefore, the dosages used in this experiment fall within these ranges and are considered were appropriate and sufficient.

Effects on Fetus

But how does nicotine administration affect development of the fetus? As mentioned above, nicotine administration during pregnancy results in lower birth weight and retarded growth in offspring. However, when nicotine is administered during pregnancy at dosages too low to bring on these gross morphologic effects, cognitive and behavioral deficits can still be measured (Fung, 1989; Navarro et al., 1989b; Slotkin, 1992). This fact indicates that the brain is particularly sensitive to the effects of nicotine during development. One method used to track alterations in brain development is ornithine decarboxylase (ODC), a key enzymatic marker for repair of cell damage (Gillette et al., 1991; Slotkin, 1998; Vendrell et al., 1991). Chronic infusion of 6 mg/kg/day of nicotine to rats causes pronounced and widespread increases in ODC activity levels during

gestation and throughout early postnatal life and is associated with actual loss of brain cells as indicated by lower levels of DNA in each brain region (Navarro et al., 1989b; Slotkin et al., 1997; Slotkin et al., 1987b). This deficit in DNA levels caused by prenatal nicotine exposure persists throughout the brain "growth spurt" period (Slotkin et al., 1987b) and recovery of cell numbers occurs only after neurogenesis has been completed, indicting that neurons have been replaced by glia (Slotkin, 1998). Additionally, it has been demonstrated that nicotine administration can increase levels of the protooncogene *c-fos* (Nomikos et al., 1995; Slotkin et al., 1997). This gene triggers apoptosis, or programmed cell death, in otherwise healthy cells (Slotkin, 1998) further reducing numbers of brain cells and delaying neuronal maturation (Roy et al., 1994).

A second possible method by which nicotine alters neural development is to change "cellular tuning" of individual and populations of neurons. During the rat's 22-day gestation period, the last two weeks are a time of neuron mitosis and migration. This is a time of "cellular tuning" during which basal neurotransmitter levels are adjusted and responses to those levels of transmitter are set (Slotkin, 1992). During this time neural cells are dividing and making connections with other cells in the brain. Once connections are made and nerve impulses are transmitted, mitosis stops and differentiation takes place (Seidler et al., 1994; Slotkin, 1998). Slotkin (1997) reported that prenatal nicotine elicits delayed neurotoxicity by altering both the timing of cellular differentiation and programming of cell death. Specifically, exposure to nicotine during this period causes cells to prematurely cease active division and undergo differentiation.

Memory

History

The study of memory has its roots as far back as ancient Greece when the philosopher Plato made his bird-brain analogy (Eysenck et al., 1995). In this analogy, memory was likened to a birdhouse, the memories themselves being the birds. During the process of recall, one need only enter the birdhouse and catch the correct bird. Errors in recall were attributed to catching the wrong bird. In this model, memory is a monolithic system, having no separate parts devoted to encoding, storage, or retrieval of the information to be remembered. Likewise, there were no attempts made to isolate different types of memory; memory was memory. This model for memory lasted for centuries.

The first attempt to suggest that memory might not be such a monolithic system came when William James used the term "primary memory" (James, 1890) to refer to those short-lasting ideas or thoughts that are present in the mind in the absence of the events that triggered them. However, the intensive study of short and long term memory did not occur until Hebb (1949) raised the possibility of two different memory systems: long- and short-term memory. A few years later, Broadbent (1958) produced his seminal information processing model of short-term memory. In this model, short-term memory consisted of two subsystems: the S system, capable of storing sensory information from many sources for a very short period of time, and the P system which had a limited capacity for processing information. This model was further modified by Atkinson and Shiffrin's multi-store model (Atkinson et al., 1968). In this model,

there are three memory stores: a sensory store, a short-term store, and a long-term store. Theses stores are differentiated by longevity of the memory trace, and amount of information that can be stored. The sensory store can hold a variable amount of information, but can hold that information for only an extremely short period of time, on the order of milliseconds (ms). The short-term memory store can hold a small number of items (ideally 7 ± 2) for a short amount of time, approximately several tens of seconds (Miller, 1956). Additionally, short-term memory is susceptible to interference and decay (Baddeley, 1986). In other words, the information that is being encoded can be mis-encoded by the short-term memory store by presentation of conflicting or interfering stimuli, and for that information to be used, it must be rehearsed, or refreshed constantly or the memory trace decays and the memory is lost. The long-term memory store can hold a seemingly infinite amount of information for an extremely long time (i.e., years). The multi-store model, based closely on the computer model of information processing, became a most popular and influential model to explain memory processes in the 1970's, yet, it too has its problems. For example, nowhere in this model is the location of any of these stores explained. There could be only one short-term memory store that processes and holds all short-term memories or there could be several that are responsible for storing memory information of a specific type or based on a specific sensory modality. Additionally, as stated in the model, information is transferred into long-term stores through short-term memory, yet the model does not explain the existence of amnesia patients who have little or no short-term memory ability, yet can still

process certain information into long-term stores (Baddeley, 1995).

To address some of the shortcomings of the multi-store memory model, Baddeley and Hitch (Baddeley, 1995; Baddeley, 1986; Baddeley et al., 1974) suggested the working memory model. In this model, the unitary short-term memory store was replaced by a three-part, inter-functional working memory store. This working memory store is postulated to consist of a central executive that acts as an attentional controller to direct cognitive and attentional resources to two slave systems: an articulatory or phonological loop and a visuospatial scratchpad or sketchpad. The articulatory or phonological loop is primarily responsible for encoding verbal or spoken information, particularly in the acquisition of language. The visuospatial sketchpad is primarily responsible for encoding and refreshing of visual and spatial information (Baddeley, 1995; Eysenck et al., 1995). Separating the functions of these two working memory subsystems explains how patients with specific neurological problems can still learn new tasks and encode new information. For example, patients with a defective phonological loop are thought to still have a functional central executive and visuospatial scratchpad and are not expected to show complete inability to learn and store information into the long-term store (Baddeley, 1995; Schacter et al., 1995).

By the 1970s, the theory that memory consists of multiple stores was well-established. It was even accepted that each of those stores consisted of multiple substores, or subunits (Norman, 1970; Schacter et al., 1995). The task then was to identify the underlying neurobiological substrates for each of these

stores. This section discusses the neurobiological processes underlying the visuospatial sketchpad of the working memory model because this portion of working memory lends itself well to investigation in an animal model. Both of the mazes used in this experiment — water maze and radial-arm maze — test visually-cued memory performance (Decker, 1995; Hodges, 1996; Morris, 1981; Olton et al., 1977). The visuospatial sketchpad, like working memory itself, is divided into two different subsystems located in two different brain regions: a system for object (or feature) processing and a system for spatial processing (Sawaguchi et al., 1994; Wilson et al., 1993). It is hypothesized that mazes task the spatial processing system. Radial-arm maze is a food-motivated task and water maze utilizes an "escape from an aversive situation" paradigm (*see Methods below*). Both types of maze were used to increase the likelihood that findings reflect spatial memory *per se* rather than reflecting either appetitive changes or stress responses as a result of prenatal nicotine exposure. The passive avoidance shuttlebox test was included to evaluate more general cognitive performance (Decker, 1995). Therefore, this experiment examined the effects of prenatal exposure to nicotine on two types of spatial working memory tasks as well as a task of general cognitive performance.

Relevant Neuroscience

Studying memory in an animal model immediately presents a certain number of difficulties at the outset. For example, one cannot ask a monkey to learn a list of words or digits (digit-span), one of the most widely used forms of human memory functioning (Eysenck et al., 1995). Another argument against

the utility of using animals to study cognition is that, according to Skinner (1984), cognition and all other such "private" events cannot be directly observed and, therefore, provide no "explanatory value." However, Terrace (1984) points out that both in human and animal cognition it is assumed that the normal state of affairs is unconscious activity and thought and therefore that memory can be studied in animals. Indeed, additional processes must be postulated in order to account for an individual's consciousness of activities, perceptions, thoughts, and so on.

With regard to working memory, paradigms exist that can be used with animal subjects. The classic delayed-response task in animals is as follows. The experimental subject is shown a food (reinforcing) item. The food item is then hidden from view by an opaque screen for a variable delay of several seconds. The subject is then allowed to choose the location of the food out of two or more locations. While the food is out of sight, the subject must hold the location of the food item in mind for several seconds in order to correctly choose its location. In order to choose the correct location of the food, the experimental subject animal must choose a location based on the memory of the food, and not based on the stimulus of the food itself. Because the location of the food item is randomly moved between locations with each trial, prior experience with the test cannot predict the location of the food. Therefore, the animal must use memory of the location of the food for its choice.

Goldman-Rakic and colleagues have studied working memory extensively using a variant of this task. In the Goldman-Rakic model, working memory is

defined in its most elementary form, as the ability to keep events in mind for short periods of time, and has been studied in non-human primates by delayed-response paradigms (Goldman-Rakic, 1995). In this paradigm, an oculomotor version of this classic paradigm is used. In the oculomotor version, the animal is trained to fixate on a point on a computer screen. The animal remains fixated on that spot during a brief (0.5 s) presentation of a stimulus (a spot of color at a specific orientation: e.g. 45° from the fixation point) and for a 3-5 second delay period, after which the animal must look in the target direction to receive a reward. This paradigm allows control over latency to react, exact, single-cell recording of events in the frontal cortex, and precise control over location of presentation of stimulus in the visual field.

Using the oculomotor delayed-response paradigm and single-cell recording of neurons in the prefrontal cortex, Goldman-Rakic and colleagues (Funahashi et al., 1989; Goldman-Rakic, 1990; Goldman-Rakic, 1993; Goldman-Rakic, 1995; Goldman-Rakic et al., 1991; Goldman-Rakic et al., 1990; Sawaguchi et al., 1994) have identified memory fields, defined as maximal firing of a neuron to the representation of a target, in one or a few locations of the visual field, with the same neuron always coding the same location. Not only do these cellular circuits hold memories for specific locations, but they are mutually inhibitory to locations oriented at 90°. That is, for a cell-circuit that fires maximally during the delay period for a stimulus at 225°, that same network of cells decreases firing rates below baseline during the delay for a stimulus at 135° or 315°. Just as in the visual system, this allows the memory system to be

precisely tuned and focused for each particular stimulus. Finally, repeated recordings from the same network of cells show that the increase in firing rate is consistently correlated with the onset and offset of the memory task. That is, firing increases as soon as the stimulus is removed, and decreases once the response has been made.

The neurotransmitter circuitry (Goldman-Rakic, 1990; Goldman-Rakic, 1993; Goldman-Rakic, 1995) as well as cortico-cortical connections (Goldman-Rakic et al., 1991) have been partially identified by Goldman-Rakic and colleagues in primates. Particularly of interest in spatial working memory is the connection of the hippocampus with the prefrontal cortex. Early work of Olton and others have identified the key role that the hippocampus plays in spatial memory processing (Olton, 1983; Olton et al., 1979a). Goldman-Rakic and colleagues have identified largely bidirectional projections between the hippocampus and areas of the prefrontal cortex in primates (Goldman-Rakic et al., 1984). Additionally, functional studies have identified similarities between behavioral effects of hippocampal damage (Zola-Morgan et al., 1985) and prefrontal cortex damage (Brozoski et al., 1979; Goldman et al., 1970). Similar results have been reported during delay-requirement tasks when the prefrontal cortex (Funahashi et al., 1989) or hippocampus (Watanabe et al., 1985) has been damaged. Similar neuroanatomic structures exist in the rat (Kolb, 1990) and the rat is an adequate model for examining prefrontal cortex (Kolb, 1990b), specifically cognitive and memory performance tasks (Kolb, 1990a; Kolb et al., 1994). Major input to the prefrontal cortex arises from the medial dorsal nucleus

of the thalamus and additional inputs arise from the ventral mesencephalic tegmentum (VMT), the nucleus accumbens, substantia nigra, and the CA1 region of the hippocampus (Kolb, 1990a).

Among other transmitter systems (*e.g.*, cholinergic, noradrenergic), it appears that the dopaminergic system plays a key role in the ability of the animal to perform spatial working memory tasks. Many of these dopaminergic prefrontal cortex neurons arise from the striatum, VMT, and substantia nigra (Kolb, 1990a; Thierry et al., 1988). Numerous investigators have shown that direct stimulation of these areas will release dopamine in the prefrontal cortex (Garris et al., 1993; Glowinski et al., 1988; Thierry et al., 1988). This model has been borne out empirically. Experiments that have examined performance in primates and rodents using various types of mazes and visuo-spatial tasks have found that when dopamine levels are increased in the frontal cortex memory performance is improved (Levin, 1988; Packard et al., 1994; Puglisiallegra et al., 1994a; Puglisiallegra et al., 1994b; White, 1988; White et al., 1993). Conversely, when dopamine neurons are released from tonic endogenous opioid peptide inhibition (Izquierdo et al., 1980) or from morphine-based inhibition (Castellano et al., 1994) memory performance also improves. However, when dopamine levels are too high, memory performance can be compromised (Murphy et al., 1996; Verma et al., 1996). When dopamine levels are reduced (Brozoski et al., 1979), either by means of prenatal exposure to a toxin (Lakshmana et al., 1994), or by selective dopamine neuron ablation using 6-hydroxydopamine (Grigoryan et al., 1996; White, 1988), memory performance is compromised but can be

restored by addition of dopamine or dopamine-releasing agents such as amphetamine (White, 1988) or nicotine (Grigoryan et al., 1996). Likewise, when examining the role that specific dopamine receptor subtypes play in memory performance, it is clear that stimulation of D1 receptors enhances memory performance (Hersi et al., 1995; Sawaguchi et al., 1988; Williams et al., 1995), while antagonism of these receptors inhibits it (Broersen et al., 1994; Broersen et al., 1995; Didriksen, 1995; Doyle et al., 1993; Sawaguchi et al., 1991; Sawaguchi et al., 1994). The picture, however is not as clear for D2 receptors (Arnsten et al., 1995; Puglisiallegra et al., 1994a; Puglisiallegra et al., 1994b). Stimulation of D2 receptors seems to impair memory performance (Arnsten et al., 1995; Bushnell et al., 1993; Uchihashi et al., 1994; Verma et al., 1993), whereas antagonism seems to enhance it or have no effect (Galkina et al., 1996; Sawaguchi et al., 1994; Zarrindast et al., 1992). It is thought that low doses of D2 agonists stimulate inhibitory autoreceptors, whereas higher doses stimulate post synaptic D2 receptors that have an excitatory effect (Zarrindast et al., 1992), but this improvement is accompanied by "hallucinatory-like" behaviors and dyskinesias (Arnsten et al., 1995). Based on the preceding discussion, it is clear the dopamine in the prefrontal cortex plays a key role in spatial working memory performance, particularly D1 receptors, and that alterations in this system may underlie changes in memory performance. This experiment examined changes in D1 receptor number in the medial prefrontal cortex because of its potential role in memory performance.

Nicotine has been reported to play an important role in memory

performance in subjects that either were compromised or displayed memory performance baselines that were impaired, as compared with adult controls. Specifically, animals that showed improved memory in response to nicotine either were: very young (Smith et al., 1996); very old (Arendash et al., 1995a; Arendash et al., 1995b; Levin et al., 1996a; Meguro et al., 1994; Widzowski et al., 1994); treated with nicotinic or dopaminergic system antagonists (Levin et al., 1996; Nitta et al., 1994; Widzowski et al., 1994); or neurosurgically altered (Grigoryan et al., 1996; Levin, 1993a; Levin et al., 1993c). Nicotine's actions to improve memory appeared to consist of restoring performance to baseline levels after damage or compromise. This finding is mirrored in the human attentional literature in that the clearest cognition-enhancing nicotine effects are evident as amelioration of attentional deficits in compromised individuals such as Alzheimer's patients (Levin et al., 1994; Newhouse et al., 1988; Sahakian et al., 1991) and attention deficit hyperactivity disorder (ADHD) patients (Levin et al., 1993; Levin et al., 1994).

Nicotine appears to act on memory by means of cholinergic innervation of areas that synapse in the frontal cortex, such as the VMT, nucleus accumbens and striatum (Kolb, 1990a), as well as via nicotine's actions to release dopamine postsynaptically (Balfour, 1994; Balfour et al., 1993; Levin, 1992; Levin et al., 1994; Levin et al., 1995; Summers et al., 1994; Summers et al., 1995). Therefore, it was hypothesized that nicotine administration during gestation would alter memory performance in the offspring due to alterations in the nicotinic cholinergic receptor system and its down-stream effects on the

dopaminergic system such as changes in receptor number (Fung et al., 1989; Gelbard et al., 1990; Muneoka et al., 1997; Navarro et al., 1988). Alterations in these systems may be revealed by alterations in memory performance in the offspring. It also was hypothesized that, as a result of prenatal nicotine exposure, changes in memory performance would be accompanied by changes in D1 receptor density in the area of the prefrontal cortex associated with spatial working memory performance: the medial prefrontal cortex (Broersen et al., 1994; Broersen et al., 1995; Murphy et al., 1996).

"Nicotinic cholinergic receptors are involved intimately in the regulation of catecholaminergic function in the CNS, so it is not surprising that... dopaminergic synaptic transmissions also are affected adversely by fetal nicotine exposure" (Slotkin, 1998, p 935). Dopamine, in particular, is of interest because of its role in memory performance. Prenatal nicotine decreased the total number of dopaminergic (DA) receptors in brains of male offspring as indexed by alterations in dopamine metabolites that persist throughout gestation and into adulthood (Ribary et al., 1989). Maternal infusion of nicotine (6 mg/kg/day) via osmotic minipump reduced tyrosine hydroxylase activity and altered catecholamine (specifically dopamine) content and utilization in cerebral cortex of offspring (Muneoka et al., 1997; Navarro et al., 1988) indicating cortical dopaminergic system inactivity as a result of prenatal exposure to nicotine. This effect was found immediately after birth but tended to disappear over the next few weeks, but reappeared by early adulthood (Navarro et al., 1988) indicating that prenatal exposure to nicotine causes alterations in dopamine

neurotransmission, but also that this effect changes over the life of the offspring. Additionally, infusion of 6 mg/kg/day nicotine during pregnancy reduced number of D2 receptors in ventral tegmental area (VTA) as well as striatum, but not nucleus accumbens or frontal cortex and increased numbers in substantia nigra (Richardson et al., 1994). However, maternal infusion of a very low dose of nicotine (1.5 mg/kg/day) for 5 days increased the number of nicotinic receptor binding sites and elevated dopamine levels in the striatum of male offspring (Fung et al., 1989). A two-week infusion of this very low dose was associated with an increase in striatal nicotinic and spiperone (D2/5HT_2 antagonist) binding sites and was associated with a potentiation of locomotor hyperactivity induced by nicotine injection (Fung et al., 1988). The two-week infusion was also associated with an enhanced ability of striatal tissue slices from the nicotine-exposed pups to synthesize [^{3}H]dopamine from [^{3}H]tyrosine and with an increased the rate of striatal dopamine turnover in male offspring (Fung, 1989). This effect was not found in nucleus accumbens (Fung et al., 1991; Fung et al., 1992). These conflicting results may be a result of the very low dose that was utilized by these investigators. This experiment used higher dosages and it was hypothesized that the findings would be consistent with those of researchers mentioned above who used 6 mg/kg/day nicotine. Further, it was hypothesized that because nicotine alters dopaminergic neurotransmission in areas that synapse in the medial prefrontal cortex, and that because dopamine in this area is involved in memory performance, prenatal exposure to nicotine would alter memory performance.

