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Pharmacology, Biochemistry and Behavior 81 (2005) 54–64

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Neonatal ethanol and nicotine exposure causes locomotor activity changes in preweanling animals

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Received 5 August 2004; received in revised form 11 February 2005; accepted 17 February 2005
Available online 19 April 2005

Abstract

Sprague–Dawley rats were used to investigate the effects of neonatal ethanol (ETOH) and nicotine (NIC) exposure on activity levels in preweanling offspring. Male and female pups received daily oral intubations of ethanol ((ETOH) 5 g/kg/day), nicotine ((NIC) 12 mg/kg/day), ethanol and nicotine ((ETOH+NIC) 5 g/kg/day+12 mg/kg/day) or isocaloric maltose (control) on either postnatal days (PND) 1–7 or PND 8–14. A non-treated control group was also included. Peak blood ethanol concentrations (BECs) measured in a separate subset of animals ranged from 167 and 344 mg/dl depending upon neonatal treatment and period of exposure. Subjects were tested in an open field apparatus on PND 19–21. Animals exposed to ETOH or ETOH+NIC on PND 1–7 were hyperactive relative to the other treatment groups. In contrast, animals exposed to NIC or ETOH+NIC during PND 8–14 were hypoactive relative to other treatment groups. Males appeared more sensitive than females on measures of anxiety (distance traveled in the center of the open field) but this also varied dependent on neonatal treatment and period of exposure. These findings suggest that the third trimester is a critical period for ETOH and NIC effects on offspring activity although the pattern of effects on activity are different depending on when drug exposure occurred during the neonatal period.

Keywords: Prenatal ethanol exposure; Prenatal nicotine exposure; Fetal alcohol effects

1. Introduction

Smoking and drinking behavior are highly correlated. Between 80% and 95% of alcoholics smoke, and 70% of alcoholics are heavy smokers, compared with the 10% in the population as a whole (Patten et al., 1996; Collins and Marks, 1995; DiFranza and Guerrera, 1990). Recent data from the National Survey on Drug Use and Health found that 9% of pregnant women reported drinking alcohol in the past month with 3% reporting binge alcohol use (SAMHSA, 2004). While the proportion of women that continue to drink during pregnancy has declined in recent years, the proportion that binge drink has not changed (Ebrahim et al., 1999) and individuals that tend to drink heavily also smoke more heavily (Collins et al., 1996). This high comorbidity between smoking and alcohol consumption may be particularly significant for the developing fetus.

Clinical studies have examined the potential interaction between maternal smoking and alcohol consumption with the primary emphasis on measures of fetal growth; usually birth weight. Smoking, in combination with maternal alcohol consumption, produced greater reductions in birth weight than either of these drugs alone (Shu et al., 1995; Haste et al., 1991); with heavy smoking and alcohol consumption having a greater impact (Peacock et al., 1991). This effect appeared selective to alcohol and smoking during pregnancy even when controlling for other variables such as illicit drug use (Jacobson et al., 1994). Whether exposure to ETOH and cigarette smoke in utero has longterm behavioral consequences is less clear. One research group has suggested a synergistic effect on infant learning (Martin et al., 1977; Streissguth et al., 1981) and more recent clinical studies have begun to assess the

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doi:10.1016/j.pbb.2005.02.002

possible role of maternal smoking in combination with alcohol consumption during pregnancy on various childhood psychopathologies, particularly attention-deficit hyperactivity disorder (ADHD) (Hill et al., 2000; Mick et al., 2002).

Animal models, most notably rodent models, have also been used to address the potential interactions of prenatal exposure to ETOH and NIC. Aversive conditioning in an operant task was more significantly impaired with prenatal exposure to both drugs although performance by ETOH offspring appeared improved in an appetitive task with NIC coexposure (Martin et al., 1982). In contrast with this limited literature, the effects of ETOH or NIC alone during pregnancy have been far more extensively studied. ETOH exposure during development has been shown to cause response perseveration and inhibition deficits, hyperactivity, low body weight, and poor performance on learning and memory tasks (Riley et al., 1979; Rockman et al., 1989; Abel, 1978, 1979; Blanchard et al., 1987). While studies assessing prenatal NIC exposure are less conclusive, prenatal NIC exposure has been associated with hyperactivity (Ajarem and Ahmad, 1998; Fung and Lau, 1988) and a variety of cognitive impairments (e.g. Sorenson et al., 1991; Levin et al., 1993, 1996; Vaglenova et al., 2004).

Research examining potential temporal windows of vulnerability for ETOH induced damage to the CNS has engaged animal investigators for several years. The "brain growth spurt" is a period of brain development that begins during the third trimester in humans. Due to species differences in when birth occurs relative to CNS development in humans and rodents (Dobbing and Sands, 1979), third trimester rodent models involve an exposure paradigm in which drugs are administered directly to the neonatal rat. Data from this model have shown that the postnatal days on which the animal is exposed, in addition to the pattern of exposure, are critical factors in determining the extent of behavioral deficit and type of damage to the CNS following neonatal ETOH exposure (Goodlett and Johnson, 1997, 1999; West et al., 1989).

Additionally, there is emerging evidence that there may be temporal windows of vulnerability for NIC administration during the brain growth spurt, however little behavioral evidence exists. Studies assessing nicotinic receptor subtypes show an increase in $\alpha4\beta2$ and $\alpha7$ during the second postnatal week (Zhang et al., 1998). Further, Miao et al. (1998), and Narayanan et al. (2002), have shown an upregulation of nicotinic receptors following neonatal NIC treatment which is long-lasting when the exposure occurs on PND 8–14. Behavioral studies provide further support that this 3rd trimester is a sensitive time for nicotinic effects on the developing brain as measured by cognitive performance (Ankarberg et al., 2001) and activity levels (Thomas et al., 2000; Nordberg et al., 1991; Eriksson et al., 2000).

