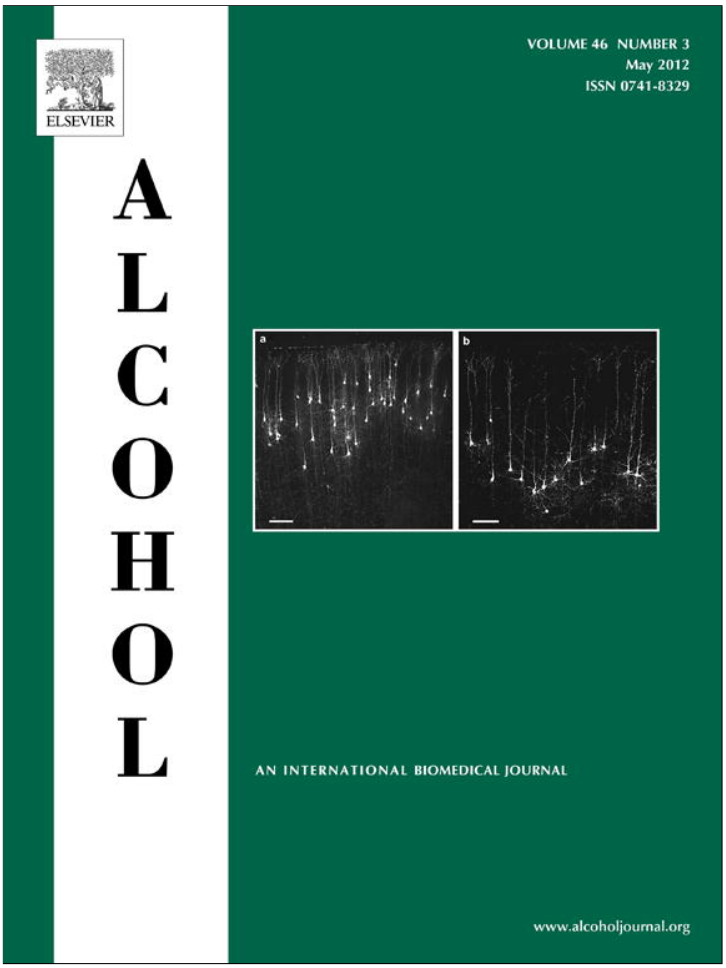

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Ontogenetic differences in ethanol's motivational properties during infancy

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ABSTRACT

Pairing a conditioned stimulus (CS) with ethanol generally produces aversion for that CS in adult rodents. However, infant rats (PD1-PD3) exposed to ethanol demonstrate appetitive reinforcement to ethanol (Nizhnikov, Varlinskaya, Petrov, & Spear, 2006; Petrov, Varlinskaya, & Spear, 2003). This sensitivity to the appetitive properties of ethanol during infancy may be transient, as during the second postnatal week rat pups tend to exhibit conditioned aversions to flavors paired with ethanol. The present study examined changes in the motivation properties of ethanol through ontogeny and the neurobiology underlying these changes. Rat pups were exposed to a taste conditioning procedure on PD4 or PD12. Rat pups were intraorally infused with 2.5% of their body weight of saccharin solution (0.1%) and immediately after injected intraperitoneolly (i.p.) with one of six doses of ethanol (0.0–2.0 g/kg). A day later pups were given saccharine infusions and percent body weight gain was used as an index of ethanol's reinforcing effects. PD4 pups expressed appetitive reinforcement to ethanol, as indicated by greater saccharin intake, as compared to control counterparts and to the older PD12 pups. Subsequent experiments revealed that PD4 pups were less sensitive to the aversive properties of the drug than PD12 pups. The older pups found high doses of ethanol aversive while PD4 rat pups did not condition aversions to this dose of ethanol after a single trial. A similar pattern of results was observed between the low doses of ethanol and the highest doses of a kappa opioid agonist. The PD12 animals did not condition to the kappa opioid agonist, while the younger rats expressed an appetitive response. These results illustrate an ontogenetic change in the motivational properties of ethanol, with sensitivity to its appetitive properties declining and responsiveness to the aversive properties increasing with age during early infancy.