As discussed in the previous section, working memory can be studied in an animal model. Further, the dependent variables used in the present experiment also tested a form of spatial working memory (e.g., the water maze and radial-arm maze) as well as an index of non-spatial memory (passive avoidance) and are widely used to test memory performance in the rodent model.

Dopamine

Physical Chemistry

Dopamine ($C_8H_{11}NO_2$) is a catecholamine; *i.e.*, a catechol base (a benzene ring with two -OH groups attached at carbons 3 and 4), with an ethylamine ($-CH_2—CH_2-NH_3$) group attached to carbon 1. Dopamine also can be described as phenylethylamine with two -OH groups attached at carbons 3 and 4 (*see Figure 1*) .

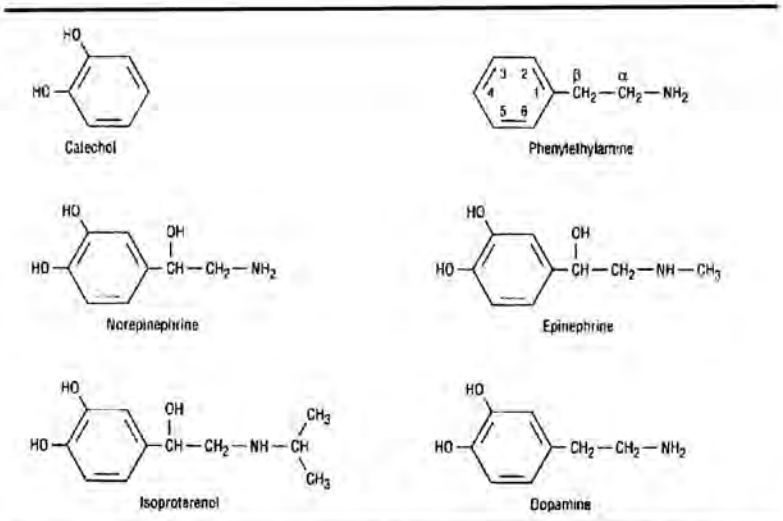


Figure 1 Structure of dopamine and related compounds

Dopamine is a white or almost white crystalline powder. It is freely

soluble in water, in methyl alcohol, and in solutions of alkali hydroxides; soluble in alcohol; sparingly soluble in acetone and in dichloromethane; practically insoluble in ether and in chloroform. The United States Pharmacopeia (USP) gives the pH of a 4% solution of dopamine hydrochloride (the form in which it is administered clinically) as between 3.0 and 5.5.

Synthesis and Degradation

Dopamine synthesis is the first step in the synthesis of all catecholamines in the body and constitutes over 50% of all catecholamines found in brain (Cooper et al., 1991). Synthesis starts with transport of the amino acid tyrosine across the blood-brain barrier into the neuron. Tyrosine is then converted to L-dihydroxyphenylalanine (L-dopa) by tyrosine hydroxylase, the rate-limiting step in the formation of all catecholamines. L-dopa is then converted to dopamine by

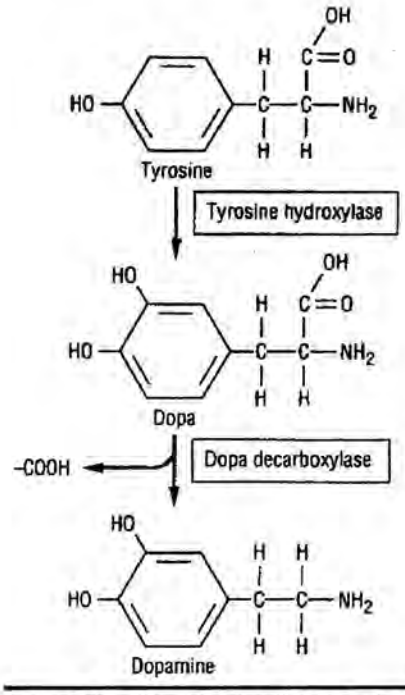


Figure 3 Synthesis of dopamine

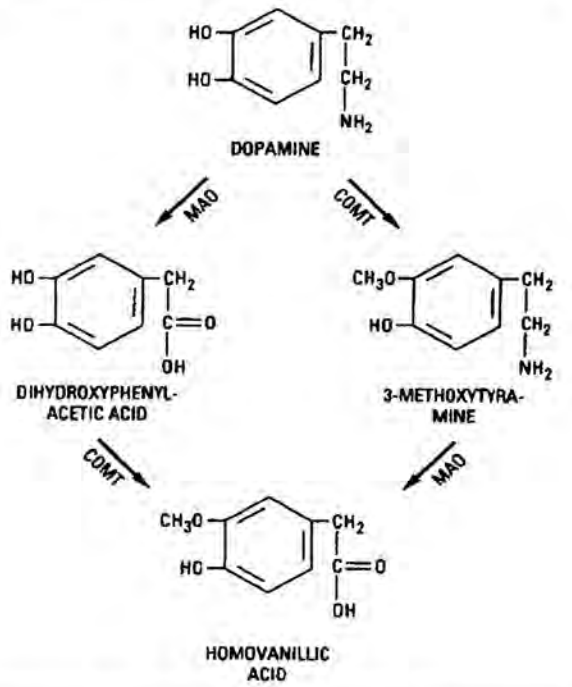


Figure 2 Metabolism of dopamine

the enzyme L-aromatic amino acid decarboxylase and transported into synaptic vesicles. In the formation of the other main catecholamines dopamine is converted to norepinephrine by dopamine β-hydroxylase, and then to epinephrine by phenylethanolamine-*N*-methyltransferase (*see Figure 2*).

Dopamine is degraded or metabolized in two ways: extracelluarly to homovanillic acid (HVA) by catechol-O-methyltransferase (COMT), and intracellularly (after re-uptake into the pre-synaptic neuron by the high-affinity dopamine transporter) to 3,4-dihyoxyphenylacetic acid (DOPAC) by monoamine oxidase B (MAO_B) (*see Figure 3*).

G-Proteins

Dopamine couples to a family of receptors known collectively as G-protein-coupled receptors (guanosine nucleotide-binding regulatory proteins) (Hedin et al., 1993; Hepler et al., 1992; Lehninger et al., 1993). G-Protein-coupled receptors are one of the most diverse families of receptor systems known. This family of receptor systems mediates actions that can: be short- or long-lasting; regulate ion channels; result in changes in growth hormone expression, gene expression, metabolism and other disparate outcomes.

G-proteins are heterotrimer complexes consisting of an α, β, and γ subunit. The α subunit has a single, high affinity site that binds guanosine triphosphate and guanosine diphosphate (GTP and GDP, respectively). When bound to GDP, the α subunit is inactive and binds tightly to the βγ subunit. When GTP binds to the α subunit, it separates from the βγ subunits and is active

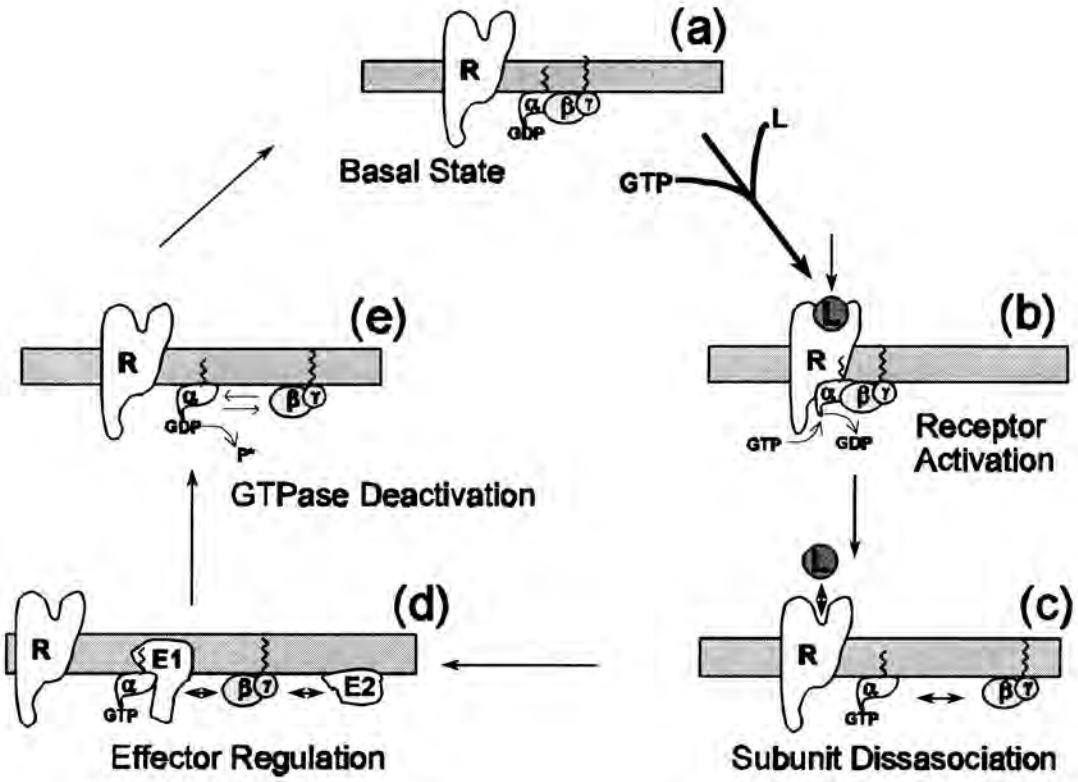


Figure 4 G-Protein-mediated transmembrane signaling.[Y] In the basal state (a), G proteins exist as heterotrimers with GDP bound tightly to the α subunit; the receptor (R) is unoccupied. Upon ligand (L) binding (b), the receptor interacts with the heterotrimer to promote a conformational change and dissociation of the GDP from the guanine nucleotide-binding site; at normal cellular concentrations of guanine nucleotide, GTP fills the site immediately. Binding of GTP to α induces a conformational change with two consequences (c). The G protein dissociates from the receptor, freeing the receptor for another liaison with a neighboring quiescent G protein. GTP binding also reduces the affinity of α for βγ, and subunit dissociation occurs. This frees α-GTP to fulfill its primary role (d) as a regulator of effectors (E1). At least in some systems, the free βγ subunit complex may also interact directly with the effector and modulate the activity of the active α subunit, or it may act independently at a distinct effector (E2). The α subunit possess an intrinsic GTPase activity (e). The rate of this GTPase activity determines the lifetime of the active species and the associated physiological response. The α-catalyzed hydrolysis of GTP leaves GDP in the binding site and causes deactivation of the active complex. The GTPase activity of α is, in essence, an internal clock that controls an on/off switch. The GDP bound form of α has high affinity for βγ; subsequent reassociation of α-GDP with βγ returns the system to the basal state (a).

[Y] Adapted from Hepler et. al 1992.

and can then regulate various effector systems. After a short amount of time, the intrinsic enzyme (GTPase) activity of the α subunit hydrolyzes the GTP to GDP. The α subunit then returns to reassociate with the βγ complex where it is stabilized in association with the receptor (*see Figure 4*).

Physiologic Roles

Dopamine receptors are divided into two families: D1 or D1-like and D2 or D2-like. The D1 receptor family includes D1 and D5 which share amino acid sequence homology. Both of these receptors are associated with G-proteins that increase the production of cAMP upon receptor stimulation. The D2 family is comprised of D2, D3, and D4 receptors. All of these receptors share amino acid sequence homology and are associated with G-proteins that inhibit or decrease the production of cAMP as well as activating potassium (K) channels and can inhibit Ca^{2+} channel activity. The D2 receptor family is most often found presynaptically (autoreceptors) and serve to decrease DA production and release and modulate DA turnover (Cooper et al., 1991).

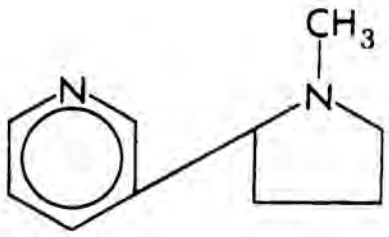
Nicotine

Tobacco-smoking is a means to self-administer the addictive drug, nicotine. The chemical name of nicotine is (S)-3-1-Methylpyrrolidin-2-yl)pyridine with the chemical formula $C_{10}H_{14}N_2$ (*see Figure 5*). Nicotine base is a colorless to pale yellow oily liquid that turns brown and gains an acrid burning odor on exposure to air. It is a naturally-occurring alkaloid obtained from the dried leaves of the tobacco plant, *nicotiana tabacum, nicotiana rustica,* and related species (*Solanaceae*). Tobacco leaves contain 2 to 8% of nicotine combined as malate

or citrate. Nicotine dihydrochloride ($C_{10}H_{14}N_2 \bullet 2HCl$), the salt form of nicotine, is a deliquescent white crystal which is extremely hygroscopic.

Nicotinic Cholinergic Receptors

The self-administration of nicotine via puffs on a tobacco-containing product results in nicotine entering the body and reaching the brain within 15 seconds (USDHHS, 1988). Once in the body, nicotine has a half-life of about two hours. The drug acts throughout the body at nicotinic cholinergic receptors (nAChRs) at muscle end plates and in autonomic ganglia, and in the brain at nAChRs that regulate and modulate many neurotransmitter and neuroendocrine systems. It is thought that nAChRs in the brain are primarily responsible for biologic rewarding effects of nicotine (Balfour, 1994)



NICOTINE

Figure 5 Structure of nicotine

The nAChRs all are similar in basic physical structure. They consist of five subunits arranged in a circle to form a channel. When an appropriate agonist binds to the receptor, the channel opens and allows the flow of positively charged ions (e.g., Ca^{2+} or Na^{+} or other cations, depending on the receptor subtype). The five α-subunits consist of different types. The α-subunits (ranging from α2 to α9) contain sites where agonists (i.e., acetylcholine,

nicotine) bind. The β-subunits (β2 to β4) are believed to be primarily structural in function. These nAChRs are distributed presynaptically in certain regions of the brain, including the ventral tegmental area (VTA), nucleus accumbens (Nacc), medial habenula, interpenduncular nucleus, hypothalamus, and hippocampus.

There are several subgroups of nAChRs that differ in structure, binding affinity for nicotine, and function. The subgroups are defined according to subunit composition. Most binding sites in the mammalian brain consist of the $(\alpha4)_3(\beta2)_2$ nAChR subset (Lindstrom et al., 1995). These receptors are found presynaptically and modulate neurotransmitter release (Lindstrom et al., 1995; Nakayama et al., 1994). A small percentage of brain nAChRs are composed in part of α7 and α8 subunits. This subset of nAChRs appears to act as agonist-gated ion channels that allow calcium to enter the cell and initiate various second messenger cascades (Lindstrom et al., 1995). The function of many of these combinations is not yet known.

Nicotine's Effects on Neurotransmitter Systems

Via actions at presynaptic $(\alpha4)_3(\beta2)_2$ nAChRs, nicotine alters activity of many different neurotransmitter systems, including those of dopamine, norepinephrine, endogenous opioids, and serotonin. Most of the work done on mechanisms underlying nicotine addiction has focused on the dopaminergic system (Corrigall, 1991) because enhancement of dopamine neurotransmission in specific brain areas is believed to be fundamental to drug reward or euphoriant effects (Di Chiara et al., 1993; Koob, 1992; Nestler, 1992), including effects of cocaine, heroin, amphetamines, and alcohol. In addition, cellular and

molecular changes that occur in these brain areas as a result of repeated drug self-administration may contribute to drug dependence and addiction (Balfour, 1994; Koob et al., 1988a; Koob et al., 1988b). Regions of the brain that mediate reward effects are phylogenetically old and respond to natural stimuli, addictive drugs, and electrical stimulation. The natural stimuli that evoke dopamine release by neurons that originate in the VTA and terminate in the NAcc are associated with behaviors that are essential to the survival of the individual and the species such as feeding, sexual behavior, birth, care of offspring, and some social behaviors (e.g., mutual grooming) (Salamone, 1994).

Tolerance to nicotine's effects is, in part, mediated by increased numbers of nAChRs (Lindstrom et al., 1995) and also depends on cellular changes in the mesolimbic dopamine system that occur with repeated nicotine self-administration (Flores et al., 1992). These changes are similar to those that occur in response to other drugs of abuse and may include increased synthesis of the enzyme tyrosine hydroxylase (the rate-limiting step in dopamine manufacture), increased rates of spontaneous firing activity in VTA neurons, and changes in the structure of cell bodies, dendrites, and axons (Beitner-Johnson et al., 1992; Gold et al., 1992; Koob et al., 1988a; Ritz et al., 1993). In the NAcc chronic addictive drug use alters G-protein levels and second messenger cascades, biochemicals that control rates of neurotransmitter synthesis and release. These neuronal-level changes reflect the fact that the mesolimbic dopaminergic neurons adjust to the repeated presence of nicotine in a compensatory way by developing tolerance.

Summary

The present doctoral dissertation research examined the effects of prenatal exposure in rats to two dosages of nicotine or saline on measures of working memory (both spatial and non-spatial), sensory gating, activity and body weight in adolescent and adult offspring. Body weight was included to verify drug administration. Locomotion was added to examine prenatal exposure effects on overall activity levels and also so that these levels could be used as a behavioral control for non-spatial memory (passive avoidance). Finally, this experiment examined dopamine D1 receptor density in the medial prefrontal cortex, an area identified as playing a role in visuospatial memory performance. This area was examined to determine if nicotine administration affected dopamine receptor density, and the extent to which any changes in this area could be correlated with changes in memory performance.

Hypotheses

Hypothesis 1: Exposure to nicotine during gestation will result in reduced body weight of both offspring and dams.

Rationale: It has been well established that nicotine administration reduces the body weight of rats (e.g., Grunberg et al., 1984; Grunberg et al., 1986; Saah et al., 1994; Winders et al., 1990). Nicotine administration during gestation has been shown to reduce the body weight of offspring (e.g., Bertolini et al., 1982; Cutler et al., 1996; Levin et al., 1998; Slotkin et al., 1993; Sobrian et al., 1995). This variable has been included to confirm effective administration of nicotine.

Hypothesis 2: Prenatal exposure to nicotine will result in impaired radial-arm maze performance (fewer correct choices made before an error is made, more total errors made, and longer latency to complete).

Rationale: Previous experiments report that prenatal nicotine exposure results in general cognitive impairment (e.g., Cutler et al., 1996) including deficits in radial-arm maze performance (Yanai et al., 1992). Other investigators, however, have reported moderate or no effects on radial-arm maze performance (Cutler et al., 1996; Levin et al., 1993b; Levin et al., 1996b; Sorenson et al., 1991). These divergent findings may be a result of different routes of administration or of low dosages of nicotine. It is hypothesized that by utilizing appropriate route and dosage levels, this experiment will reveal deficits in radial-arm maze performance.

Hypothesis 3: Prenatal exposure to nicotine will result in impaired water maze

performance (longer latencies to find the hidden platform).

Rationale: This hypothesis follows from rationale for hypothesis 2 and is based on previous work (Cutler et al., 1996; Levin et al., 1993b; Levin et al., 1996b; Yanai et al., 1992) that reported little or no effect of prenatal exposure to nicotine. Again, these experiments utilized potentially inappropriate routes of administration and dosages of nicotine and it is hypothesized that by utilizing appropriate route and dosage levels, this experiment will reveal deficits in water maze performance. Additionally, previous reports have indicated that water maze performance is compromised by altering dopaminergic and nicotinic cholinergic systems (e.g., Brandeis et al., 1989; McNamara et al., 1993).

Hypothesis 4: Prenatal exposure to nicotine will result in impaired passive avoidance performance (shorter latencies to cross, or larger proportion of subjects crossing).