A very limited number of studies have begun to investigate the potential interaction of ETOH and NIC using this 3rd trimester model. While there is currently little evidence for a synergistic or interactive effect on neuroanatomical or behavioral indicators, neonatal ETOH and neonatal NIC produce neuroanatomical and behavioral changes (Chen et al., 1998, 1999; Girard et al., 2001). Furthermore, the presence of NIC has been reported to significantly reduce blood alcohol concentrations in neonatal rats (Chen et al., 2001) and prenatal or neonatal ETOH exposure appears to alter subsequent response to NIC (Rogers et al., 2004; Nagahara and Handa, 1999).

The purpose of the current study was to examine the effects of neonatal ETOH and/or NIC administration on locomotor activity. Drugs were administered via oral gavage as the route of administration. Although NIC is not typically administered orally in animal models, Narayanan and colleagues have reported an upregulation of nicotinic receptors in pups fed from a lactating dam given NIC providing evidence that oral NIC reaches neonatal rat CNS (Narayanan et al., 2002). To look at developmental windows of vulnerability, litters were administered drugs on either PND 1–7 or PND 8–14. These days were chosen based on existing data showing significant changes in both cholinergic (Rawat, 1977) and glutamate receptor subtypes (Williams et al., 1993) during the first two postnatal weeks that could be influenced by ETOH, NIC or this dual drug combination.

2. Methods

2.1. Breeding procedure

Subjects were neonatal Sprague–Dawley rats. Parent animals were purchased from Harlan Labs (Indianapolis, IN), and were singly housed (males) or group housed (females) in a colony room (12:12 light/dark cycle, temperature and humidity controlled). Males were individually placed with 3–5 females overnight in large group cages, with removal of the male in the morning. Pregnant females were individually housed and transported to a separate room designated as a nursery where they received water and laboratory chow ad libitum. As parturition approached, females were checked twice daily for newborn offspring. Birth was considered postnatal day 0. On postnatal day 1, litters were weighed and randomly culled to 10 pups, keeping 5 males and 5 females whenever possible. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

2.2. Neonatal drug administration

One male and one female pup from each litter was assigned to one of five treatment groups; ETOH 5 g/kg/day

(ETOH), nicotine tartrate 12 mg/kg/day (NIC), ETOH 5 g/kg/day+NIC 12 mg/kg/day (ETOH+NIC), maltose (1 mg/ml) as an isocaloric control and a non-intubated control. These doses were chosen following extensive pilot work to determine doses that minimized pup mortality and body weight differences but yet reached clinically relevant BECs. Animals were marked with a non-toxic marker for identification purposes. Each day, the litters were briefly separated from the dam and each pup was weighed and placed on a heating pad until all pups had received drug treatments. A piece of PE-10 tubing (Clay Adams) connected to a 1 cc syringe was lubricated with corn oil and then gently inserted into the pup's mouth, down the esophagus and into the stomach. Once the tubing reached the stomach, an infusion of milk solution designed to mimic rat milk (West and Hamre, 1985) was administered (.0278 ml/g bw). This procedure is relatively non-invasive as measured by signs of distress (overactivity during administration or vocalizations), normal weight gain, and very low mortality. No acute physiological response (e.g. difficulty in respiration or distress) was observed among any of the pups following administration of these drugs via this route. Pups were intubated twice per day at 13:00 and 15:00 h on either PND 1–7 or PND 8–14. Pups do the majority of their suckling in the morning (Lee and Williams, 1977), thus intubations were given in the afternoon in order to minimize body weight differences between treatment groups as much as possible. The daily dose was split into two separate feedings as pilot studies showed that giving the 5 g/kg dose in a single bolus produced significant body weight deficits, especially in pups intubated PND 1–7.

2.3. Blood ETOH concentration (BEC)

Neonatal animals from four additional litters were intubated daily between 1400 and 1600 from PND 1–4 or PND 8–11 for measurement of blood ethanol concentrations. Four groups were included in this design: PND 1–4, ETOH and ETOH+NIC, PND 8–11, ETOH and ETOH+NIC. Blood was collected by making a 1 mm cut at the tip of a subject's tail on PND 4 or 11 and collecting 20 μl of blood at each sampling. The BEC curve was established by collecting samples at 30, 60, and 120 min, and 24 h following ETOH administration. Plasma was separated and frozen at −70 °F freezer for 1 week until subsequent BEC assay using an Analox AM 1 Alcohol Analyzer (Analox Instruments). Standards were run prior to and after every 20 samples. There were 7–11 animals per group and exposure period in each cell of the experimental design.

2.4. Locomotor activity

Beginning on PND 19, animals were tested singly in an open field apparatus, (30.48 × 30.48 cm). Pre-weanling animals were used in this study following previous reports showing that this age is sensitive for alcohol-related effects on hyperactivity (Kelly et al., 1987c; Melcer et al., 1994). Pups were separated from their dam and put on heating pads in their home cage until all pups in the litter were tested (approximately 1 h or less). Each subject was individually placed in a holding cage and brought into the test room for 1 min habituation prior to placement in the activity chamber. Locomotor activity was measured by a Polytracker™ Video Imaging System (San Diego Instruments) interfaced with an IBM computer for 20 min daily (in 10 min blocks) for three consecutive days. Testing was conducted in a darkened room with fans to provide white noise.

The dependent variables recorded included total distance traveled (as a function of block and day) and two variables related to anxiety; latency to enter the center of the open field (10.6 cm × 10.6 cm), and distance traveled in the center. There were 7–10 animals/group/sex/exposure period in each cell of the experimental design thus representing 7–10 litters in each cell.

3. Results

3.1. Neonatal body weights

Postnatal Days 1–7: Animals intubated PND 1–7 had growth curves that differed depending upon gender and neonatal treatment (day × ETOH [$F(6,462)=50.79$]; day × NIC [$F(6.462)=17.94$; day × sex, [$F(6,462)=7.77$, $p<0.0001$]) (data not shown). Subsequent univariate analyses showed that animals treated with ETOH or ETOH+NIC gained weight more slowly relative to the other treatment conditions beginning on PND 2 [$F(1,83)=11.73$, $p<0.001$], accepted p value of 0.012 to correct for repeated analyses. NIC exposed animals also showed a reduction in weight gain relative to controls but this was not significant until PND 5 [$F(1,83)=12.04$, $p<0.001$]. Males weighed more than females on PND 1 [$F(1,83)=22.42$, $p<0.0001$] and PND 4 [$F(1,83)=8.04$, $p<0.01$].

Postnatal Days 8–14: Similar to animals treated on PND 1–7, animals treated with ETOH or ETOH+NIC on PND 8–14 lagged behind other groups in weight gain, (day × ETOH, [$F(6,402)=2.12$, $p<0.05$]) (data not shown). Subsequent univariate analyses showed that the difference between the groups that received ETOH and all other groups was not significant until PND 11, [$F(1,73)=9.12$, $p<0.005$].

3.2. Blood ETOH concentration (BEC)

Data were analyzed using a repeated measures ANOVA with group, gender and age at drug exposure as factors. As original analyses showed no main effect or interactions with gender that were significant, data were collapsed across this factor ($N=7$–11 group/period of

exposure). The overall analysis revealed a significant group × period of exposure × time of drug administration interaction [$F(3,99)=12.45$, $p<0.0001$] (see Fig. 1). Subsequent univariate analyses showed that administration of these drugs in combination during the second postnatal week resulted in lower BEC then ETOH alone at the 30 and 60 min time points ([$F(3,35)=13.075$, $p<0.0001$, Tukey's HSD test, $p<0.0001$][$F(3,35)=28.649$, $p<0.0001$, Tukey's HSD test, $p<0.0001$], respectively. There were no differences at these time points when exposure was during the first postnatal week. However, at the 2-h time point, exposure to both ETOH+NIC during both postnatal weeks resulted in lower BEC than the animals treated with ETOH in their respective exposure ages [$F(3,35)=54.73$, $p<0.0001$, Tukey's HSD test, p's<0.01].

In addition to BEC differences between animals treated with ETOH vs. ETOH+NIC, there were also BEC differences in animals treated with ETOH during the first postnatal week versus the second postnatal week. Animals that were treated with ETOH had a significantly higher BEC when treated during the second postnatal week than during the first postnatal week at the 60 min and 2-h time points ([$F(3,35)=28.649$, $p<0.0001$, Tukey's HSD test, $p<0.02$][$F(3,35)=54.73$, $p<0.0001$, Tukey's HSD test, $p<0.0001$]). Animals treated with ETOH+NIC during the first versus second postnatal weeks differed at the 2-h time point only where animals treated during the second week had a significantly lower BEC than animals treated during the first week ([$F(3,35)=54.73$, $p<0.0001$, Tukey's HSD test, $p<0.0001$]).

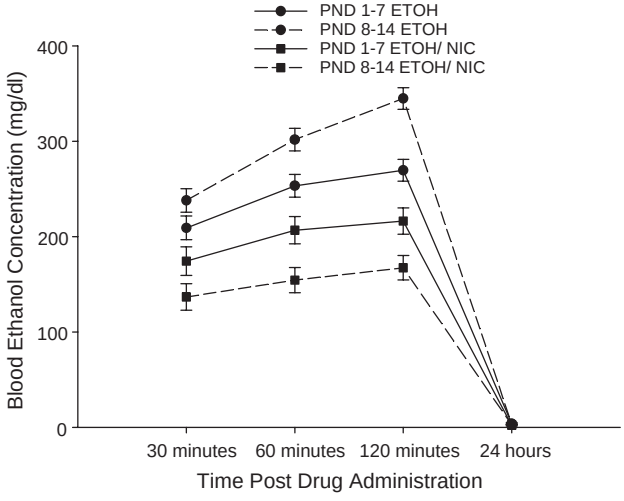

Fig. 1. Mean blood ETOH concentration (± SEM) collapsed across sex as a function of neonatal drug treatment either during the first or second postnatal weeks. ETOH treated animals had higher BECs than ETOH+NIC treated animals, an effect that was more pronounced in animals intubated during the 2nd postnatal week.

3.3. Locomotor activity: statistical analyses

Activity data were initially analyzed using a repeated measures analysis of variance (ANOVA) with neonatal treatment, (ETOH 5 g/kg/day, NIC 12 mg/kg/day, ETOH 5 g/kg/day+NIC 12 mg/kg/day, maltose, and non-treated control), period of exposure (PND 1–7 or PND 8–14) and gender as grouping variables, day as a repeated measure, and block within day as a nested factor. Separate analyses were then examined for each period of exposure (i.e. PND 1–7 vs. PND 8–14) due to multiple four-way interactions which made interpretation difficult. There were no differences across the two control groups, and so these control groups were collapsed into a single control group and 2 × 2 ANOVAs were conducted using ETOH and NIC as grouping variables to directly assess potential interactions of ETOH and NIC. For all 2 × 2 ANOVAs, data were collapsed across day, sex, or block and re-analyzed only when differences were not significant.