Rationale: Previous reports have indicated that prenatal exposure to nicotine results in impaired or altered avoidance (both passive and active) performance (Bertolini et al., 1982; Genedani et al., 1983; Peters et al., 1982).

Hypothesis 5: Prenatal exposure to nicotine will result in altered acoustic startle response and will impair PPI (smaller amounts of inhibition on trials with pre-pulse).

Rationale: A previous report (Popke et al., 1997) has indicated that prenatal nicotine exposure may alter acoustic startle performance. Additionally,

this measure can be considered an animal model for attention, information processing or sensory-gating (Acri et al., 1991; Acri et al., 1994; Caine et al., 1992; Swerdlow et al., 1992), and it follows from epidemiologic findings in humans that report impaired attentional and sensory-gating in children of smoking mothers (Denson et al., 1975; Landesman-Dwyer et al., 1979; Streissguth, 1984) that similar results may be found in rats exposed to nicotine prenatally.

Hypothesis 6: Prenatal exposure to nicotine will result in increased locomotor activity.

Rationale: This hypothesis is based on previous findings in rats (Fung et al., 1988; Richardson et al., 1994; Tizabi et al., 1997) and in humans (Denson et al., 1975; Kristjansson et al., 1989; Sorenson et al., 1991) that prenatal exposure to nicotine resulted in higher levels of activity.

Hypothesis 7: Prenatal exposure to nicotine will result in altered D1 binding density in the medial prefrontal cortex.

Rationale: This hypothesis is based on the compilation of several factors: 1) previous reports that prenatal exposure to nicotine alters catecholaminergic systems (Fung, 1989; Levin et al., 1998; Lichtensteiger et al., 1987; Navarro et al., 1988; Ribary et al., 1989); 2) chronic stimulation of receptor systems results in altered receptor density (Cooper et al., 1991; Fung et al., 1991); 3) nicotine stimulates the DA system (Balfour, 1994; Balfour et al., 1993; Fung et al., 1991; Fung et al., 1992).

Hypothesis 8: A significant inverse correlation between D1 receptor density and

memory performance will be revealed.

Rationale: This hypothesis follows from hypothesis 7 and from previous work that indicates D1 receptors in the medial prefrontal cortex are involved in working memory performance (e.g., Goldman-Rakic, 1995; Sawaguchi et al., 1991; Sawaguchi et al., 1994).

Hypothesis 9: Effects of prenatal exposure to nicotine will be revealed differently based on age of testing.

Rationale: This hypothesis is based on previous reports in humans that effects of prenatal exposure to cigarette smoke may be long-lasting but may also disappear by adulthood (e.g., Denson et al., 1975; Fogelman et al., 1988; Fried et al., 1992; Fried et al., 1990).

Methods

Subjects:

Subject animals were male and female offspring of 15 timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). This particular strain of animals has been chosen in order to replicate and expand on previous work investigating effects of prenatal nicotine (Levin et al., 1993b; Popke et al., 1997; Tizabi et al., 1997). Animals were maintained in a temperature (23°C) and humidity (roughly 50% relative humidity) controlled environment. Light-dark cycles were 12-12 hours (lights off at 0700). Standard laboratory rat chow (Harlan Teklad, 4% Mouse/Rat Diet 7001) and tap water were available continuously in the home cage except immediately prior to and during radial-arm maze testing (see below) when food was restricted such that each rat maintained 80-85% maximum body weight (*see Figure 13*).

Fifteen timed-pregnant female rats were delivered to the animal housing facility on gestational day 2 (GD2) and were implanted on GD4 (before embryo implantation to the uterine wall) with osmotic minipumps (see below) containing either saline or one of two doses of nicotine (n=5 per group). One dam in the 12 mg/kg group was either not pregnant or spontaneously resorbed her fetuses. Fetal resorbtion has been reported to occur under high doses of nicotine (Levin et al., 1998; Slotkin, 1992). All remaining dams delivered on GD22. Two dams in the 12 mg/kg group rejected their offspring after parturition and all offspring from those dams died. Previously, dams have rejected their offspring in earlier experiments in this laboratory and in pilot studies (unpublished data) that used

lower doses of nicotine or that did not utilize nicotine at all. Therefore, it is unlikely that the dams rejected their offspring in this experiment because of nicotine administration. Litters from the remaining dams were cross-fostered 24 hours after parturition between dams within each treatment group to control litter size (n=11 per dam) and to assure continuation of nicotine administration via breast milk until the end of the effective pump-life of the minipump (21.1 days). The sex of each of the offspring was determined and each was weighed at the time of cross-fostering. Offspring were weaned on post-natal day 23 (PND 23) and moved to same-sex cages of 4-6 animals each. On PND 35 three males and three females were chosen quasi-randomly (based on body weight) from each dam for experimentation and housed two per cage. All offspring from the remaining 12 mg/kg dams (11 males and 11 females) were used. Final count for the experiment was 82 rats — 15 males and 15 females each from the 0 and 6 mg/kg groups and 11 males and 11 females from the 12 mg/kg group. All 82 animals were tested on all measures. Testing occurred during adolescence (after PND 40) and during adulthood (after PND 60). On any given testing day, only one measure was performed to reduce any potential confounds that performing multiple tests/handling session may (see *Results* section below for exact dates of each measure). This sample size was based on previous empirical investigations (unpublished pilot data), personal communications with other laboratories conducting similar work (similar experiments have been performed with as few as 7 offspring per group; personal communication, Edward Levin, Ph.D., Duke University, NC, 1997), and on power analyses to

ensure that sufficient statistical power was achieved.

Drug:

Nicotine dihydrochloride (0, 6, or 12 mg/kg/day — expressed as base) was dissolved in physiologic saline and placed in Alzet osmotic minipumps (Model 2002, Alza Corp., Palo Alto, CA). These minipumps were used because they are small (3.0 cm) and cause no discernable discomfort or distress to the rat. The minipumps administer their contents at a constant rate (0.48 µl per hour) throughout the delivery period (21.1 days), and avoid problems associated with repeated daily injections. This type of minipump also has been used successfully in previous investigations of adult and prenatal nicotine administration (Grunberg, 1982; Levin et al., 1993b; Murrin et al., 1987; Navarro et al., 1988; Ribary et al., 1989; Richardson et al., 1994; Seidler et al., 1992; Slotkin et al., 1987b).

Surgery:

Dams were randomly assigned to drug groups (n=5). Minipumps were surgically implanted. Animals were anesthetized by inhalation of Metophane™ (methoxyflurane, Pitman-Moore, Mundelein, IL). Each animal was placed into a bell jar containing gauze soaked with 2-4 ml of Metophane™. The jar was located in a ventilated hood to isolate fumes. The rat was removed from the jar when a tail pinch produced no reflex (approximately 1.5-2.0 min). A 4 cm square patch was shaved on the back of the rat. This area was then swabbed with a betadine solution (The Purdue Frederick Co., Norwalk, CT). A 2 cm horizontal midline incision was made between the withers and a subcutaneous pocket was

formed into which the minipump was inserted with the flow moderator cephalad. Incisions were closed with 9 mm stainless steel Autoclip™ wound clips (Stoelting, Wood Dale, IL). Clips are designed to fall out after several days. Pumps were not removed.

Apparatus

Video Tracking System

Each test session on the radial-arm maze and water maze was recorded using a computerized video tracking system (Polytrack System, San Diego Inst., San Diego, CA). This system consisted of a video camera mounted above the maze connected to a computer interface. Using this system each maze was traced into the computer. The system then tracks light objects against a dark background, plots the object's path onto the screen, and records, several parameters, including path length, and test session latency (*see Figure 6).*

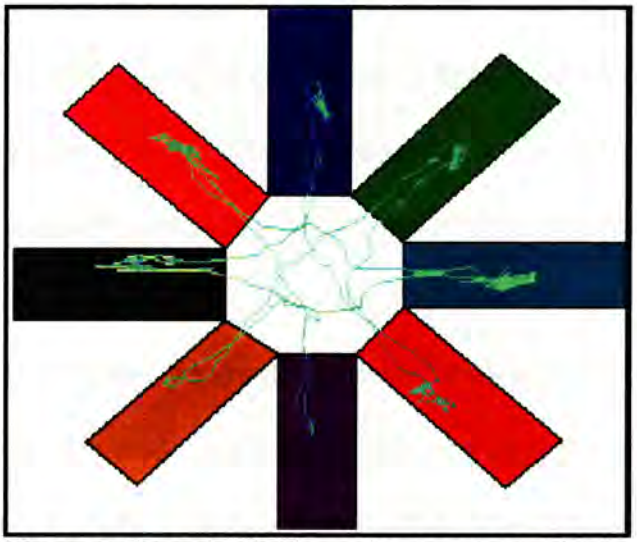


Figure 6 Example of video software output. This is an example of RAM performance, the green line denotes the path taken by the animal

Mazes

The idea that animals, particularly rats, can and do use spatial information to orient themselves in the world, and that they form cognitive maps of the world is not a new one. Tolman posited that "in the course of learning something like a field map of the environment gets established in the rats brain" (Tolman, 1943, p 192). An example of the importance of spatial memory in animal survival is the Hawai'ian honeycreeper (Kamil, 1978). This small Hawai'ian bird feeds on the nectar of flowers. Each flower only provides a small amount of nectar upon each visit the bird makes to it. To maximize the amount of food that the bird can derive from a day's feeding, the bird must remember where each flower is in its territory and visit each one several times throughout the day. This foraging behavior is common to many species of animals and is an ethologically valid measure of memory in animals (Olton, 1977).

The mazes were placed in a room with extra-maze visual cues of two types: large extra-maze objects and luminosity gradients. As explained by Kraemer and colleagues (1996), extra-maze visual cues and luminosity gradients across the maze space are presumed to provide critical spatial information to the rat necessary for accurate maze performance (Morris, 1981; Olton et al., 1979b). Large extra-maze objects consisted of experimental equipment and black-and-white squares of different patterns placed on several walls adjacent to the mazes. A luminosity gradient was created by placing spotlights around the radial-arm maze such that the "south" (arbitrary designation) wall was a brightly-lit wall painted sand color in high-gloss paint, the

"east" and "west" walls were not lit, providing a general "southward" luminosity gradient across the maze space. A luminosity gradient was created around the water maze using spotlights such that the "south" wall was brightly lit, the "east" wall was not as brightly lit and had a large black door that occluded the entire "north" half of the wall, the "north" and "west" walls were open to the rest of the room showing distal objects placed around the room (*i.e.*, other large experimental equipment, computer, experimenter, *etc.*). The experimenter stood in the same place during all water maze trials. This additional set of stimuli may be particularly important when considering the overall poor visual acuity of unpigmented rats. The luminosity gradient (*see Figure 7*), in addition to

Figure 7 Placement of the mazes and lights in the experimental room. Note the light sets used to create luminosity gradients (the overhead flourescent lights were not used during testing) and the large black door mentioned in the text

extremely large and salient visual cues (the presence or absence of a wall, the large black door in an, otherwise uninterrupted, wall) may be functionally superior to those cues which a human may find particularly salient (Olton, 1977).

Radial-Arm Maze

Testing occurred in an eight-arm radial-arm maze constructed of opaque, non-reflective black Plexiglas walls placed on a circular 121 cm diameter non-reflective black plastic mat. The radial-arm maze consisted of a 36 cm diameter central area with eight 43 x 12 .5 x 30 cm arms (l x w x h) extending radially. Food cups were placed at the distal end of each arm. The protocol followed the method of Olton (1976) as modified by Levin (1996a). Specifically, beginning on PND 35 food was restricted such that male and female animals received 15 g or 12 g of food each, respectively. On PND 38 the animals were introduced to the maze and allowed to explore it for 5 min. Food rewards consisted of one-half to one-third piece of Kellogg's Froot Loops® breakfast cereal (Kellogg's, Battle Creek, MI). Preliminary investigation revealed that smaller pieces were more reinforcing (*i.e.*, rats took less time to consume each individual reward and spent less time searching around and under the food cups and moved on to the next arm more quickly). Food rewards were placed in the food cups at the end of each arm. Each trial began by placing the rat in a 25 x 25 cm cardboard cylinder in the center of the maze. After 10 s, the cylinder was lifted and the rat was permitted to move about the maze until all eight arms had been entered and food rewards eaten or until 5 min had elapsed. A correct entry was counted when the rat entered the arm, proceeded to the end, and removed the food

reward. Because food was not replaced once eaten, an error was counted when the rat entered an arm which it had previously eaten the food. The number of correct entries made before an error was made (entries to repeat), the total number of subsequent errors and latency to find all food rewards were recorded. Testing occurred once daily for 7 days beginning on PND 37 and again on PND 62 for 4 days. On the last day of testing, food hoppers were filled completely at the end of the day and rats were allowed to eat *ad libitum*. Entries to repeat, number of errors, and latency to complete were analyzed using repeated-measures multivariate analyses of variance (MANOVA), with time as the within-subjects factor and drug and sex as the between-subjects factor. When significant differences between sexes were revealed, analyses were repeated for each sex separately. Additional univariate analyses of variance (ANOVA) were performed at each time point when overall significance was revealed using the repeated-measures MANOVA. Differences between groups was determined using the Tukey-HSD or Dunnett's *t post hoc* analyses.

Water Maze

Experiments were conducted in a Morris water maze (Morris, 1981)following the procedure of Levin and colleagues (1996) and Lindner and colleagues (1992). The water maze was a circular, dark blue plastic tank 200 cm in diameter (180 cm in diameter at water level) and 50 cm high. The escape platform was a dark acrylic 12.5 cm square platform mounted on a clear 30 cm high acrylic base. The maze space was conceptually divided into four quadrants (*i.e.*, north-west, north-east, *etc.*). The platform was placed in one of four

quadrants in the maze, equally distant (50 cm) from each orthogonal wall, on each of the test days. The maze was filled with 30°C (±1°C) water until the escape platform was 1 cm below the surface; deep enough not to be seen or cause ripples from surface agitation, but shallow enough so that the rat was out of the water once it stood on the platform. The task was a delayed matching to position task. Specifically, each trial consisted of two blocks separated by 60 s. Previous experiments using the water maze (Cutler et al., 1996; Lindner et al., 1992) have determined that various intervals between trials (e.g., 15, 30, 60, or 80 s) do not significantly affect performance on the second trial. Therefore, the latency of 60 s was chosen for logistical reasons. In the first block, each rat was placed in the water facing the wall in a randomly chosen quadrant (using the random number generator function in Corel, Quattro Pro™) and allowed to search for the escape platform which was marked by a bright white acrylic box that extended above the surface of the water by 2.5 cm and below the surface by 10 cm. After the rat found the marked platform, it was allowed to stay on it for 15 s. All rats found the marked platform within the allowed time. Then the rat was removed from the maze and placed in a 20 gal plastic bucket with several dry towels in it for 60 s. The holding bucket was moved each day to match the position of the escape platform. This was done so that the rat would not learn the position of the bucket and perseverate in that quadrant rather than proceeding to the platform. During the delay, the white acrylic box was removed from the escape platform. The rat was then placed back in the water in the same starting position and allowed to search for the, now hidden, platform. If the

rat found the platform within 3 min, it was immediately removed from the maze and placed in a cage with a dry towel in it. If the rat failed to find the platform within 3 min, then it was guided there, and allowed to remain for 15 s before being removed. All rats were toweled dry at the end of each session and returned to their home cages. Latency to reach the marked platform, the unmarked platform, and distance traveled to each was recorded. Testing began on PND 50, occurred once daily for 5 days, and again beginning on PND 69. Latency to find the unmarked platform data were analyzed using repeated-measures MANOVA, with time as the within-subjects factor and drug and sex as the between-subjects factor. When significant differences between sexes were revealed, analyses were repeated for each sex separately. Additional MANOVAs were performed at each time point when overall significance was revealed using the repeated-measures MANOVA. Differences between groups was determined using the Tukey-HSD *post hoc* analysis.

Passive Avoidance

Shuttlebox passive-avoidance (often referred to as inhibitory avoidance) is considered to be an index of general cognition or memory performance in animals (Decker, 1995; Doyle et al., 1993; Riekkinen et al., 1993). In this task, the animal must inhibit a naturally occurring drive to move out of a lit space into a dark space. Bertolini and colleagues (1982) used cigarette smoke inhalation to pregnant dams administered twice per day. They reported no significant differences in number of rats that reached criterion. This study is flawed, however, because of the route of administration. Not only was nicotine not

administered in a robust fashion, but there is no way to identify the dosage of nicotine administered. Additionally, as mentioned above, smoke inhalation also administers a host of other, potentially harmful substances (*e.g.*, carbon monoxide) that may affect cognition.

Animals were trained using a protocol used previously by the author (Rahman et al., 1996) that is based on published reports (Decker, 1995; Decker et al., 1993) utilizing an automated avoidance training system (Gemini, San

Figure 8 Shuttlebox - passive avoidance apparatus

Diego Instruments, San Diego, CA). The apparatus consisted of two 21 x 25 x 17 cm chambers separated by a vertically-sliding door (*see Figure 8*). Lighting in the chambers was provided by a 50 watt bulb located 3 cm above the translucent plastic ceiling. Scrambled, constant-current shocks (0.8 mA for 1 s) were delivered through a grid floor. Control of the door, lighting, and shock was provided by means of an interfaced 486 computer running proprietary software ("PA," San Diego Instruments, San Diego, CA). Training and testing procedures were identical and began by placing the animal in one chamber of the darkened

apparatus. After a delay of 60 s, the 50 watt light came on in the start chamber and the door opened to the other, darkened chamber. When the rat crossed completely into the darkened chamber, the door closed, a 0.8 mA shock was delivered for 1 s, and latency to cross was recorded. The rat was then immediately removed from the chamber. Because passive avoidance can only be performed once to control for learning effects on subsequent test days, rats were trained on the procedure on PND 55 and tested 24 hours later. this particular timing for the test was chosen as a mid-point between the two testing periods used for the other behavioral measures. Shock was not administered on the test day. All rats crossed into the darkened chamber on the training day. If the rat did cross into the other chamber on the test day, it was removed after 300 s. Because the latency data are bounded and non-normally distributed they could not be analyzed parametrically. The data were recoded into binary data — that is, whether the rat did or did not cross within the 300 s time — and were analyzed using Chi-square analysis. Additionally, the actual latency data were analyzed using the Kruskal-Wallis one-way analysis of variance by ranks test a nonparametric equivalent to one-way (Siegel et al., 1988, p. 206) that tests whether *k* independent samples are from different populations.

Acoustic Startle

The Acoustic Startle Reflex (ASR) and pre-pulse inhibition (PPI) of the ASR are behavioral responses believed to index central processes related to information processing (Caine et al., 1992; Swerdlow et al., 1992) and possibly attention (Acri, 1994; Acri et al., 1995; Acri et al., 1991; Acri et al., 1994). The

acoustic startle reflex is a characteristic sequence of involuntary, muscular responses elicited by a sudden, intense acoustic stimulus (Davis, 1984). Jumping in response to an unexpected car backfire is an everyday example of the startle reflex. The reflex is present in all mammals, including humans and rats, and is considered an index of reactivity to external acoustic stimuli. In addition, because the reflex can be elicited using the same stimuli across species (Swerdlow et al., 1994), the paradigm has face validity for generalizing from an animal model to human issues.

Pre-pulse inhibition (PPI) of the acoustic startle reflex (ASR) occurs when the startling stimulus is preceded by a non-startling acoustic stimulus by a short interval (about 100 msec). The presence of the pre-pulse results in measurably reduced startle amplitude (Braff et al., 1978; Graham, 1975). In the everyday example of a car backfire, the ability of this loud noise to startle would be reduced if the listener also heard the engine sounds immediately preceding the backfire. This reduction in startle amplitude is pre-pulse inhibition of the ASR. As with the ASR, the phenomenon of pre-pulse inhibition occurs in humans and in rats. Pre-pulse inhibition is believed to index an innate sensory-cognitive-motor "gating" mechanism that operates at a non-volitional or pre-cognitive level and underlies the organism's ability to select relevant stimuli from the environment while screening out irrelevant information (Swerdlow et al., 1992). PPI also has been interpreted to reflect processes associated with attention (Acri et al., 1991; Acri et al., 1994), and in humans PPI is negatively correlated with distractibility (Karper et al., 1996).

Acoustic startle reflex amplitudes and pre-pulse inhibition were measured in a Coulbourn Instruments Acoustic Response Test System (Coulbourn Instruments, Allentown, PA) (*see Figure 9*). The Acoustic Response Test System consisted of four weight-sensitive platforms inside a sound-attenuated chamber (*see Figure 10*). Subjects' movements in response to stimuli were measured as a voltage change by a strain gauge inside each platform and were converted to grams of body weight change following analog to digital conversion. Responses were recorded by an interfaced computer as the maximum response occurring within 200 msec of the onset of the startle-eliciting stimulus. Each rat was individually placed in a 8 x 8 x 16 cm open air cage. The open air cages are small enough to restrict extensive locomotion but large enough to allow the subject to turn around and make other small movements. Each open air cage then was placed on one of the four platforms. The platforms were arranged radially around central speakers in the floor and ceiling of the chamber. A ventilating fan provided an ambient noise level of 56 dB. Following placement of four animals in the chamber, a 3-minute adaptation period occurred in which no startle stimuli were presented. Although it has been reported that some rats emit ultrasonic vocalizations during startle testing (Haney et al., 1994; Vivian et al., 1993), there is no evidence indicating that vocalizations alter startle responses. However, to ensure minimal effects of vocalizations should they occur, subjects were balanced across treatment groups within each testing session.

Startle stimuli consisted of 112, 122 or 98 dB noise bursts of 20 msec duration sometimes preceded 100 msec by 68 dB 1 KHz pure tone (pre-pulse).

Decibel levels previously have been verified by a Larson-Davis Sound Pressure Machine Model 2800 (unweighted scale; re: 0.0002 dynes/cm^2). Each stimulus has a 2 msec rise and decay time such that onset and offset are abrupt and in a square-wave pattern, a primary criterion for startle (Cassella et al., 1986). There were eight types of stimulus trials, and each trial type was presented eight times, for a total of 64 trials. Trial types were presented in random order to avoid order effects and habituation. Inter-trial intervals ranged randomly from 10 - 30 sec. Trial types included: (1) 112 dB stimulus, (2) 112 dB stimulus preceded by a 68 dB pre-pulse, (3) 122 dB stimulus, (4) 122 dB stimulus preceded by a 68 dB pre-pulse, (5) 98 dB stimulus, (6) 98 dB stimulus preceded by a 68 dB pre-pulse, (9) 68 dB pre-pulse only, and (8) no stimulus. The testing period lasted approximately 30 min. Open-air cages were washed with soap and warm water and dried after each use. Males and females were tested in separate test chambers. Treatment groups were balanced within each chamber for each measurement session.

Figure 9 Acoustic startle apparatus

Figure 10 Startle platforms

Trials during which no stimuli are presented were used to control for normal subject movements on the platform. This information is necessary in

order to accurately calculate platform displacement that occurs in response to specific noise stimuli. To derive these values, platform displacement on the no-stimulus trials (i.e., the body weight of each subject) was from platform displacement in response to the various noise stimuli, leaving only the amount of platform displacement related to the stimulus. Additionally, in order to acclimate the subjects with the experimental procedure, the subjects were placed in the ASR apparatus on two separate days and sounds were presented as explained above, however, the data for acclimation days were not analyzed. Several days later on PND 48 and PND 67, true measures of ASR were performed and data were kept and analyzed. This protocol has been empirically derived in this laboratory in order to reduce the amount of spurious activity (or freezing) during the procedure without habituating the response (unpublished empirical data). Amplitude of startle (in grams), amount PPI and percent PPI were calculated and analyzed using ANOVA.

Locomotor

Locomotor activity is a sensitive, reliable measure commonly used to evaluate effects of drugs and stress on behavior. It was used in the present

Figure 11 Locomotor apparatus

experiment to assess prenatal nicotine effects on open field activity. In addition, locomotion was used to control for the potential confound of activity level on memory performance tasks that required lack of movement (PA) (Decker et al., 1993). Subjects were placed individually in an Omnitech Electronics Digiscan infrared photocell system (Test box model RXYZCM) which consisted of a 40 X 40 X 30 cm clear plexiglass arena surrounded by an array of infra-red photo-beams (*see Figure 11*). Males and females were tested separately. Spontaneous locomotor was automatically gathered and transmitted to a personal computer via an Omnitech Model DCM-8-BBU analyzer. Animals were monitored continuously for a 2-hour period with data recorded as cumulative activity over 5 minute time periods. Testing occurred on PND 46 and PND 60. Animals were acclimated to the experimental procedure, as described above, but these data were not analyzed. Analyses of variance were performed on: horizontal activity (number of beams broken), total distance (in cm), number of discrete movements, vertical activity (number of vertical beam breaks), number of discrete vertical movements, time spent vertical, distance traveled and time spent in the center of the open field and margins. Each sex was analyzed separately because it has previously been reported that normal, untreated male and female rats emit different patterns of locomotor activity (Faraday et al., 1998b). Additionally, locomotor activity data were entered into a multiple regression and correlation (MRC) analysis along with sex and drug treatment as predictor variables for passive avoidance performance data to determine if these variables significantly accounted for variance in passive avoidance data.

Neurochemistry

Dopamine Receptor Autoradiography

Rat brains were immediately removed after decapitation and snap-frozen by immersion into powdered dry ice and were stored at -80°C until sectioning. Consecutive coronal brain sections (18µm thick) were cut using a motor-driven cryostat (Microm HM 505E, Carl Zeiss Inc., Thornwood, NY) at -20°C and thaw-mounted onto chrome-alum gelatine-coated glass microscope slides and stored not more than one week at -80°C until assay. Sections were made 3.2-2.6 mm anterior to bregma as identified by Garris and colleagues (1993). Autoradiography was performed on one slide with four sections for each animal.

Dopamine D1 receptor autoradiography was performed using the selective D1 agonist [^{125}I]SCH 23982 (R(+)-8[^{125}I]-iodo-7-hydroxy-2,3,4,5-tetrahydro-3methyl-5-phenyl-1H-3-benazepine, New England Nuclear Life Science Products, Boston, MA) using the method developed by Dawson and colleagues (1990) with modifications (Bonhomme et al., 1995; Leslie et al., 1987; Leslie et al., 1991; Picciotto et al., 1997; Schambra et al., 1994). Specifically, slide-mounted sections were allowed to come to room temperature, removed from the storage box and pre-incubated for 30 min at room temperature in a 50 mM Tris-HCl (pH 7.4) solution containing 120 mM NaCl, 5 mM KCl, 2mM CaCl, and 2mM $MgCl_2$. Sections were then incubated at room temperature for 60 min using the above solution to which 0.1 nM [^{125}I]SCH 23982 had been added. Additionally, a concentration of 50 nM mianserine (RBI, Natick, MA) was added to the incubation solution in order to block binding to 5-HT receptors.

Nonspecific binding was determined in the presence of 1μM of the selective D1 agonist ("cold") SCH 23990. Incubation was terminated with three 5-minute washes of ice-cold 50 mM Tris. Sections were then dipped in ice-cold distilled water before being dried. Sections from each drug group were assembled and apposed to Hyperfilm-3H (Amersham, Sweden) in an X-Ray film cartridge for 4 days. Standards of known radioactivity were prepared and placed on a slide with each film. After exposure films were developed by hand using Kodak-GBX developer and fixer (Kodak, Rochester, NY). Optical density of autoradiograms (*see Figure 30*) was determined using proprietary software designed for this purpose (Tommerdahl et al., 1985). Optical density per area of cortex examined (mm^2) were analyzed using analyses of variance.

Statistical Analyses

Behavioral data were analyzed using repeated-measures multiple analyses of variance (MANOVA) with time as the within-subject factor and drug condition and sex as the between-subjects factor. Univariate analyses of variance (ANOVA) were used at each time point on the behavioral tests and on receptor density data to determine which drug groups differed significantly. Multiple regression correlation analyses (MRC) were used to determine if drug condition significantly predicted subsequent behavior or receptor binding (Cohen et al., 1983). Because some hypothesized effects were non-linear, *a priori* planned comparisons were performed between all groups as necessary (Hays, 1994, pp. 421-471). Post hoc analyses (Tukey-HSD or Dunnett's *t*) were used when significance was revealed to determine which groups differed significantly

(Hays, 1994, p. 462; Winer, 1962, pp. 201-203). Because passive avoidance performance data were non-parametric, they were analyzed using the Chi-Square test for *k* independent samples (Siegel et al., 1988, p. 191). All tests were two-tailed and conducted with an α-level ≤ 0.05.

Body Weight

Figure 12 presents mean maternal body weight data. Dams that had received nicotine weighed less that those that did not. Repeated-measures analyses of variance (ANOVA) on maternal body weight from gestational day 3 (GD 3) through GD 22 (day of parturition) revealed that body weight increased over Time [$F(1,12)=238.017$, $p<.001$], that animals receiving drug had body weights that differed from control animals [$F(2,12)=4.473$, $p<.05$], and revealed a significant Time X Drug interaction [$F(2,12)=8.184$, $p<.01$]; that is, animals that had received drug gained weight at a different rate over time than those that did not receive drug. Univariate analyses of variance performed on maternal body

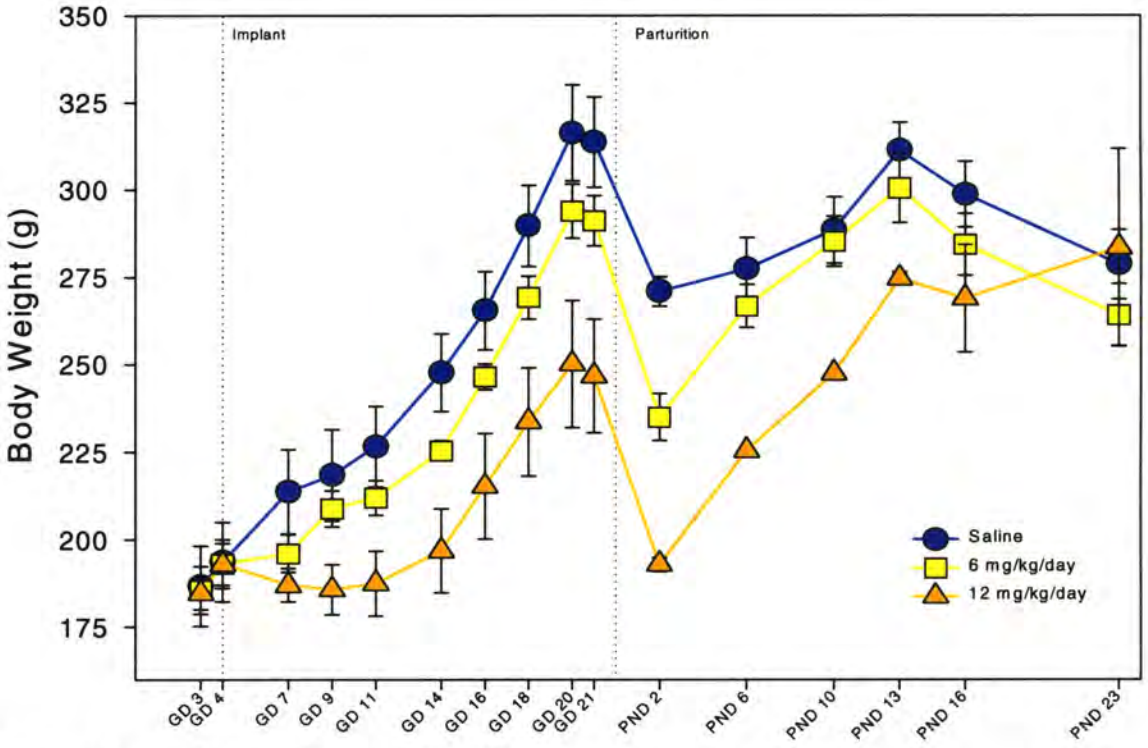


Figure 12 Mean maternal body weight

weight at each day revealed a main effect for drug on all days beginning on GD11 (*see Table 3 for F values*) through post natal day 6 (PND 6). Tukey HSD *post hoc* analyses revealed that the dams receiving 6 mg/kg/day nicotine weighed less than saline controls on all days on which the overall analysis detected significant differences. Those dams receiving 12 mg/kg/day nicotine weighed less than all other dams (*see Figure 12*). After parturition, on PND 2 and PND 6 dams receiving 12 mg/kg/day nicotine weighed significantly less than dams receiving 6 mg/kg/day nicotine and controls. On PND 2 dams receiving 6 mg/kg/day nicotine weighed significantly less than saline controls.

Figure 13 presents mean offspring body weight. Because males gain weight at a different rate than females, all analyses were performed separately for each sex. Repeated-measures ANOVA on male offspring body weight from

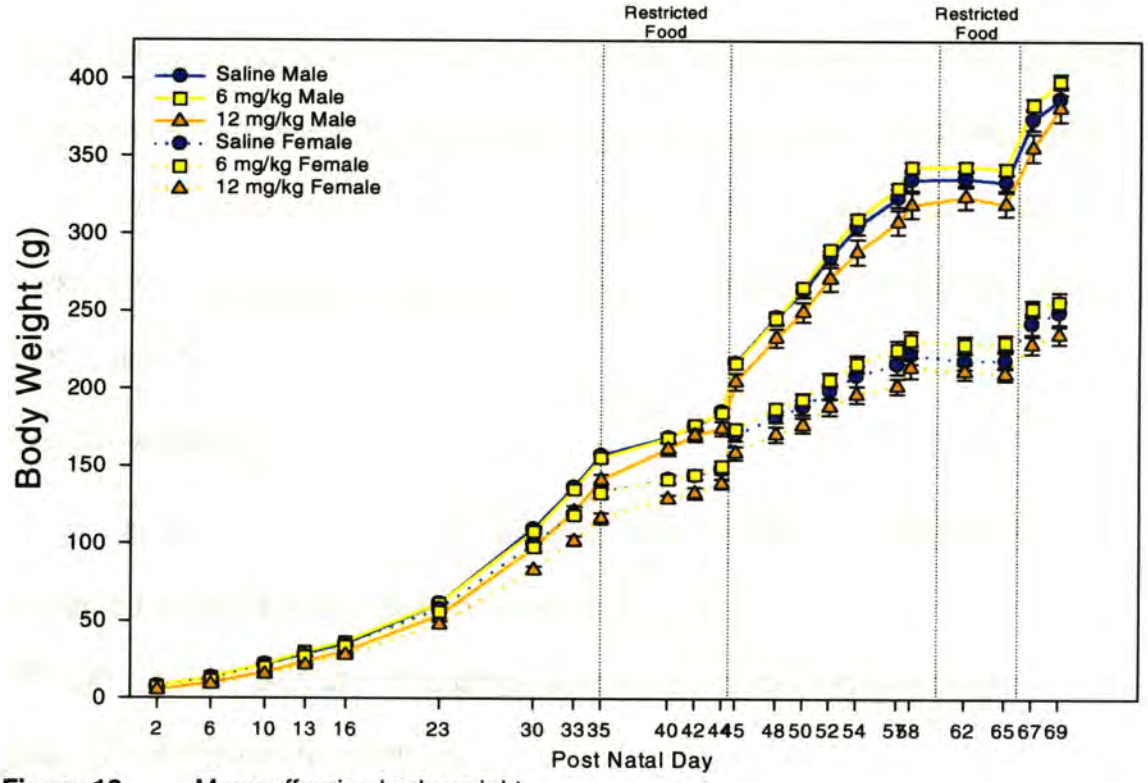


Figure 13 Mean offspring body weight

PND 2 through PND 69 revealed a significant main effect for Time (animals gained weight over time) [$F(1,37)=9669.766$, $p<.001$] and a main effect for Drug (offspring that had been exposed to drug weighed differently than those that had not been exposed to drug) [$F(2,37)=5.246$, $p<.01$]. Repeated-measures ANOVA on female offspring body weight from PND 2 through PND 69 revealed a significant main effect for Time [$F(1,39)=4271.253$, $p<.001$] and a main effect for Drug [$F(2,39)=5.049$, $p<.05$]. Univariate ANOVAs on male offspring body weight revealed significant main effects for Drug on: PND 2-35; PND 44-58; and PND 65-67 (*see Table 4 for F values*). Days on which significance was not revealed were days during which food was restricted as described above in the Methods section for Radial-Arm Maze. At all time points where significance was revealed, the offspring that had been exposed to 12 mg/kg/day nicotine weighed less than the other groups (*see Table 4 for exact comparisons*). Univariate ANOVAs on female offspring body weight revealed significant main effects for Drug on: PND 2-48; PND 57, 65, and 67 (*see Table 5 for F values*). Again, at all time points where significance was revealed, the 12 offspring that had been exposed to 12 mg/kg/day nicotine weighed less than the other groups (*see Table 5 for exact comparisons*).

Radial-Arm Maze

Repeated-measures multivariate analyses of variance (MANOVA) revealed a main effect for Sex on radial-arm maze performance [$F(1,74)=11.271$, $p<.001$]. Therefore, all data from radial-arm maze were analyzed separately for each sex.

Figure14 presents entry to repeat data from the radial-arm maze for males. No significant differences were revealed during adolescent testing (PND 42-44) other than a main effect for Time [$F(1,37)=7.076$, $p<.02$] indicating that all animals improved performance over time. Repeated measures ANOVA on adult performance (PND 62-65) revealed a significant main effect for Time [$F(1,37)=18.663$, $p<.001$] and a main effect for Drug [$F(2,37)=23.408$, $p<.01$]. Univariate ANOVAs at each time point revealed a trend on PND 62 for Drug [$F(2,37)=.088$, $p=.058$] and a significant main effect for drug on PND 63 [$F(2,37)=3.289$, $p<.05$] and PND 64 [$F(2,37)=4.393$, $p<.05$]. Dunnett's *t post hoc* analyses revealed that the offspring that had been exposed to 12 mg/kg/day

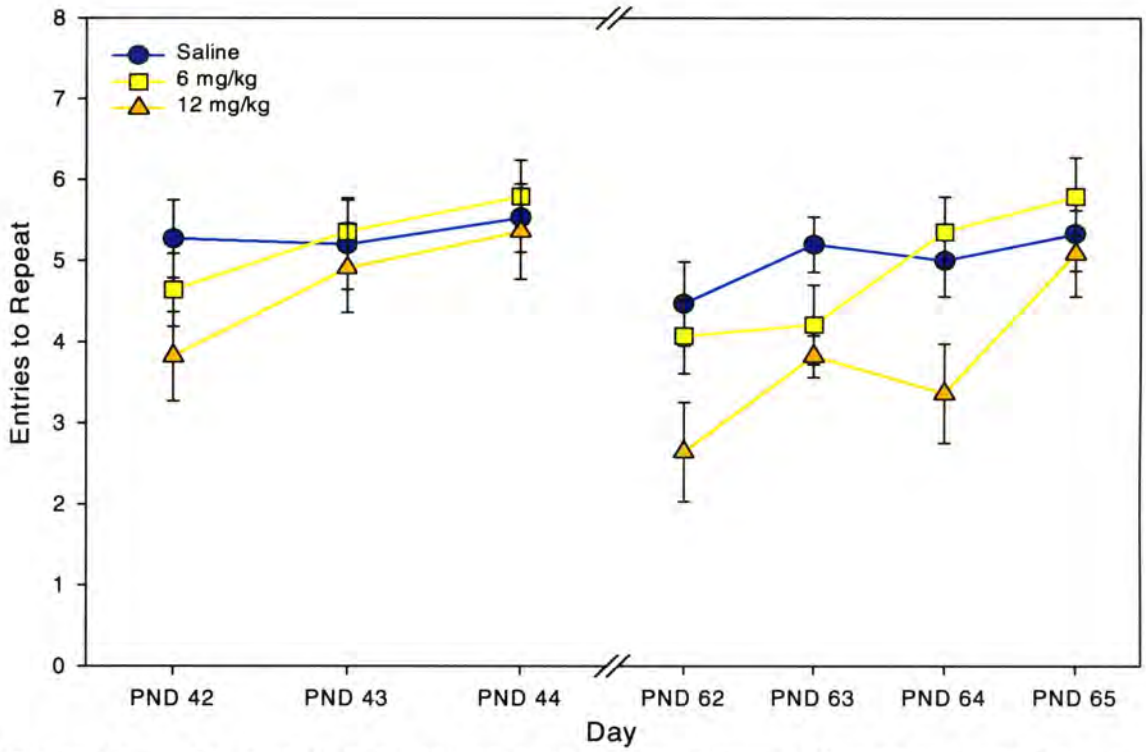


Figure 14 Number of correct entries made by male offspring before an error was made during Radial-Arm Maze (RAM) testing

nicotine made significantly fewer correct choices than the control group in PND 62, 63, and 64.