3.4. Distance traveled

3.4.1. Drug exposure during PND 1–7

Fig. 2a shows activity as a function of neonatal treatment collapsed across day, sex, and block. Overall, animals treated with ETOH or ETOH+NIC during the first postnatal week traveled more distance (i.e. increased activity) across the 3 days of testing than animals treated with NIC or controls (main effect of ETOH, [$F(1,77)=6.27$, $p<0.01$]. These results were not due to a failure to habituate to the test environment by the ETOH or ETOH)+NIC treated animals since linear contrasts showed no difference in the slope of the lines in animals treated with ETOH or ETOH+NIC compared to NIC treated or control animals (block × ETOH, [$F(1,83)=3.47$, $p>0.05$], data not shown). There was also a day × block × NIC interaction, [$F(2,154)=6.40$, $p<0.005$]. To probe this three-way interaction, separate analyses were conducted that showed a block x NIC interaction was significant on the first day of testing but not Day 2 or Day 3 [$F(1,83)=29.18$, $p<0.0001$]. On the first day of testing, NIC exposed offspring were less activity during the first 10 min block relative to all other treatment groups (Tukey's HSD, $p<0.05$; see Fig. 2b). No significant group differences were observed in the 2nd block of testing on day 1. As expected, there were main effects of day [$F(2,154)=4.28$, $p<0.05$] and block [$F(1,77)=213.07$, $p<0.0001$] showing that animals, independent of group, decreased activity across test days and within each test session.

3.4.2. Drug exposure during PND 8–14

Animals intubated on PND 8–14 with NIC or NIC+ ETOH displayed hypoactivity relative to non-nicotine treated animals [NIC main effect, [$F(1,67)=6.04$, $p<0.05$]

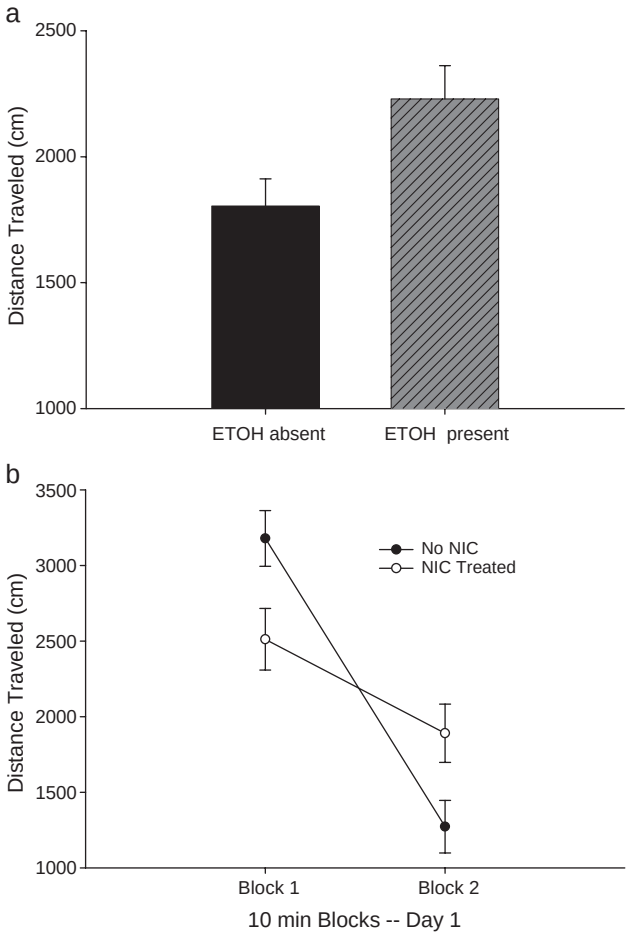

Fig. 2. (a) Mean distance traveled (± SEM) as a function of neonatal drug treatment during the first postnatal week. ETOH and ETOH+NIC animals displayed hyperactivity when tested PND 19–21. (Data collapsed across day, block, and sex with ETOH absence or presence as factors. Individual group means: ETOH: 2271.3 ± 190.5, NIC: 1603.5 ± 174.6, ETOH+NIC: 2188.1 ± 181.4, Control: 2005.9 ± 124.9). (b) Mean distance traveled (± SEM) as a function of neonatal drug treatment during the first postnatal week. NIC and ETOH+NIC treated animals displayed hypoactivity during the first 10 min of testing on day 1. (Data collapsed across sex with NIC absence or presence as factors. Individual group means (block 1, block 2): ETOH: 3320.0 ± 347, 1428.7 ± 291.0, NIC: 2068.4 ± 319.0, 1590.0 ± 267.0, ETOH+NIC: 2955.6 ± 331, 2189.9 ± 277, Control: 3037.1 ± 228, 1116.0 ± 191).

as shown in Fig. 3. Main effects of day [$F(2,134)=13.18$, $p<0.0001$] and block [$F(1,67)=133.84$, $p<0.0001$] were also observed.

3.5. Latency to enter and distance traveled in the center

3.5.1. Drug exposure during PND 1–7

3.5.1.1. Latency to enter the center. The ANOVA revealed a significant ETOH x NIC interaction [$F(1,77)=7.00$, $p<0.01$] (see Fig. 4). Data were collapsed across day and sex for post-hoc tests. Tukey's HSD post-hoc test showed that NIC treated animals had longer latencies to enter the center than all other groups, ETOH+NIC treated animals did not differ from either the ETOH treated or control offspring (p's <0.05).