Figure 15 presents entry to repeat data for female offspring. There were no significant effects during adolescent testing. Repeated-measures ANOVA on adult performance data revealed a main effect for Time [$F(1,39)=14.179$, $p<.001$] and a Time X Drug interaction [$F(2,39)=3.602$, $p<.05$], that is, animals that had been exposed to drug showed different rates of improvement over time than those animals that had not been exposed to drug. Individual ANOVAs performed on each adult testing day revealed no significant drug effects.

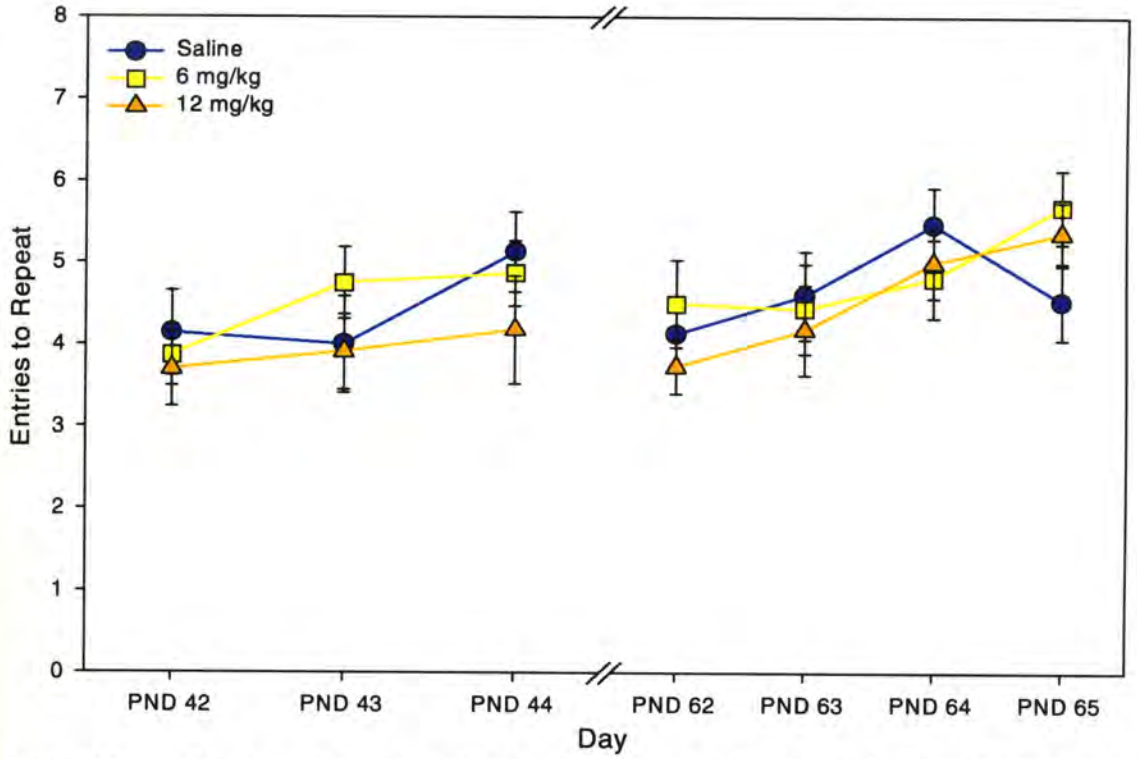


Figure 15 Number of correct entries made by female offspring before an error was made during RAM testing

Figure 16 presents number of errors made by males. There were no significant findings for this variable during adolescent or adult testing.

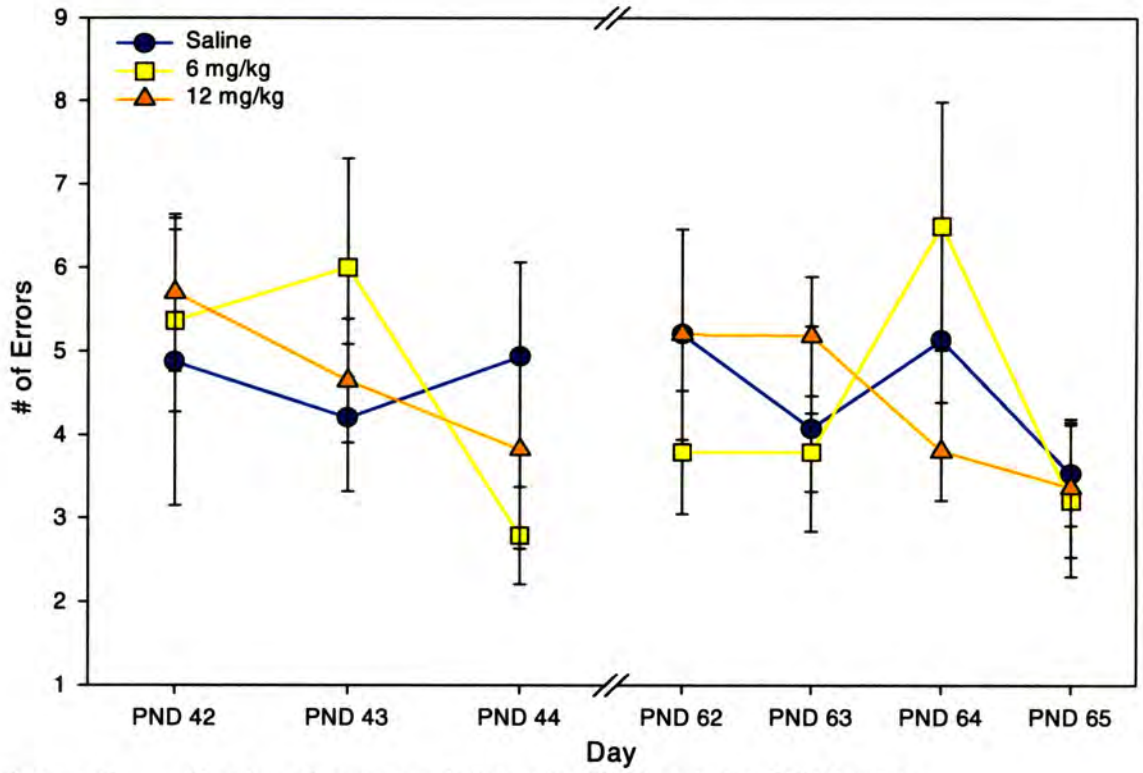


Figure 16 Number of errors made by male offspring during RAM testing

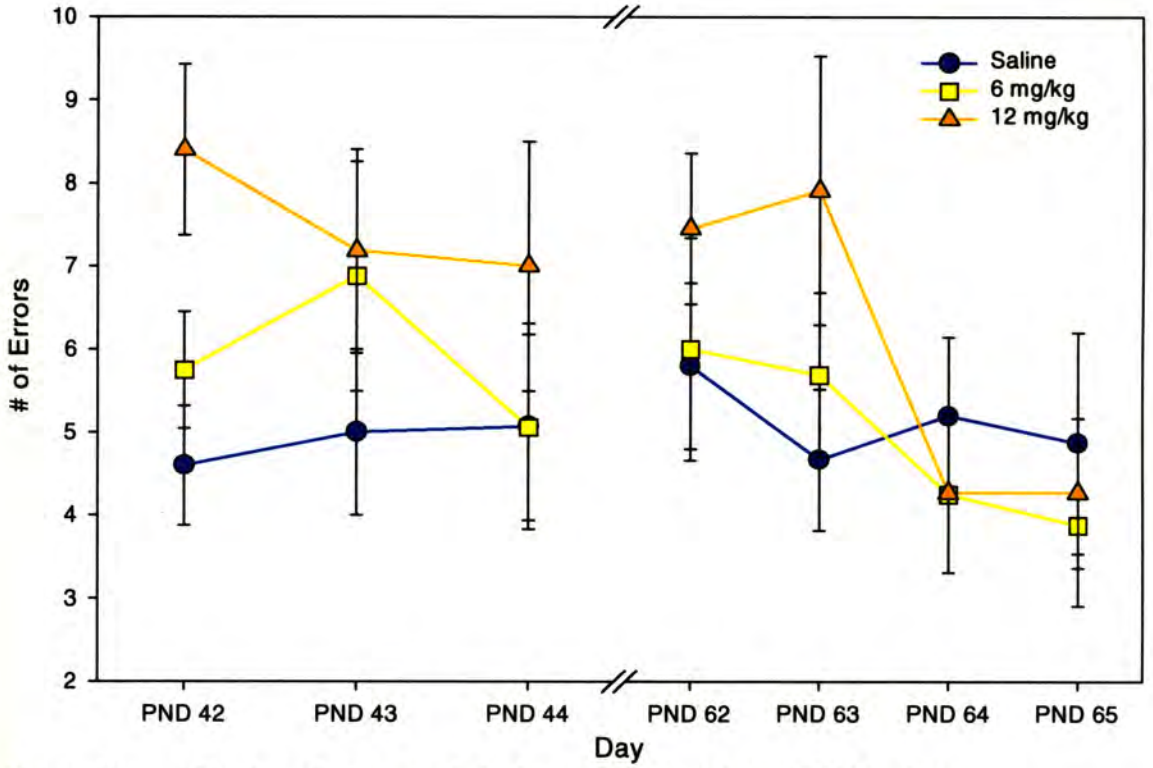


Figure 17 Number of errors made by female offspring during RAM testing.

Figure 17 presents number of errors made by females. Repeated-measures ANOVA revealed a main effect for Drug [$F(2,38)=3.550$, $p<.05$]. Univariate ANOVAs revealed that females differed significantly on PND 42 [$F(2,38)=5.143$, $p<.05$], where the offspring that had been exposed to 12 mg/kg/day nicotine made more errors than the other groups. There were no significant effects during adult testing.

Figure18 presents male latency to complete data. Repeated-measures ANOVA revealed a main effect for Time for adolescent performance, all offspring improved their performance by completing the task more quickly as the experiment progressed during adolescent testing, [$F(1,37)=92.653$, $p<.001$]. During adult testing (PND 62-65), there was a significant main effect for Time [$F(1,37)=29.633$, $p<.001$] and for Drug [$F(2,37)=4.693$, $p<.02$] such that the

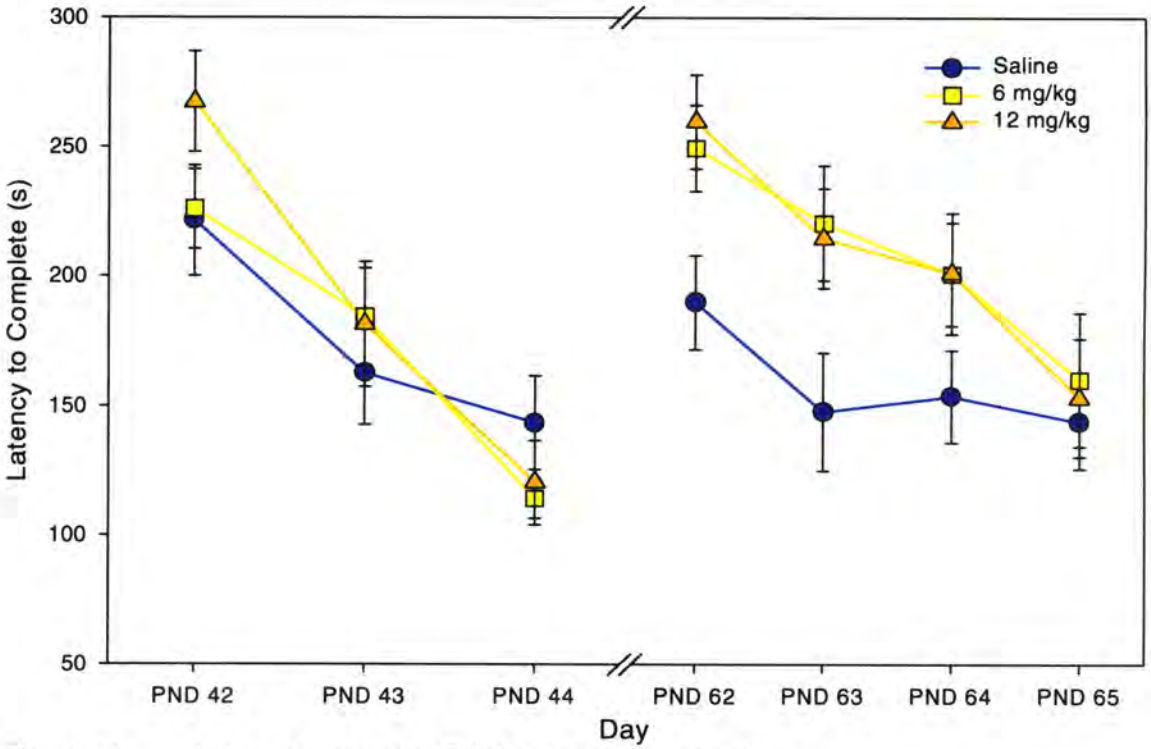


Figure 18 Latency to complete RAM trials for male offspring

animals that had been exposed to drug took longer to complete the task than the animals that had not, although all animals improved performance over time. Univariate ANOVAs revealed that males differed significantly on PND 62 [$F(2,37)=4.639$, $p<.02$] and PND 63 [$F(2,37)=3.580$, $p<.05$]. Dunnett's *t post hoc* analyses indicated that the saline control offspring completed the task more quickly than the offspring exposed to both 6 and 12 mg/kg/day nicotine on PND 62, and more quickly than the offspring exposed to 12 mg/kg/day nicotine on PND 63.

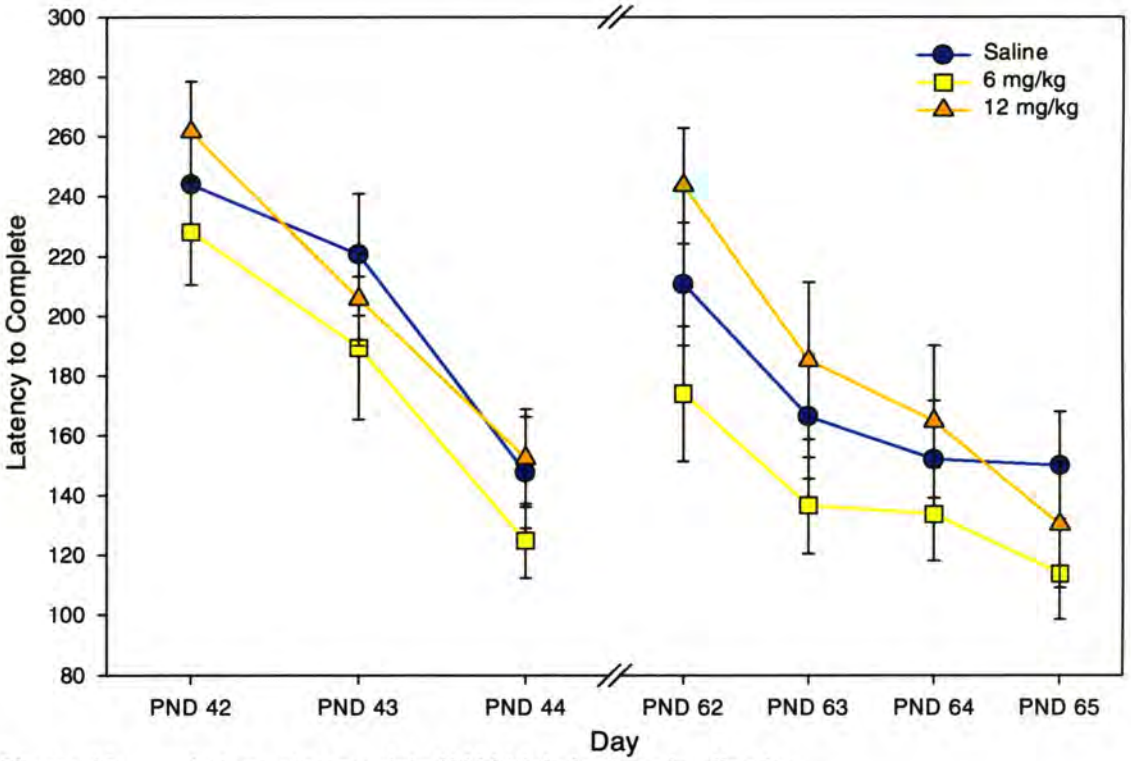


Figure 19 Latency to complete RAM trials for female offspring

Figure 19 presents female latency to complete data. All offspring completed the task more quickly as the experiment progressed; repeated-measures ANOVA revealed a main effect for Time [$F(1,37)=88.772$, $p<.001$] for adolescent testing and a main effect for Time [$F(1,39)=50.294$, $p<.001$] for adult

testing. There were no effects for drug on this variable.

Water Maze

Repeated-measures MANOVA for latency to find the unmarked platform in water maze revealed a main effect for Time [$F(1,72)=24.240$, $p<.001$] and a significant Time X Sex X Drug interaction [$F(1,72)=3.173$, $p<.05$]. That is, over time male and female animals responded to the drug differently. In order to examine individual components of this interaction, the sexes were analyzed separately.