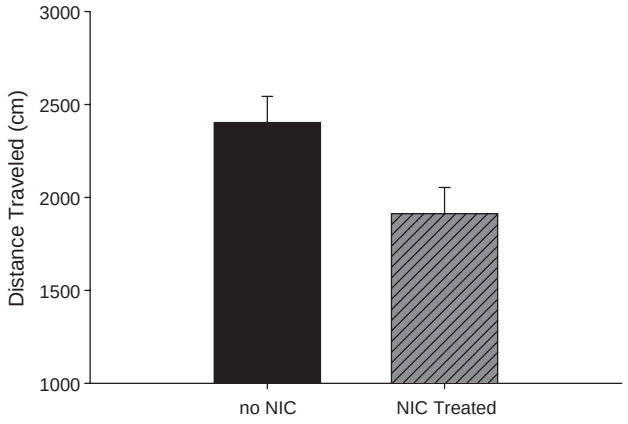

Fig. 3. Mean distance traveled (± SEM) as a function of neonatal drug treatment during the second postnatal week. NIC and NIC+ETOH animals showed hypoactivity when tested PND 19–21. (Data collapsed across day, sex, and block with NIC absence and presence as factors. Individual group means: ETOH: 2577.8 ± 236.9, NIC: 1740.3 ± 205.196, ETOH+NIC: 2084.0 ± 193.5, Control: 2226.4 ± 152.5).

3.5.1.2. Distance traveled in the center. The overall ANOVA revealed a significant interaction of day × sex × ETOH [$F(2,154)=3.14$, $p<0.05$] (see Fig. 5). Separate ANOVAs with sex and ETOH as factors were completed for each test day to probe this interaction. These analyses showed a significant sex × ETOH interaction for Day 1 but not Day 2 or Day 3 of testing (sex × ETOH interaction for Day 1 [$F(1,81)=8.30$, $p<0.05$]). Males treated with ETOH or ETOH+NIC traveled significantly more distance in the center on Day 1 of testing than males treated with NIC or controls ([$F(1,40)=10.049$, $p<0.005$]), whereas females ETOH or ETOH+NIC treated animals did not show this pattern. Additionally, in the overall ANOVA, there was a block × sex × ETOH interaction [$F(1, 154)=5.68$, $p<0.05$]. For post-hoc tests, distances traveled in the center were

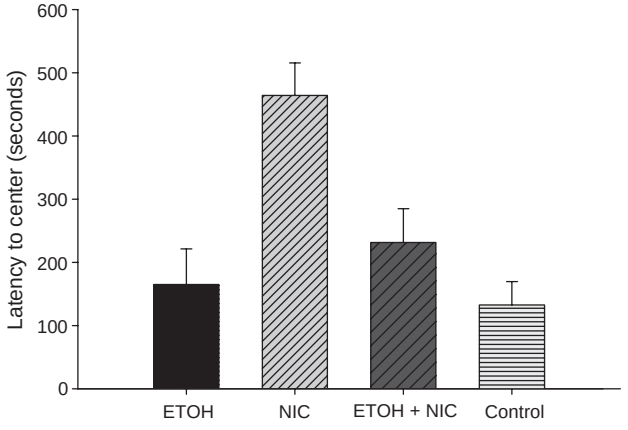

Fig. 4. Mean latency to enter the center of the activity chamber (± SEM) as a function of neonatal treatment during the first postnatal week. NIC treated animals took longer to enter the center than all other groups. (Data collapsed across day, block and sex).

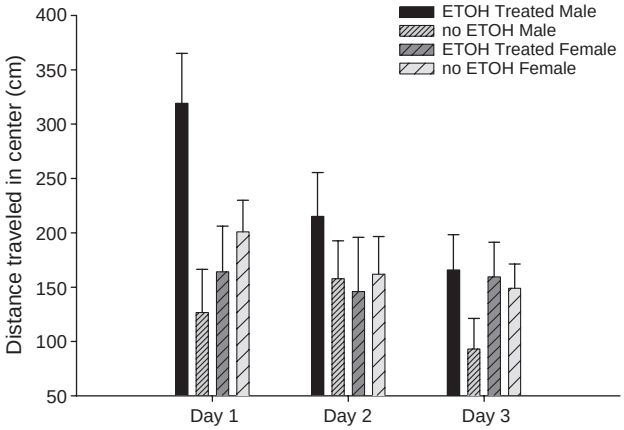

Fig. 5. Mean distance traveled in the center of the testing chamber (± SEM) as a function of neonatal drug exposure during the first postnatal week. Males treated with ETOH or ETOH+NIC traveled more distance in the center on day 1 than all other groups. (Data collapsed across block. Individual means day 1 males only: ETOH: 381.6 ± 69, NIC 86.2 ± 69, ETOH+NIC 269.1 ± 61.6, Control 146.9 ± 48.7).

summed across the three days of testing for each block of testing, and an ANOVA was completed for each block with sex and ETOH as factors. These analyses showed a significant sex × ETOH interaction for block 1 of testing, but not block 2 [$F(1,81)=7.912$, $p<0.05$]. This effect again was due to males. Males treated with ETOH or ETOH + NIC traveled significantly more distance in the center each day on the first block of testing than NIC or control animals [$F(1,42)=8.0$, $p<0.01$] and again, this pattern was not observed among females (data not shown).

3.5.2. Drug exposure during PND 8–14

3.5.2.1. Latency to enter the center. Unlike animals treated PND 1–7, there were no differences between groups for latency to enter the center for animals intubated on PND 8–14.

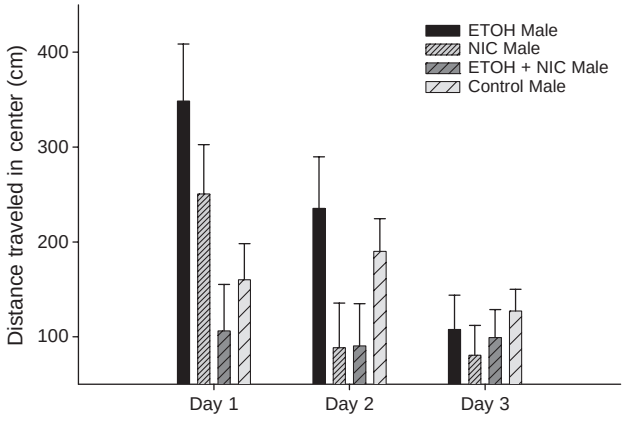

Fig. 6. Mean distance traveled in center (± SEM) as a function of neonatal drug exposure during the second postnatal week. Males treated with ETOH+NIC traveled less distance in the center than ETOH males on Day 1 and ETOH+NIC and NIC males traveled less distance than ETOH and Control males on Day 2 of testing.

Table 1
Body weights (in g ± SEM)

Group	PND 1–7	PND 8–14
ETOH	46.6 ± 1.4	44.8 ± 1.5
NIC	48.7 ± 1.3	47.0 ± 1.3
ETOH plus NIC	42.9 ± 1.3	43.7 ± 1.3
Maltose	51.5 ± 1.2	50.0 ± 1.4
NTC	51.5 ± 1.3	47.8 ± 1.4

3.5.2.2. Distance traveled in the center. The repeated measures ANOVA revealed a significant day × sex × ETOH × NIC interaction [$F(2,134)=3.88$, $p<0.05$] (see Fig. 6). Post-hoc analyses were completed for each day with ETOH and NIC as factors and were conducted separately on males and females. On Day 1 of testing, a significant ETOH × NIC interaction was revealed for males only [$F(1,34)=10.9$, $p<0.005$]. Post-hoc Tukey's tests revealed that ETOH+NIC treated males traveled less distance in the center than the ETOH treated animals [Tukey's HSD, $p<0.02$] although there were no other group differences. On Day 2 of testing, both the NIC and ETOH+NIC treated males traveled less distance than ETOH and Control males (univariate ANOVA with ETOH and NIC as factors [$F(1,34)=7.28$, $p<0.01$]). No differences were displayed by females (data not shown).

3.6. Body weight at time of testing

Body weight was assessed prior to testing on PND 19 and analyzed via a univariate ANOVA with group, sex, and age at drug exposure as factors. These data revealed a main effect of both group [$F(4,140)=10.36$, $p<0.0001$] and gender [$F(1,140)=18.14$, $p<0.0001$] (see Table 1). Tukey's HSD post hocs showed that animals treated with ETOH weighed less than those given isocaloric maltose ($p<0.01$). Animals treated with ETOH+NIC weighed less than all other groups except the ETOH group (p's <0.01). Additionally, as predicted, males weighed more than females.

4. Discussion

Neonatal exposure to ETOH and/ or NIC during the "brain growth spurt" produced different effects on activity depending on when drug exposure occurred. Offspring intubated on PND 1–7 with ETOH or ETOH+NIC were hyperactive compared to control or NIC treated animals. In contrast, offspring treated on PND 8–14 displayed very different behavior. NIC or ETOH+NIC treated animals displayed hypoactivity relative to all other treatment groups. Thus, there were clearly differences in sensitivity to these drugs on activity levels as a function of when drug exposure occurred. ETOH + NIC treated animals appeared more like ETOH treated animals when drug exposure occurred on PND 1–7 and more like NIC treated animals when drug exposure occurred on PND 8–14.

Initially, it was proposed that there might be an interactive effect of ETOH and NIC on activity and that this might be dependent upon when drug exposure occurred. For the most part, this does not appear to be the case. There was at least one subtle change in activity that was observed with exposure to both drugs simultaneously, namely males treated with ETOH+NIC on PND 8–14 traveled less distance in the center than either group treated with each of these drugs alone but this was only observed on the first day of testing. Otherwise, the combination of ETOH and NIC more likely resembled one or the other of the treatment conditions depending upon when exposure occurred.

Hyperactivity following prenatal or neonatal ETOH exposure is one of the most commonly reported findings in the literature on the effects of ETOH on development. Prenatal ETOH exposure has been shown to produce hyperactivity in a variety of species including rhesus monkeys (Schneider et al., 2001), guinea pigs (Gibson et al., 2000; Catlin et al., 1993), rats (Bond, 1988; Vorhees and Fernandez, 1986; Means et al., 1986; Ulug and Riley, 1983; Abel, 1982) and mice (Mothes et al., 1996) as well as humans providing further support for the validity of animal models for studying the behavioral effects of prenatal alcohol exposure.

Our data also supports existing data that neonatal ETOH exposure in rats produces hyperactivity. Melcer et al. (1994) treated neonatal rats PND 4 through 9 with either 4 or 6 g/kg/day of ETOH and showed that animals receiving the high dose of ETOH demonstrated hyperactivity when animals were tested on PND 18. Furthermore, rats treated with ETOH during the neonatal period with a slightly larger dose than used by Melcer and colleagues (6.6 g/kg/day) were still hyperactive compared to controls when tested on PND 90 (Kelly et al., 1987c). Our results extend the findings in the literature by reporting that ETOH induced hyperactivity also occurred when subjects were exposed to ETOH on PND 1–7 although not when exposure was from PND 8–14. These studies suggest that there may indeed be a critical window or windows for the effects of neonatal ETOH exposure for producing hyperactivity. Further studies should address whether ETOH exposure during the first postnatal week causes long-lasting changes in activity levels.