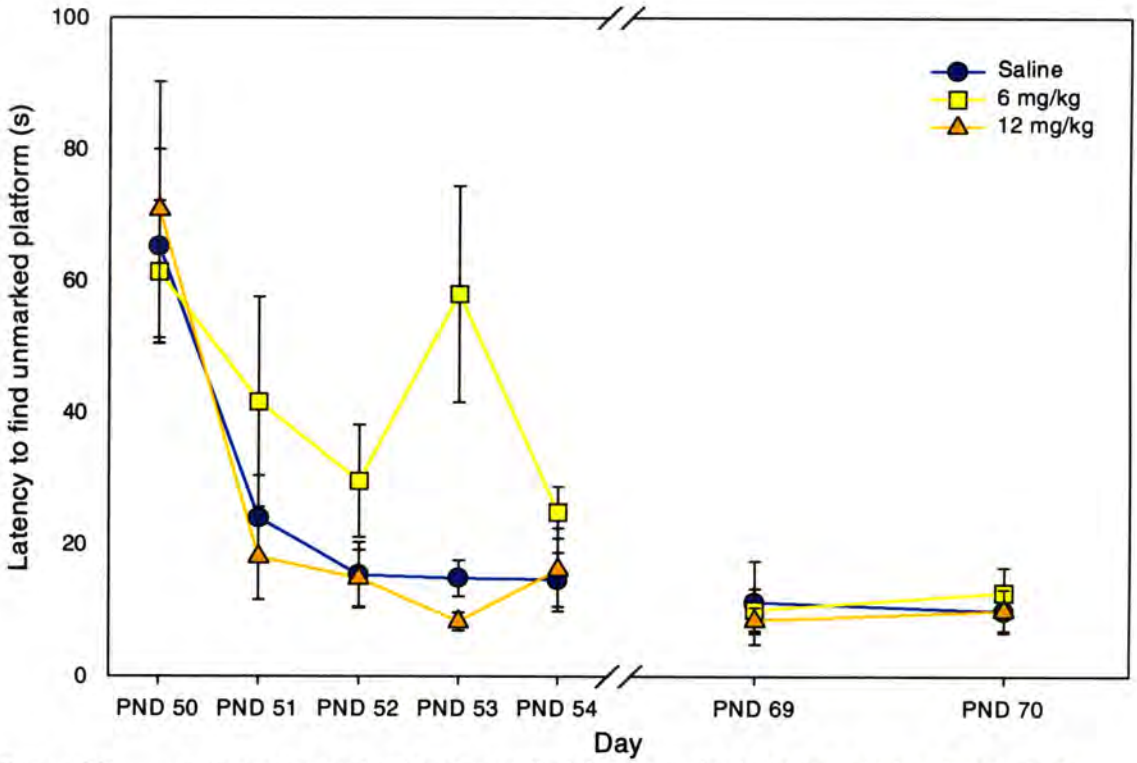


Figure 20 Latency for male offspring to find the unmarked platform during water maze (MW)

Figure 20 presents latency to find the unmarked platform for males. Repeated measures ANOVA on adolescent performance revealed a significant main effect for Time [$F(1,35)=19.251$, $p<.001$] and for Drug [$F(2,35)=5.608$,

$p<.01$] such that males improved performance over time, and drug affected performance. The offspring that had been exposed to 6 mg/kg/day nicotine took significantly longer to find the platform than either the saline control group or the offspring exposed to 12 mg/kg/day nicotine. Univariate ANOVA revealed a main effect for Drug [$F(2,37)=7.067$, $p<.01$] on day 4 of adolescent testing (PND 53). There were no effects during adulthood.

Figure 21 presents latency to find the unmarked platform for females. Repeated Measures ANOVA of adolescent performance revealed a significant main effect for Time [$F(1,37)=6.638$, $p<.05$] such that all females improved performance over time, but there were no significant effects for Drug on this variable. There were no effects during adulthood.

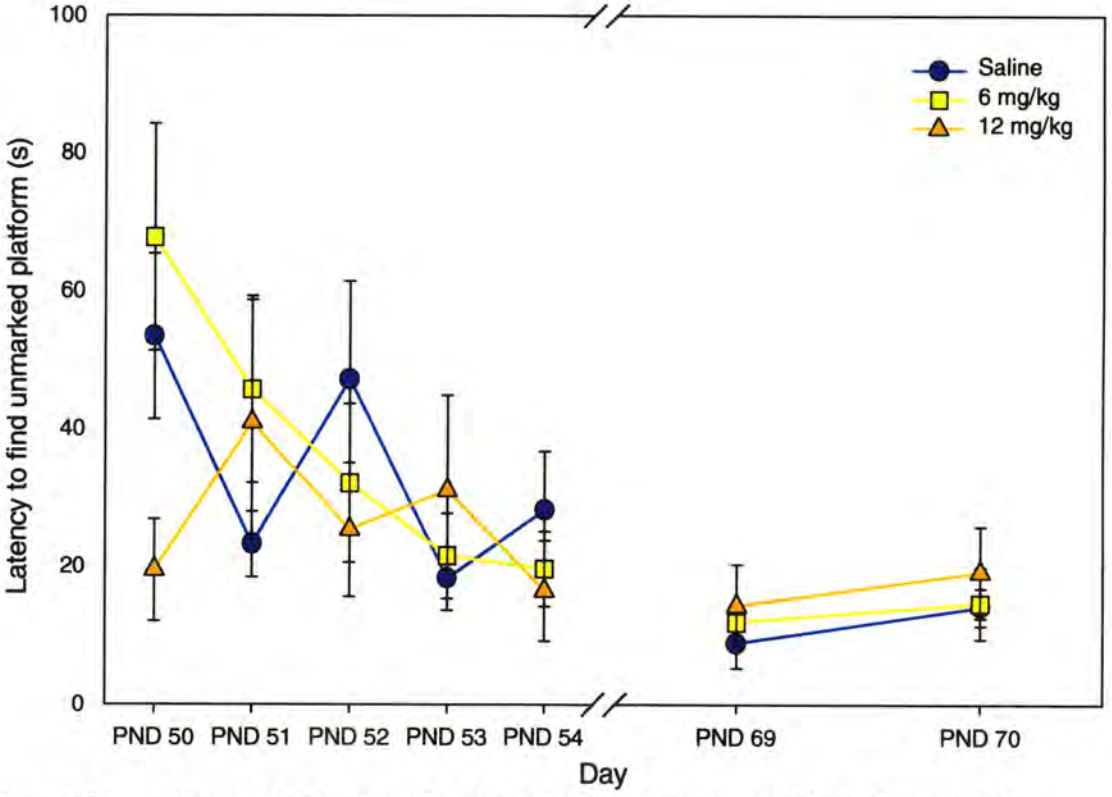


Figure 21 Latency for female offspring to find the unmarked platform during WM

Passive Avoidance

Data for Passive Avoidance were recoded into a binary format: "yes", the animal successfully performed the PA task — it did not cross into the dark side; or "no", the animal did not successfully perform the task — it did cross. Table 6 presents chi-square analyses of the passive avoidance data. The analyses revealed that a significantly larger proportion of the male offspring that had been exposed to 6 mg/kg/day and to 12 mg/kg/day nicotine performed the task correctly. All other groups were equally distributed between the two criteria. Additionally, when the non-transformed latency data were analyzed (*see Figure 22*) by the Kruskal-Wallis test (a non-parametric ANOVA equivalent) a significant overall main effect for Sex [χ^2 =8.273 *df*=1, *p*<.01] and for Drug [χ^2=6.550, *df*=2, *p*<.05] were revealed. When the sexes were analyzed separately, a main effect for drug was revealed in males only [χ^2 =7.464, *df*=2, *p*<.05.

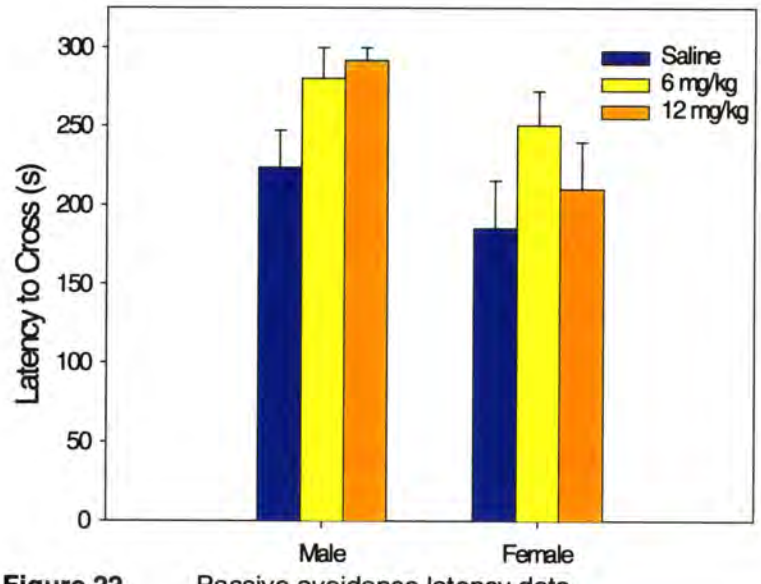


Figure 22 Passive avoidance latency data

Multiple regression correlation (MRC) analyses were performed to ascertain the amount of variance of passive avoidance performance that was accounted for by sex, drug, and locomotor activity (used as a predictor variables). This was done in order to determine to what extent each predictor variable contributed to passive avoidance performance. A step-wise, hierarchical MRC was performed in which each predictor variable was entered in order (sex, drug, locomotor). Sex and prenatal drug exposure accounted for a significant proportion of the variance of passive avoidance performance, [$F(2,79)=4.692$, $p<.05$ $R^2=.106$]. However, when locomotor activity was entered, it did not contribute significantly to the proportion of the variance of passive avoidance performance. The overall R^2 increased only to $R^2=.107$, which was not a significant contribution to the overall variance, $t_{(78)}=.248$, $p=.805$. This finding indicates that passive avoidance performance could not be explained by the each animal's locomotor activity.

ASR-PPI

Figure 23 presents amount of startle amplitude to the 122 dB stimulus when presented with a pre-pulse on PND 67. Univariate ANOVA revealed a significant main effect for Sex [$F(1,76)=13.285$, $p<.001$] and Drug [$F(2,76)=6.317$, $p<.001$]. When the sexes were analyzed separately, a significant main effect for Drug was revealed in females only [$F(2,39)=4.646$, $p<.05$]. Tukey-HSD *post hoc* analysis revealed that the female offspring that had been exposed to 12 mg/kg/day nicotine startled significantly more than the other offspring.

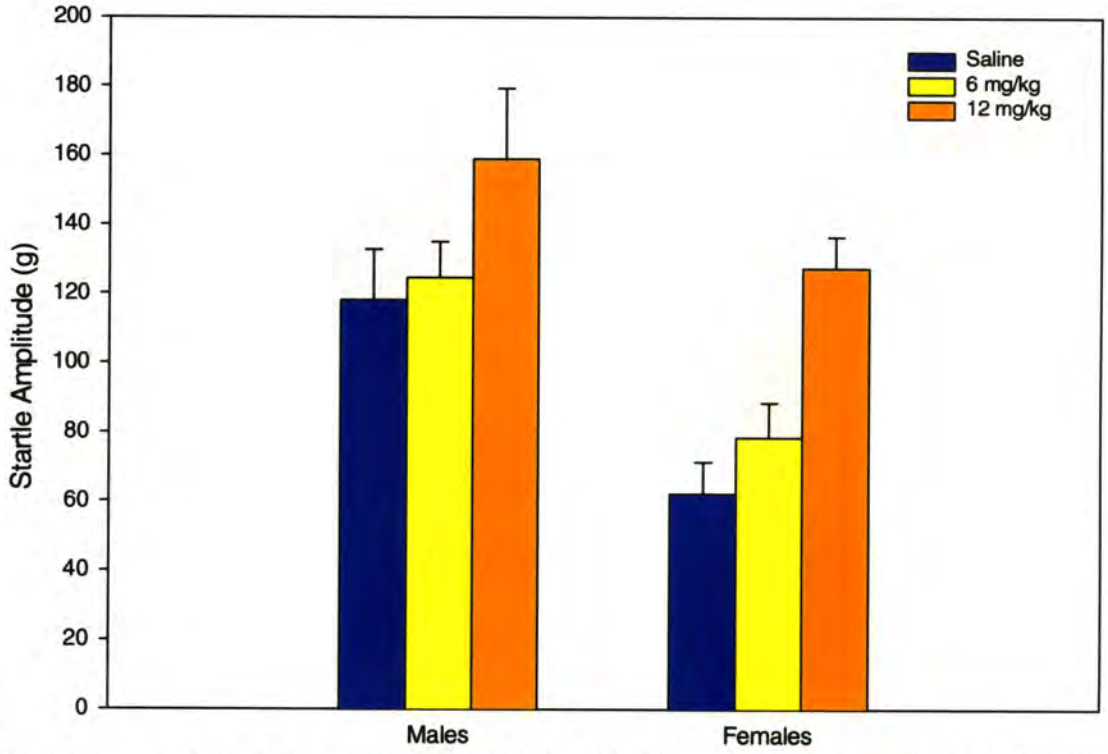


Figure 23 Amount of startle amplitude to the 122 dB stimulus when presented with a pre-pulse on post natal day (PND) 67

Figure 24 presents amount PPI to the 98 dB stimulus on PND 67. Univariate ANOVA did not reveal any significant differences. However, because the data were non-linear, *a priori* comparisons were performed and significant differences were revealed as follows: male offspring that had received 6 mg/kg/day nicotine showed significant impairment of PPI as compared to controls, $t_{(37)}=2.477$, $p<.05$; and female offspring that had received 6 mg/kg/day nicotine showed significantly improved PPI as compared to controls, $t_{(39)}=-2.368$, $p<.05$ and to offspring that had received 12 mg/kg/day nicotine $t_{(39)}=-2.006$, $p<.05$.

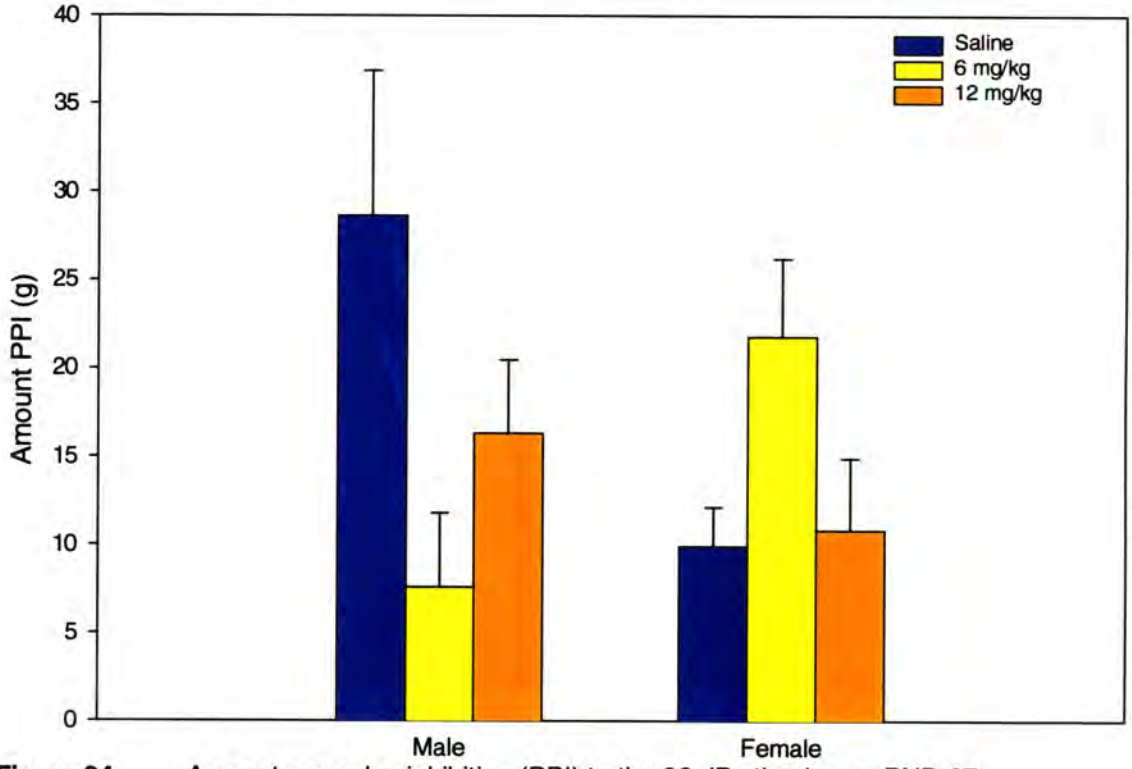


Figure 24 Amount pre-pulse inhibition (PPI) to the 98 dB stimulus on PND 67

Locomotion

All analyses for locomotor activity were conducted separately for each sex because females tend to move different amounts than males (Faraday et al., 1998b). All significant differences were revealed during adolescent testing (PND 46). These differences were not found during adult testing (PND 60). Figure 25 presents number of movements data, that is, the number of discrete movements made during the observation period. Female offspring that had been exposed to both dosages of drug made significantly fewer movements than control offspring, [$F(2,39)=4.276$, $p<.05$].

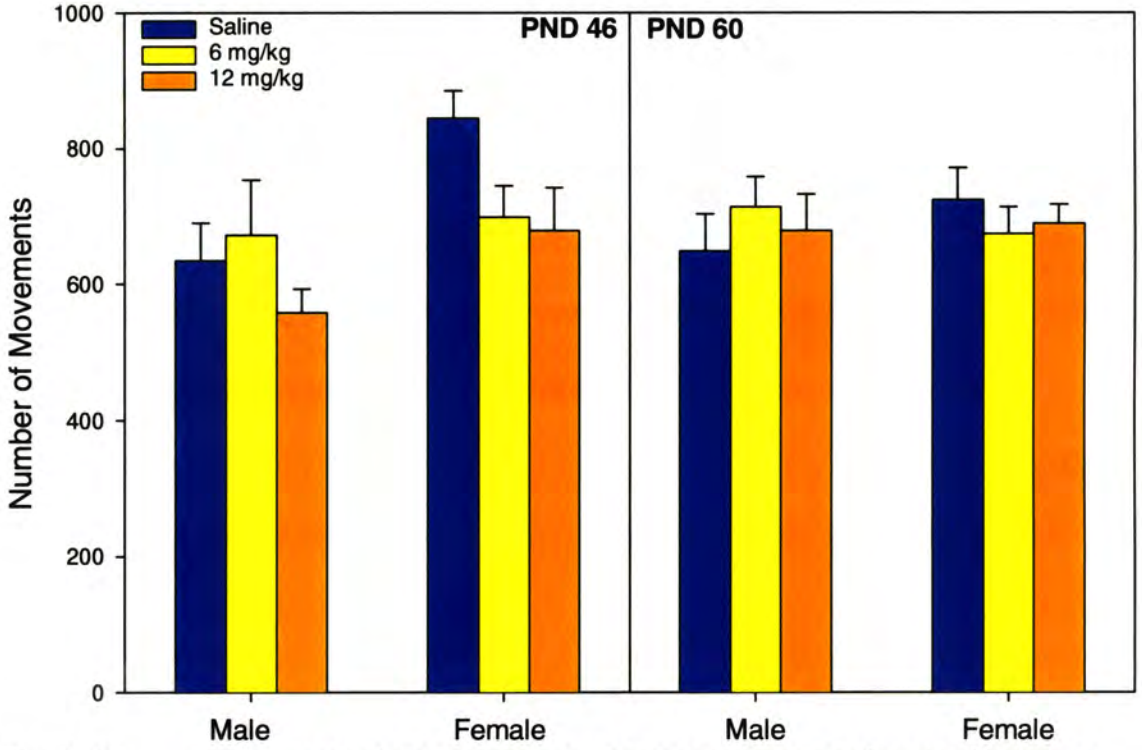


Figure 25 Total number of discrete movements made by male and female offspring on PND 46 and PND 60

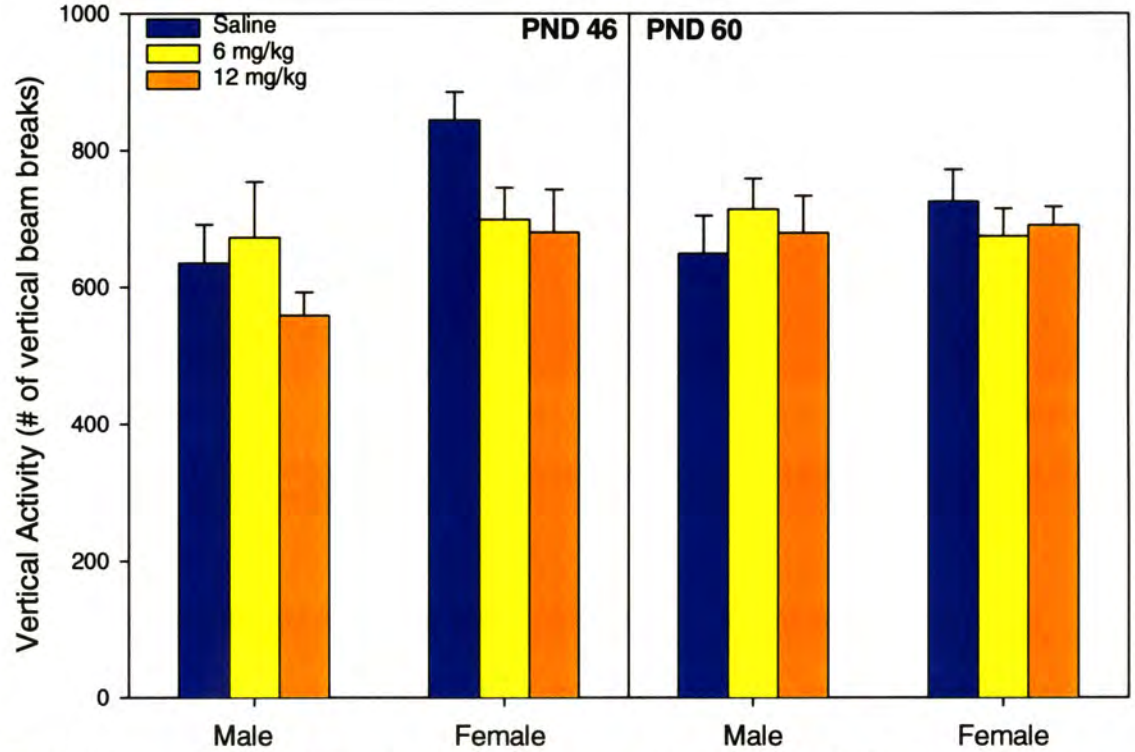


Figure 26 Total vertical activity of male and female offspring on PND 46 and PND 60

Figure 26 presents vertical activity data, that is, number of vertical beam breaks that occurred during the observation period. Female offspring that had received 12 mg/kg/day nicotine made significantly fewer vertical beam breaks than the other offspring, [$F(2,39)=4.276$, $p<.05$].