The alcohol exposure model used in these experiments is considered a binge exposure model, characterized by high peak blood ETOH concentrations. Previous data has shown that greater behavioral deficits including hyperactivity are produced with a binge exposure than when the same dose of ETOH is administered in small amounts over 24 h (Kelly et al., 1987b,c; West et al., 1989). It is important to note that while a high peak BEC is needed to produce hyperactivity according to the literature, our data suggests that this was not the only factor in producing hyperactivity. Although the highest BEC were observed in our pups treated on PND 8–14, these animals did not show hyperactivity. Thus, these data provide further support for different windows of vulnerability to alcohol's effects on behavior.

While the mechanisms for increased locomotor activity due to neonatal ETOH exposure have yet to be elucidated, several sources have suggested that the effects of ETOH on the hippocampus may play a role. As the hippocampus is a late-developing brain region (Bayer and Altman, 1995), ETOH exposure during the neonatal period has been shown to be particularly damaging to the hippocampus in comparison to many other brain regions (Goodlett and Peterson, 1995; Goodlett and Johnson, 1997; Thomas et al., 1998). Hippocampal lesions have been shown to produce similar characteristics as those demonstrated by animals exposed to ETOH prenatally, such as response inhibition deficits (Riley et al., 1986), spatial learning and memory deficits (Morris et al., 1982; Bannerman et al., 1999; Praag et al., 1998), and hyperactivity (Kelly et al., 1987c; Bond, 1988). Thus, hippocampal damage may be one mechanism to explain the hyperactivity observed in our study.

Hippocampal damage by neonatal ETOH exposure may also affect the neural circuitry to other parts of the brain involved in regulating locomotor activity levels. These pathways include glutamatergic neurons from the hippocampus that project to the nucleus accumbens and synapse on the dopamine (DA) neurons in that region (Kelley and Domesick, 1982). In further support of this hypothesis, prenatal ETOH exposure has been shown to directly reduce the function of DA producing neurons in the other regions of the mesolimibic DA pathway including substantia nigra and ventral tegmental areas (Shen et al., 1999), could potentially affect activity levels.

One previous study has reported hypoactivity with NIC exposure during this second neonatal week. NIC exposure on PND 10–16 (in mice) resulted in long-term hypoactivity when testing occurring in adulthood (Nordberg et al., 1991). To the best of our knowledge, the current study is the first to show a similar hypoactivity in pre-weanling aged rats following NIC administration during PND 8–14. In contrast, rats administered NIC orally between PND 4–9 displayed increased locomotor activity when tested on PND 18 (Thomas et al., 2000). It is interesting to note that one study that included NIC treatment during either the first, second or third neonatal week showed hyperactivity (as adult mice) when NIC treatment occurred in either the first or third week and hypoactivity if NIC exposure was during the second postnatal week (Eriksson et al., 2000). There clearly appears to be a growing literature suggesting that the timing of the NIC administration may be critical in predicting activity outcome following NIC exposure with hypoactivity observed in rodents if exposure occurs during the second postnatal week. It should be noted that in the current study, neonatal NIC during the first week did not produce hyperactivity when NIC exposure occurred alone so there is still some discrepancies in the literature that need to be resolved.

Zhang et al. (1998) have also provided evidence that the CNS appears particularly sensitive to NIC administration during the second postnatal week. The number of nicotinic

receptors appears to peak during the second to third postnatal weeks (Zhang et al., 1998). These levels were higher than those found in either PND 1–7 or adult brain in the cortex, hippocampus, striatum, and thalamus. Additionally, Miao et al. (1998) and Narayanan et al. (2002) have both shown upregulation of nicotinic receptors following neonatal NIC treatment although this effect only appeared long-term if NIC exposure occurred during the second postnatal week. It has also been proposed that changes in the development of low affinity nicotinic receptors due to NIC treatment PND 10–16 in mice could be critically important (Nordberg et al., 1991). In any case, these studies suggest that the developing CNS may be particularly sensitive to NIC treatment during the second postnatal week in rodents.

In addition to activity changes, neonatal exposure to ETOH and NIC also affected two potential measures of anxiety; entries into the center and distance traveled in the center of the open field (OF) and the effects observed occurred more often in males. Exploratory behaviors in a novel OF, especially entries into the center, are associated with reduced anxiety and these measures have been shown to be negatively correlated with behavioral and physiological measures of fear in rats (Archer, 1973). Typically as the rat habituates to the test chamber, ambulation in the center increases, indicating a reduction in fear and emotionality, as well as habituation to the novel environment (Walsh and Cummins, 1976). While latency to enter the center was unaffected by neonatal ETOH exposure on PND 1–7, males treated with ETOH or ETOH+NIC traveled more distance in the center than all other groups but this was limited to the Day 1 of testing. NIC offspring treated on PND 1–7 took longer to enter the center of the activity chamber compared to all other groups including animals treated with ETOH+NIC on all three days of testing, and males exposed to NIC or ETOH+NIC on PND 8–14 traveled less distance in the center. While NIC-related hypoactivity may partially explain this effect, it is clearly not the only explanation since hypoactivity was only observed during the first block of the first day of testing (in NIC exposed offspring treated on PND 1–7) and avoidance of the center was observed across all three days of testing.

Rats treated with NIC during development have been shown to exhibit more fear of open areas and a decrease in novelty-seeking behavior (Vaglenova et al., 2004). Since entering the center of the activity chamber requires both novelty-seeking behavior and a reduction in fear of open spaces, these data may help to explain our findings that NIC treated animals took longer to enter the center (PND 1–7) and NIC or ETOH+NIC treated males traveled significantly less distance there (PND 8–14). In both the pre-clinical and clinical literature, males have shown a greater sensitivity to NIC as measured by fetal growth, greater hyperactivity, and behavioral abnormalities compared to both female and non-treated subjects (Lichtensteiger and Schlumpf, 1993). Since the second postnatal week seems to be a particularly sensitive time for behavioral effects due to NIC exposure, gender effects may be even most robust during this time.

An interesting interaction was observed in blood ETOH levels depending on the age of the pup and the presence/absence of NIC. NIC lowered blood ETOH levels and this effect was more pronounced in PND 11 pups than it was in PND 4 pups. This reduction in blood ETOH levels in association with concurrent NIC exposure has been previously reported (Chen et al., 2001) although to the best of our knowledge, no one has examined the ETOH+NIC interaction in pups of different ages. While the underlying mechanism is not yet understood, Chen et al. (2001) hypothesized that slowed gastric emptying caused by nicotine's effects on the CNS (Nowak et al., 1987; Scott et al., 1993) may allow more first pass metabolism of ethanol to occur in the stomach and less ethanol to be absorbed into the blood stream (Johnson et al., 1991). This may help explain why the ETOH+NIC treated animals had lower BEC than ETOH alone treated animals.

It is intriguing that BEC levels were lower in pups that received ETOH during the first neonatal week relative to the second neonatal week. To the best of our knowledge, one other study has ontogenetically examined BEC across a wide range of ages (Kelly et al., 1987a) and their findings conflict with the findings in the current report. Following a single acute dose of ETOH, the highest peak BEC were observed on PND 4 relative to pups younger (PND 1 or 2) or older (PND 6, 8 or 10) although younger pups (PND 1 or 2) did have higher peak BEC than older pups (PDN 6, 8 or 10). There are a number of differences across these studies that could contribute to this discrepancy. First, Kelly and colleagues examined BEC after a single acute 2.5 g/kg dose of ETOH in contrast with the dose used in our study (which was higher). In addition, a second methodological difference was that our exposure regimen was repeated ETOH exposure rather than a single acute administration. There are differences in brain and blood ETOH levels depending upon whether animals receive ETOH in a single acute or a more chronic (multiple day) exposure (Silveri and Spear, 2001) and these differences may help explain the discrepancies between BEC in the current study and that reported by Kelly et al. It is interesting to note that younger rats do appear less sensitive to some of the behavioral effects of ethanol than more mature rats. For example, younger animals show shorter ETOH induced sleep-times and higher waking BEC levels relative to older rats as well as less motor impairment on a tilt plane test (Hollstedt et al., 1980; Silveri and Spear, 2001). While the ages studied in these studies are not identical to the current study, it would be interesting if the reduction in sensitivity to ETOH could be explained, at least in part, by some of these ontogenetic differences in BEC.

There were body weight differences as a function of neonatal treatment independent of when drug exposure occurred. ETOH and ETOH+NIC treated animals intubated during PND 1–7 or PND 8–14 weighed less than NIC alone

or control animals during neonatal treatment. Evidence suggests that there is a relationship between lower weight during development and hyperactivity (Saigal et al., 2001; Mick et al., 2002). However, it is unlikely that body weight differences alone can explain the current findings. ETOH exposed animals treated on PND 8–14 were not hyperactive, but still displayed lower neonatal body weights than all other groups. Still, since there may be differences in the importance of postnatal growth on subsequent activity levels if growth is retarded on PND 1–7 versus PND 8–14, we cannot rule out the potential role of neonatal growth deficits in the current findings. These body weight deficits persisted at least for the ETOH treated animals although not for the NIC treated offspring. Thus, while body weight deficits may play some role, it alone, cannot explain the current findings.

An additional issue for consideration is the possibility that the hypoactivity displayed by animals neonatally administered NIC could be due to NIC withdrawal symptoms. This was a very high dose of nicotine although it did not have any impact on mortality or pup body weight during or after dosing. Still, the NIC induced hypoactivity was more robust in animals intubated PND 8–14 versus PND 1–7. There was a shorter washout period between drug treatment and testing in animals intubated on PND 8–14 (5 days), than in animals intubated on PND 1–7 (12 days). While NIC withdrawal symptomatology is well characterized in both humans and adult rodent models (Shiffman and Jarvik, 1976; Malin, 2001), it has not been well characterized in the neonatal rat. In adults, specific somatic, affective, and behavioral signs of NIC withdrawal including hypoactivity, have been shown to peak sometime between 18 and 22 h following the cessation of NIC treatment (Malin, 2001) so it is unlikely that protracted withdrawal could explain this hypoactivity.

In summary, we reported that changes in activity levels following neonatal administration of ETOH and/or NIC depended upon when during development the drugs were administered. In the current study, animals administered ETOH or ETOH+NIC during PND 1–7 displayed hyperactivity and animals administered NIC or ETOH+NIC during PND 8–14 displayed hypoactivity when tested PND 19–21. While there was little evidence of any interactive effects of neonatal ETOH and NIC exposure, further research looking at more complex behavioral endpoints are needed.

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