Figure 27 presents number of vertical movements data, that is, number of discrete vertical movements that occurred during the observation period. Female offspring that had received 12 mg/kg/day nicotine made significantly fewer vertical beam breaks that the other offspring, [$F(2,39)=3.943$, $p<.05$].

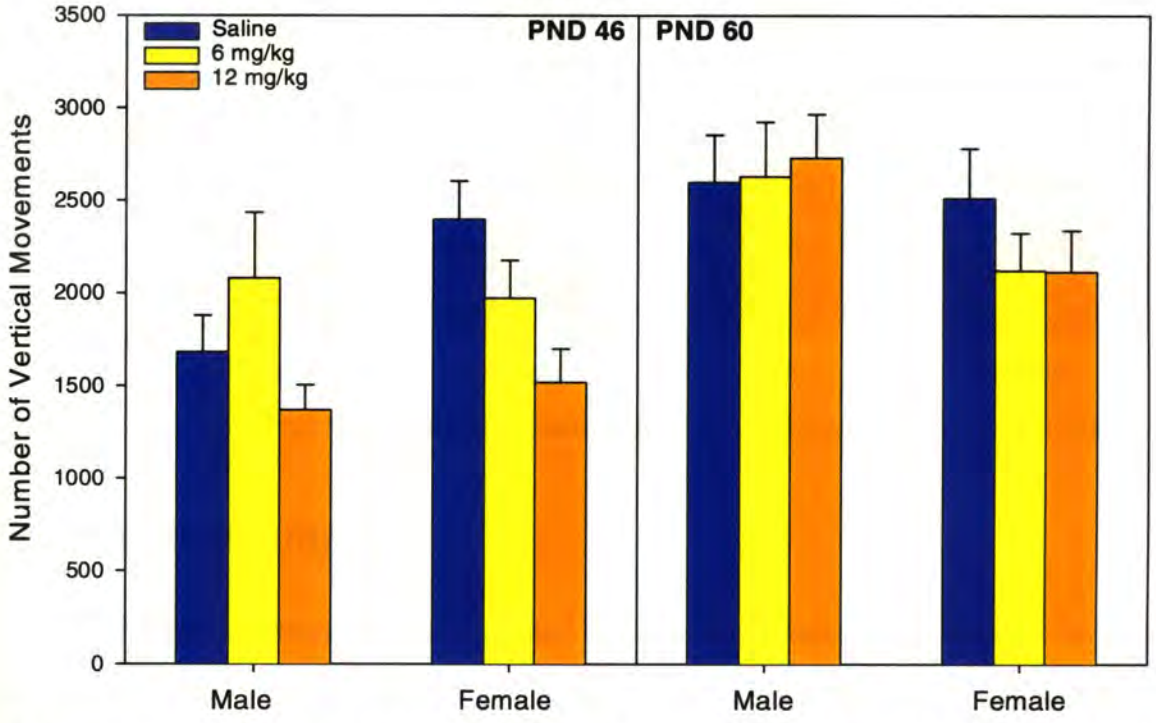


Figure 27 Total number of vertical movements made by male and female offspring on PND 46 and PND 60

Figure 28 presents data for amount of time spent vertical. *A priori* comparisons revealed that male offspring that had received 6 mg/kg/day nicotine

spent significantly more time vertical than the other groups, $t_{(37)}=2.23$, $p<.05$. Female offspring that had received 12 mg/kg/day nicotine spent significantly less time vertical than the other groups, [$F(2,39)=4.193$, $p<.05$].

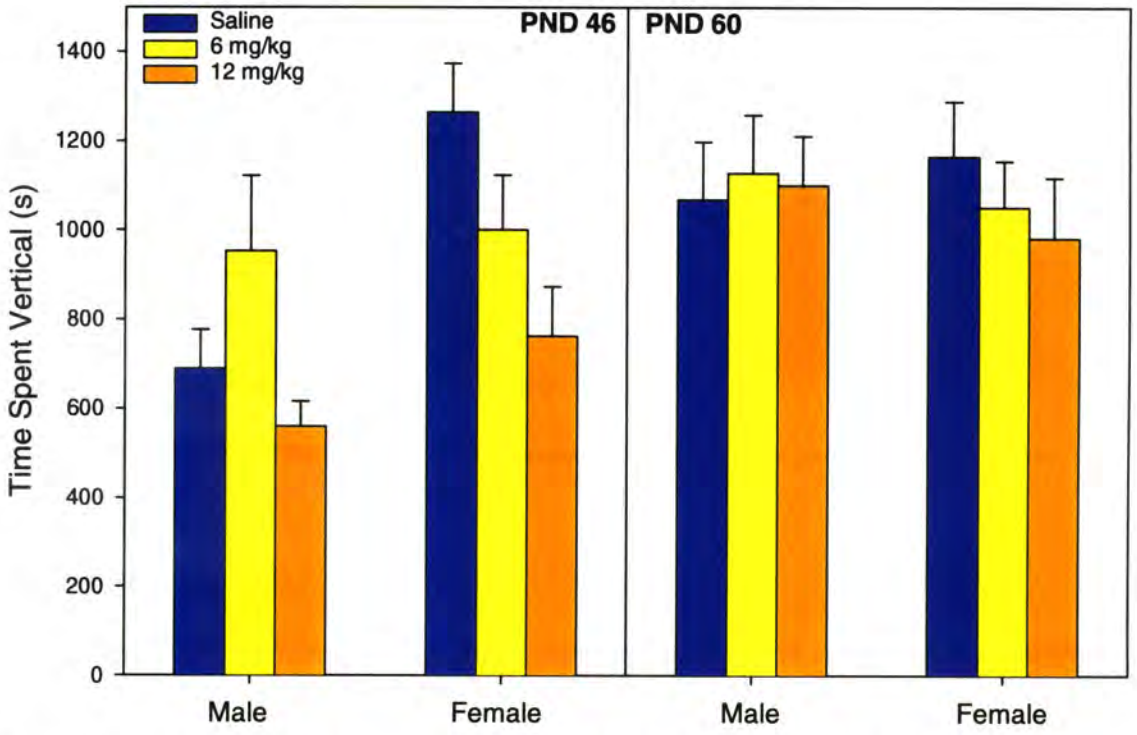


Figure 28 Total time in seconds spent vertical by male and female offspring on PND46 and PND 60

D1 Autoradiography

Figure 29 presents mean optical density, expressed as percent of cortical optical density. Optical density was analyzed using analyses of covariance (ANCOVA) in which mean optical density was the dependent variable and optical density of an area of cortex that did not show evidence of D1 binding, area analyzed (in mm^2), and piece of film as covariates. These variables were used as covariates in order to compare the area of frontal cortex analyzed to an area

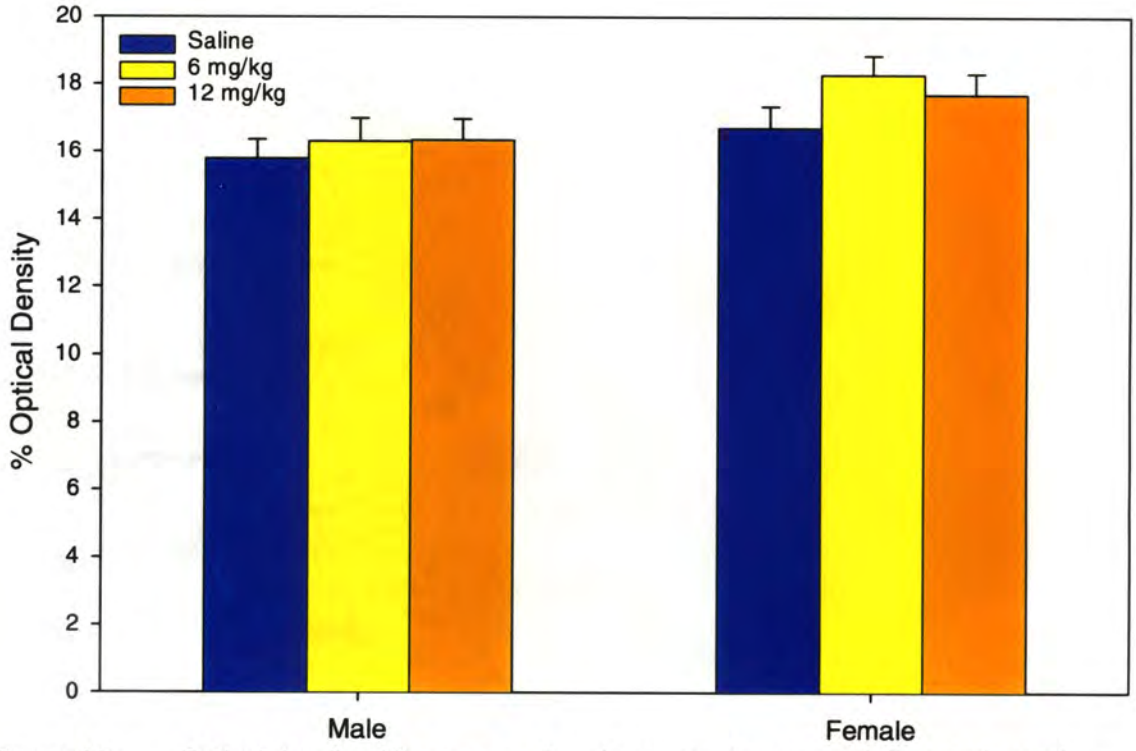


Figure 29 Optical density of the area analyzed expressed as percent of control area for male and female offspring

that did not have large amounts of D1 binding to control for gradations in film darkness, non-specific binding, and because each piece of film was treated as an individual experiment. The ANCOVA revealed a significant main effect for sex [$F(1,324)=8.194$, $p<.01$] but no effect for drug. Separate ANCOVAs for each sex did not reveal any significant findings.

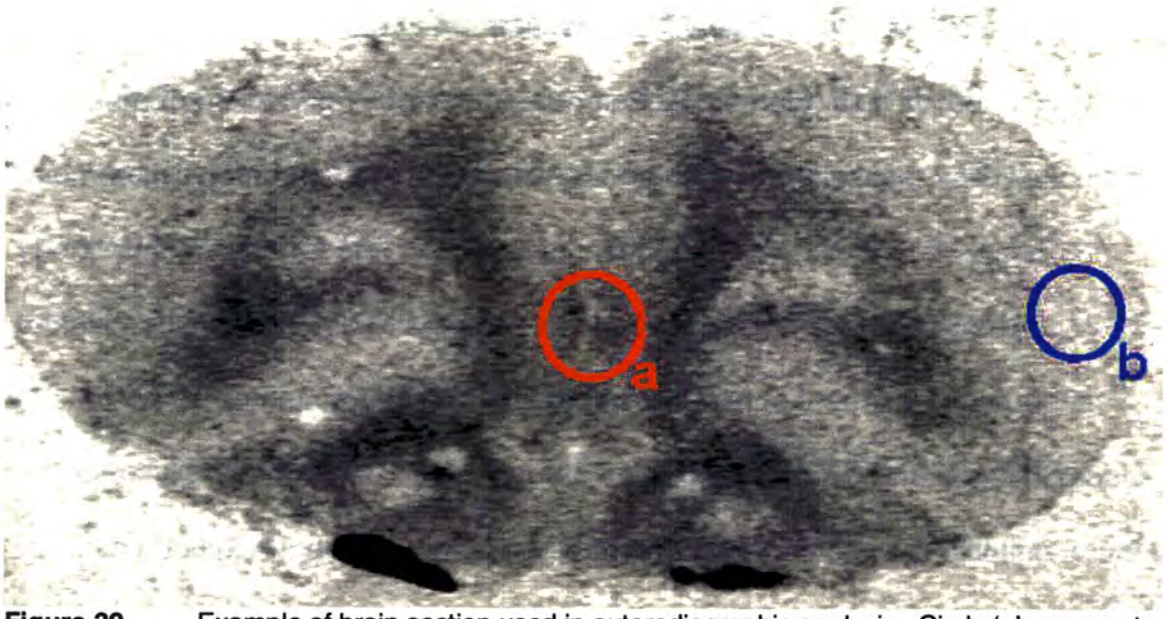


Figure 30 Example of brain section used in autoradiographic analysis. Circle 'a' represents the area that was analyzed for mean optical density, it was expressed as percent darker than circle 'b,' an area of cortex that was approximately the same size (~30 mm^2) showing low D1 binding. There were no differences in darkness of area 'a' between drug groups.

Discussion

The purpose of the present experiment was to examine effects of prenatal exposure to nicotine on spatial and non-spatial memory, activity, sensory gating and reactivity, and dopamine D1 receptor binding in adolescent and adult male and female rats. Prenatal exposure to nicotine resulted in cognitive and behavioral effects in the offspring. Some of these changes appeared in adolescence, some appeared in adulthood, and some that had appeared in adolescence disappeared by adulthood. In addition, there were sex differences in these effects. In general, offspring that had been exposed to nicotine *in utero* showed altered: body weight, memory performance, reactivity to an acoustic stimulus, sensory gating abilities, and activity levels. There were, however, no discernible changes in D1 receptor binding in the medial prefrontal cortex. Based on these findings, some of the hypotheses were accepted and others were rejected. Table 7 presents a listing of the acceptance and rejection of specific hypotheses. More specifically, Table 8 presents a summary of all of the findings broken down by dependent variable, sex and age of testing of the subjects. Each of the findings summarized in Table 8 is discussed below followed by a discussion of how these findings relate to other reports, the implications of the present findings for prenatal tobacco effects in humans, and possible future directions for research.

Body Weight

Nicotine reduced body weight of dams and offspring, as hypothesized and consistent with previous reports. Dams that had received nicotine lost weight in a dose-response manner. Those animals that received 12 mg/kg/day nicotine weighed less than those that received 6 mg/kg/day nicotine and they, in turn, weighed less than those that had received saline. This finding confirms previous reports that nicotine reduces body weight in a dose-response manner (Grunberg et al., 1984; Grunberg et al., 1986; Morgan et al., 1987; Saah et al., 1994; Winders et al., 1993) and serves to confirm that nicotine was effectively administered in the present experiment.

Additionally, as hypothesized, offspring of dams that had received nicotine during pregnancy weighed less than those that did not receive nicotine. This result confirms previous findings with animals (e.g., Cutler et al., 1996; Levin et al., 1998) and humans (e.g., Aronson et al., 1993; Brooke et al., 1989; Peacock et al., 1991). The effect of nicotine to reduce body weight occurred in male and female subjects and persisted throughout adolescence and into adulthood.

Memory Performance

Spatial Working Memory

Overall, prenatal administration of nicotine resulted in either impaired spatial working memory performance or no effect. The specific results depended on the specific task (i.e., radial-arm maze versus water maze), age of subjects, and sex of subjects.

Radial-Arm Maze

While all animals showed evidence of ability to learn the maze over time, offspring that had been exposed to nicotine took longer to attain performance levels indistinguishable from controls. Additionally, memory performance was impaired as a result of nicotine administration *in utero*. However, each sex was affected differently. Adult male offspring that had been exposed to nicotine made fewer correct choices in the radial-arm maze before an error was made and took longer to complete the task. Adolescent females that had been exposed to nicotine made more errors than control females, but this effect was not found in adults. These findings partially confirm the hypothesis that prenatal exposure to nicotine will globally impair radial-arm maze performance. The results are complex and indicate that time of testing and sex of subject are extremely important.

The present findings are at odds with the conclusion of Levin and colleagues (1996; 1993b; 1996b) that prenatal exposure to nicotine resulted in no effects on radial-arm maze performance but confirmed the findings of Yanai and colleagues (1992) who reported deleterious effects in mice. Taken together, these findings indicate that prenatal exposure to nicotine does affect radial-arm maze performance and previous null findings may be explained by too low a dosage of nicotine.

Water Maze

The results of water maze performance also depended on nicotine exposure, age, and sex of the subjects. Adolescent males that had been

exposed to nicotine tended to take longer to find the hidden, un-marked platform, but this effect disappeared by adulthood and there were no differences found for females of either age group. This effect was seen robustly only on one day of the experiment. The reason for this change in performance on one day of testing, in only one group of animals is not known. All of the behavioral measures were run in a counter balanced fashion. That is, all possible groups were represented in each "run" of a test and multiple runs were performed throughout the day until all animals had been tested. The order of animals was changed randomly each day. Therefore, on PND 53 the at least two male offspring that had been exposed to 6 mg/kg/day nicotine were tested in each run of the water maze task throughout the day. Perhaps on this day, these particular males experienced a change in adolescent hormone levels on this day, and the other males did not.

These findings disagree with the report by Cutler and colleagues (1996) that prenatal nicotine exposure does not affect water maze performance. As with the radial-arm maze findings, the difference between the present findings and Cutler and colleagues is likely a result of the fact that Cutler used a dosage of nicotine that was too low (4 mg/kg/day).

The water maze results of the present experiment, taken with radial-arm maze results, suggest that human males exposed to cigarette smoke *in utero* may have deficits in spatial memory processing that do not appear until adulthood, whereas females that had been exposed to cigarette smoke may have deficits that peak in adolescence and disappear by adulthood.

Non-spatial Memory

Passive Avoidance

Unexpectedly, passive avoidance performance was improved in males and there was no effect in females, counter to the hypothesized effect. Previous reports examining avoidance conditioning reported mostly null findings of prenatal nicotine (Bertolini et al., 1982; Genedani et al., 1983; Peters et al., 1982) but, as with previous studies examining prenatal nicotine exposure and spatial working memory, the dosages used by other investigators, likely, were too low (0.5-3.0 mg/kg).

The improvement in performance found in the present experiment was not associated with nor could it be explained by changes in locomotor activity (*see Locomotor Activity below*). This finding is particularly interesting because it demonstrates a disconnect between effects of nicotine on spatial working memory performance (mazes) and non-spatial memory (passive avoidance), and an actual reversal in the direction of the effects between these two types of memory performance. Additionally, these findings, like those for spatial working memory, demonstrate a differential response to prenatal drug exposure between the sexes. Males exposed to nicotine *in utero* were better able to inhibit a natural inclination to move away from a lit area into a dark area, whereas females and non-drug-exposed males were equally likely to cross or not cross into the dark area. This finding is particularly interesting given the animals' locomotor activity.

Locomotor Activity

There were two major reasons for examining locomotor activity: 1) as an index of overall activity, and 2) as a behavioral control for passive avoidance performance. Briefly, because passive avoidance performance is motor-activity dependent, it is important to measure and consider any changes in general motor activity.

Adolescent females tended to decrease locomotor activity in a linear fashion with increased dosage of prenatal drug exposure, counter to hypothesis and previous reports in rats (Richardson et al., 1994; Tizabi et al., 1997) and humans (Denson et al., 1975; Naeye, 1992). Adolescent males showed an inverted U-shaped dose response, that is, males prenatally exposed to the low dose of nicotine tended to exhibit higher levels of exploratory locomotor activity (e.g., vertical activity, time spent vertical (Decker, 1995)) as compared to controls and the higher dose, partially confirming the hypothesis that prenatal exposure to nicotine would increase activity of both sexes.

If the passive avoidance performance had been governed by motor activity alone, then females should have performed better on passive avoidance and should not have crossed to the dark chamber. Similarly, males exposed to 6 mg/kg/day nicotine, should have moved more quickly to the dark chamber because they exhibited higher levels of exploratory behavior than controls or the 12 mg/kg/day group. Because the passive avoidance performance was inconsistent with the locomotor findings, it must be concluded that prenatal nicotine exposure somehow improved males ability to remember not to cross

into the dark chamber and had no effect on females. Again, these findings indicate that sex of subject is extremely important in testing cognitive performance.

Acoustic Startle and Pre-Pulse Inhibition

The findings indicated that prenatal nicotine exposure had no effect on acoustic startle and pre-pulse inhibition during adolescence, and had an unexpected gender-specific effect during adulthood.

Female offspring that had been prenatally exposed to nicotine had increased reactivity (in a linear fashion with increasing dosage) to the 122 dB stimulus when presented with a pre-pulse when tested in adulthood. This finding is particularly striking because no other effects were found in reactivity to an acoustic stimulus prior to PND 67. In contrast, male offspring that had been exposed to nicotine prenatally showed no effect.

Additionally, adult males that had been exposed to the low dose of nicotine showed a decrement in ability to inhibit their reaction to a startling acoustic stimulus, in other words, an inability to gate sensory information, whereas adult females showed an enhanced ability to inhibit their responses. Again, similar to the findings for acoustic startle reactivity, the effects of prenatal exposure to nicotine did not appear until adulthood, and these effects were not only different for each sex, but were opposite in their direction. These findings suggest that while attention deficit disorder, with and without hyperactivity (ADD and ADHD, respectively), may be associated with maternal smoking in human children (Denson et al., 1975; Kristjansson et al., 1989; Naeye, 1992; Picone et

al., 1982; Streissguth, 1984), ability to gate or inhibit reactions to startling stimuli may be impaired in adult males, a defect that may not show up until adulthood. This finding may help to explain recent reports that adults are increasingly reporting symptoms similar to ADD and ADHD (e.g., Bellak et al., 1992; Dalteg et al., 1998; Shaffer, 1994). Additionally, if nicotine administration through cigarette smoking during gestation causes these deficits, then nicotine administration in adults may ameliorate or improve attentional processing (Bates et al., 1995; Kassel, 1997a; Kassel et al., 1997b; Spilich et al., 1992).

D1 Autoradiography

It was hypothesized that prenatal nicotine administration would affect dopamine receptor numbers as previously reported (Fung et al., 1989; e.g., Fung et al., 1992; Levin et al., 1998; Navarro et al., 1988). It was further hypothesized that changes would be evident in the medial prefrontal cortex because of its role in memory processing (e.g., de Bruin et al., 1994; Goldman-Rakic, 1990) and that changes in this area would be associated with changes in memory processing. Increased optical density indicating presence of dopamine receptors was found in a pattern that is in agreement with previous findings examining the amount of dopamine released in the medial prefrontal cortex as a result of electrical stimulation (Garris et al., 1993). That is, less binding at the most dorsal and ventral locations of the medial prefrontal cortex and most located in the area in between (*see Figure 30, circle 'a'*) indicating that the correct area was examined. However, there were no differences in optical density of this area between groups of offspring, even though changes in

memory processing were found as a result of nicotine administration. Possible explanations for this null finding could be: 1) the dopaminergic system adapted to alterations as a result of nicotine administration (neural plasticity); 2) dopamine alone does not account for memory processing (e.g., the cholinergic system also plays a role in memory processing (Broersen et al., 1995)); 3) the area analyzed in rats is not the area responsible for memory processing despite the reports of previous studies in rats (e.g., Aggleton et al., 1995; Broersen et al., 1995; Bubser et al., 1994; de Bruin et al., 1994) and primates (e.g., Goldman-Rakic, 1992; Sawaguchi et al., 1991; Williams et al., 1995)); 4) changes in spatial memory performance may have been a result of alterations in other areas previously shown to play a strong role in these behaviors (e.g., the hippocampus (e.g., Olton et al., 1979a; Olton et al., 1977)).

Summary and Future Directions

Taken together, the findings from this experiment indicate that prenatal exposure to nicotine results in behavioral and cognitive effects that are: 1) different for each sex, 2) dependent on age of testing, 3) not always an impairment in performance, 4) not associated with changes in D1 binding in the medial prefrontal cortex in rats. In light of these considerations, there are several possible future studies that should be completed to address each consideration.

First, it is important to determine why there are sex differences in behavioral responses to prenatal exposure to nicotine. One possibility is that the differences that were observed were a result of differences in sensitivity to

nicotine. That is, females and males may have similar responses to nicotine's effects but be differentially sensitive to particular dosages of this drug. If this is the case, then the differences that were observed reflected a shift in an underlying dose-effect curve. Alternatively, males and females may respond differently to nicotine. In locomotor activity, for example, females exhibited a decreasing linear dose response to prenatal exposure to nicotine, whereas males exhibited an inverse U-shaped dose response. Are the females shifted to the left in the dose-response spectrum (an increase in sensitivity) and only the decreasing limb of the inverted U-shaped dose response curve is seen, or is there a different function that underlies female and male responses to prenatal nicotine exposure? One way to investigate these possibilities is to repeat this experiment using a larger number of dosages (e.g., 3.0, 6.0, 9.0, and 12.0 mg/kg/day) in order to cover a larger portion of the dose-response spectrum to identify the entire curve.

Another possible explanation for this sex difference may be neuroanatomical differences in response to gonadal hormones. Kolb and colleagues (1990a) have identified structural differences between the sexes in prefrontal cortex neurons. Specifically, there are differences in the global structure of the prefrontal cortex between the sexes, and male rats show greater amounts of dendritic arborization. Grafts from female rats placed into males showed similar effects and these effects were blocked by neonatal castration (Stewart et al., 1988). Additionally, in behavioral studies conducted by Kolb (1987; 1988) lesions of the medial frontal region produced larger deficits in

females on water maze and a variant of the radial-arm maze. To follow up on this possible explanation for the sex differences in the present experiment, offspring could be gonadectomized and sex hormones could be manipulated.

Because of the differences in the findings between spatial memory tasks and the non-spatial task, future experiment should examine effects of prenatal nicotine exposure on other types of cognitive tasks such as: active avoidance, freezing, 16-arm radial-arm maze, other radial-arm maze procedures (e.g., restricting access in the hub of the maze), and other water maze procedures (e.g., underwater swim maze). The addition of these measures would broaden the investigation to the study of several different types of memory, more complicated and demanding memory tasks, and measures of learning. Perhaps this inclusion would reveal additional effects of prenatal exposure to nicotine on cognitive performance.

Another possible future study should examine each of the variables tested in this experiment, but starting at an older age. Changing the age of testing would establish if the effects truly disappear at adulthood, as in the case of water maze performance, or if they disappear because of learning effects. If the effect truly disappears by adulthood (or conversely, appears only during adulthood as found in this experiment for radial-arm maze performance), then it can be established that many effects of prenatal exposure are either long-lasting or require long periods of time to become evident. Because these variables are affected by nicotine administration during gestation, could administration of various dosages of nicotine in adulthood ameliorate the deficits found? To

answer this question, rats exposed to nicotine prenatally would be administered various dosages of nicotine or saline and tested throughout the life span.

The findings of the present research also may have implications for humans. It is particularly important to confirm that nicotine administration during gestation can cause long-term effects on offspring, and whether some of these effects may not appear until adulthood. Epidemiologic studies examining the effects of cigarette smoking in humans should examine adults who were children of smoking mothers to determine if there are untoward effects that either persist until adulthood, as Fogelman and Manor (1988) reported with intellectual achievement, or do not appear until adulthood. Such studies might be conducted in populations where cigarette smoking prevalence remains high (e.g., in low socioeconomic status communities in the United States, throughout Europe, and in Asia). Additionally, other cognitive variables (e.g., attention and specific types of memory) should be examined in adult humans. If nicotine administration via cigarette smoking during pregnancy causes deleterious effects in the offspring, then administration of nicotinic cholinergic agonists may help to ameliorate these deficits. If administration of nicotinic agonists does help to alleviate deficits induced by smoking during pregnancy, then that may explain why some people initiate smoking behavior; they are self-medicating themselves by self-administering nicotine. This finding may lead to alternate explanations for smoking behavior that may lead to additional treatment strategies for smoking cessation.

In summary, the present research examined prenatal exposure to nicotine

and found that cognitive performance was affected with most measures showing impairment but a few revealing improvement. There were sex differences in these responses and some effects were not revealed until adulthood. Future research should further examine these sex differences. In addition, it is important to examine possible, underlying mechanisms including the potential involvement of specific neurotransmitter systems (e.g., cholinergic) and specific neuroanatomic (e.g., hippocampus) involvement.

Tables

Table 1 Estimates of constituents present in tobacco and smoke

Table I

Estimates of Constituents Present in Tobacco and Smoke

Source	Number
Total found in tobacco smoke	**3875**
Total found in leaf (before burning)	**2549**
Transferred unchanged from leaf to smoke	**1135**
Formed in pyrolytic decomposition of tobacco	**2740**
Found in leaf but not transferred to smoke	**1414**

Adapted from (Dube et al., 1982, p. 47).

Table 2 Approximate number of compounds identified in some major compound classes

Table II

Approximate Number of Compounds Identified in Some Major Compound Classes

Class	Number
Amides, Imides, Lacatams	237
Carboxylic Acids	237
Lactones	150
Esters	474
Aldehydes	108
Ketones	521
Alcohols	379
Phenols	282
Amines	196
N-Heterocycles	921
Hydrocarbons	755
Nitriles	106
Anhydrides	11
Carbohydrates	42
Ethers	311
Total	4720

From (Dube et al., 1982, p. 48).

Note: The total 4720 is different from 3875 noted in Table 1 because some compounds have multiple functional groups which will place them into more than one category.

Table 3 F-Values from maternal body weight analyses

Day	Finding	F-Value	Direction of effect
GD4	*n.s.*		
GD7	*n.s.*		
GD9	*n.s.*		
GD11	*m.e. drug*	[$F(2,12)=4.827$, $p=.029$]	12 < sal
GD14	*m.e. drug*	[$F(2,12)=7.081$, $p=.009$]	12 < sal
GD16	*m.e. drug*	[$F(2,12)=5.278$, $p=0.23$]	12 < sal
GD18	*m.e. drug*	[$F(2,12)=5.902$, $p=.016$]	12 < sal
GD20	*m.e. drug*	[$F(2,12)=5.851$, $p=.017$]	12 < sal
GD22	*m.e. drug*	[$F(2,12)=7.182$, $p=.009$]	12 < sal
		parturition	
PND2	*m.e. drug*	[$F(2,7)=25.447$, $p<.001$]	6&12 < sal; 12 < 6
PND6	*m.e. drug*	[$F(2,9)=7.369$, $p=.013$]	12 < sal; 12 < 6
PND10	*n.s.*	[$F(2,9)=4.161$, $p=.053$]	n.s. (Tukey)
PND13	*n.s.*		
PND16	*n.s.*		
PND23	*n.s.*		

Table 4 F-Values from male offspring body weight analyses

PND	Finding	F-Values	Direction of effect
2	*m.e. drug*	[$F(2,37)=31.423$, $p<.001$]	12 < sal
6	*m.e. drug*	[$F(2,37)=50.428$, $p<.001$]	12 < sal; 12 < 6
10	*m.e. drug*	[$F(2,37)=39.537$, $p<.001$]	12 < sal; 12 < 6
13	*m.e. drug*	[$F(2,37)=42.006$, $p<.001$]	12 < sal; 12 < 6
16	*m.e. drug*	[$F(2,37)=23.576$, $p<.001$]	12 < sal; 12 < 6
23	*m.e. drug*	[$F(2,37)=11.152$, $p<.001$]	12 < sal; 12 < 6
30	*m.e. drug*	[$F(2,37)=10.214$, $p<.001$]	12 < sal; 12 < 6
33	*m.e. drug*	[$F(2,37)=10.394$, $p<.001$]	12 < sal; 12 < 6
35	*m.e. drug*	[$F(2,37)=9.557$, $p<.001$]	12 < sal; 12 < 6
		begin food restriction	
40	*n.s.*		
42	*n.s.*		
44	*m.e. drug*	[$F(2,37)=3.464$, $p=.042$]	*n.s.* (Tukey)
		end food restriction	
45	*m.e. drug*	[$F(2,37)=4.577$, $p=.017$]	12 < 6
48	*m.e. drug*	[$F(2,37)=3.817$, $p=.031$]	12 < 6
50	*m.e. drug*	[$F(2,37)=3.815$, $p=.031$]	12 < 6
52	*m.e. drug*	[$F(2,37)=3.443$, $p=.043$]	12 < 6
54	*m.e. drug*	[$F(2,37)=3.734$, $p=.033$]	12 < 6
57	*m.e. drug*	[$F(2,37)=3.562$, $p=.038$]	12 < 6
58	*m.e. drug*	[$F(2,37)=3.706$, $p=.034$]	12 < 6
		begin food restriction	
62	*n.s.* (p=.059)		
65	*m.e. drug*	[$F(2,37)=4.023$, $p=.026$]	12 < 6
		end food restriction	
67	*m.e. drug*	[$F(2,37)=4.128$, $p=.024$]	12 < 6
69	*n.s.*		

Table 5 F-Values from female offspring body weight analyses

PND	Finding	F-Values	Direction of effect
2	*m.e. drug*	[$F(2,39)=34.753$, $p<.001$]	12 < sal; 12 < 6
6	*m.e. drug*	[$F(2,39)=18.939$, $p<.001$]	12 < sal; 12 < 6
10	*m.e. drug*	[$F(2,39)=36.294$, $p<.001$]	12 < sal; 12 < 6
13	*m.e. drug*	[$F(2,39)=37.949$, $p<.001$]	12 < sal; 12 < 6
16	*m.e. drug*	[$F(2,39)=29.283$, $p<.001$]	12 < sal; 12 < 6
23	*m.e. drug*	[$F(2,39)=16.805$, $p<.001$]	12 < sal; 12 < 6
30	*m.e. drug*	[$F(2,39)=15.135$, $p<.001$]	12 < sal; 12 < 6
33	*m.e. drug*	[$F(2,39)=12.326$, $p<.001$]	12 < sal; 12 < 6
35	*m.e. drug*	[$F(2,39)=8.780$, $p<.001$]	12 < sal; 12 < 6
		begin food restriction	
40	*m.e. drug*	[$F(2,39)=7.707$, $p<.002$]	12 < sal; 12 < 6
42	*m.e. drug*	[$F(2,39)=4.760$, $p=.014$]	12 < sal; 12 < 6
44	*m.e. drug*	[$F(2,39)=3.733$, $p=.033$]	12 < 6
		end food restriction	
45	*m.e. drug*	[$F(2,39)=4.449$, $p=.018$]	12 < 6
48	*m.e. drug*	[$F(2,39)=4.821$, $p=.013$]	12 < 6
50	*n.s.*	[$F(2,41)=3.192$, $p=.052$]	12 < 6
52	*n.s.*	[$F(2,39)=3.130$, $p=.056$]	12 < 6
54	*n.s.*		
57	*m.e. drug*	[$F(2,39)=3.981$, $p=0.27$]	12 < 6
58	*n.s.*		
		begin food restriction	
62	*n.s.*		
65	*m.e. drug*	[$F(2,39)=4.000$, $p=.026$]	12 < 6
		end food restriction	
67	*m.e. drug*	[$F(2,39)=3.700$, $p<.034$]	12 < 6
69	*n.s.*		

Table 6 Chi-Square analyses for passive avoidance

		Chi-Square			
		Male		**Female**	
		No	Yes	No	Yes
Saline	**n**	7	8	10	5
	X^2, *p*	X^2=.067, *p*=.796		X^2=1.667, *p*=.197	
6 mg/kg	**n**	1	13	7	9
	X^2, *p*	**X^2=10.286, *p*=.011**		X^2=.250, *p*=.617	
12 mg/kg	**n**	1	10	7	4
	X^2, *p*	**X^2=7.364, *p*=.007**		X^2=.818, *p*=.366	

Table 7 Hypothesis acceptance/rejection table

Hypothesis

Prenatal exposure to nicotine will result in:		Accepted?
1)	reduced body weight	Yes
2)	impaired radial-arm maze performance	Yes, but complex
3)	impaired water maze performance	Males: Yes Females: No
4)	impaired passive avoidance performance	No (enhanced in males)
5)	altered acoustic startle response	Males: No Females: Yes
	and PPI	Males: Yes (decrement) Females: Yes (enhancement)
6)	increased locomotor activity (more so in males)	Males: complex Females: No (linear decrease)
7)	altered D1 binding density	No
8)	correlation between D1 binding and memory performance	No relationship
9)	different results at different ages of tesing	Yes

Table 8 Summary of findings

	Males		Females	
	Adolescent	**Adult**	**Adolescent**	**Adult**
body weight	↓	↓	↓	↓
radial-arm maze				
entries to repeat	–	↓	–	↓
errors	–	–	↓	–
latency	–	↓	–	–
water maze	↓	–	–	–
passive avoidance	↑		–	
locomotion	↑	–	↓	–
ASR-PPI	–	–	–	↑
amt. ASR 122 w/pp				
amt. PPI 98db	–	↓↓	–	↑↑
D1 autoradiography	–		–	

Note: Symbols denote that prenatal nicotine exposure resulted in either no effect (–), improvement or increase (↑), or decrement or decrease (↓) in the variable identified.

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