Introduction

Experience with alcohol either prenatally or during early infancy may be a contributing factor for exacerbated alcohol use during adolescence. This may in turn enhance the likelihood of alcohol-related problems in adulthood (i.e., the "alcoholism generator"; Miller & Spear, 2006). Evidence that supports this hypothesis, however, is still scarce (but see Spear, 2002). Exposure to ethanol during infancy in humans is, surprisingly, not as rare as one might think (for review see Spear & Molina, 2005) and can occur through breast-feeding by a drinking mother or when the drug is applied to the infant for medicinal purposes (e.g., as a sedative-hypnotic or anesthetic, Mennella & Beauchamp, 1991, 1993). Recent epidemiological studies confirm that humans exposed prenatally to moderate amounts of ethanol are at risk for alcohol abuse as adolescents, and subsequently as adults (Alati et al., 2008; Baer, Bar, Bookstein, Sampson, & Streissguth, 1998; Baer, Sampson, Barr, Conner, & Streissguth, 2003; Yates, Cadoret, Troughton, Stewart, & Giunta, 1998). This phenomenon has been experimentally confirmed through the use of preclinical animal models (Spear & Molina, 2005). For example, it has been shown that rats exposed to ethanol during late gestation (1.0 or 2.0 g/kg on gestational days 17–20 GD 17–20) exhibit enhanced ethanol intake at mid infancy and adolescence (e.g., Arias & Chotro, 2005; Chotro & Arias, 2007; Chotro, Arias, & Laviola, 2007; Molina, Dominguez, Lopez, Pepino, & Fas, 1999). This effect is also evident when ethanol exposure occurs during the first two-weeks of life (Hayashi & Tadokoro, 1985; Lopez & Molina, 1999), even when pups are exposed to the drug through nursing with an intoxicated dam (e.g., Pepino, Abate, Spear, & Molina, 2004; Ponce, Pautassi, Spear, & Molina, 2004, 2011).

Very young animals (postnatal day 1–3: PD1 – PD3) easily condition appetitive responses to ethanol in first order conditioning paradigms (Cheslock et al., 2001; Nizhnikov, Molina, & Spear, 2007; Nizhnikov, Molina, Varlinskaya, & Spear, 2006; Nizhnikov, Varlinskaya, et al., 2006; Petrov et al., 2003). On the other hand

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rats 1–2 weeks of age, although readily ingesting ethanol, tend to exhibit aversion to conditioned stimuli (CS) paired with ethanol (Dominguez, Lopez, & Molina, 1998; Hunt, Spear, & Spear, 1991; Molina et al., 1996; Pautassi, Godoy, Spear, & Molina, 2002). One especially clear example of this effect can be seen in experiments conducted by Arias and Chotro (2006). When 7–8 day-old rat pups were exposed to ethanol (3.0 g/kg) they exhibited a preference for ethanol 3 days later at test. On the other hand if this exposure occurred on days 10–11 an aversion was exhibited at test. This discontinuity of conditioning suggests an ontogenetic shift in the sensitivity of the infant rat to the motivational properties of ethanol. Recent studies by Nizhnikov, Pautassi, Truxell, and Spear (2009) and Pautassi, Nizhnikov, Acevedo, and Spear (2011) have found clear conditioning of place preference to ethanol (1.0 g/kg) in infant rats 13–15 days old. It seems that even though older infants are capable of responding to the positive reinforcing properties of ethanol, aversions to stimuli paired with its effects are more readily expressed.

Taking into account the established appetitive reinforcing effects of ethanol at these young ages it is plausible that the experience of appetitive conditioning with ethanol during infancy may lead to increases in drinking during adolescence or adulthood. Some results indicative of this can be found in studies by Ponce, Pautassi, Spear, and Molina (2008). In these Experiments infant rats (PD12 – PD16) readily learned to nose poke for ethanol and consume considerable amounts of the drug in this procedure. When tested 2 weeks later (P30) on ethanol intake in a free choice two bottle test, rats that had been conditioned with ethanol as reinforcer exhibited significantly greater alcohol intake than their yoked controls. Furthermore, a control group given only water or noncontingent ethanol exposure during infancy always drank more water than ethanol during the two bottle test at P30, while ethanol conditioned subjects drank more alcohol than water (Ponce et al., 2008). These results suggest that learning or conditioning associated with early ethanol experience might be an important determinant of future ethanol intake.

Several lines of evidence have implicated endogenous opioid systems in ethanol intake, as well as in its reinforcing properties (Herz, 1997; Ulm, Volpicelli, & Volpicelli, 1995). For example, animal studies have demonstrated that non-selective opioid receptor antagonists (Myers, Borg, & Mossberg, 1986; Myers & Lankford, 1996; Reid & Hunter, 1984; Samson & Doyle, 1985; Stromberg, Volpicelli, & O'Brien, 1998), as well as selective mu (Hyytiä & Kiianmaa, 2001; Krishnan-Sarin et al., 1998; Stromberg et al., 1998) and delta antagonists (Hyytiä & Kiianmaa, 2001; June et al., 1999; Krishnan-Sarin et al., 1995; Krishnan-Sarin, Portoghese, Li, & Froehlich, 1995), reduce ethanol intake in adult animals. This effect is seen across a variety of species and selected lines, as well as under a variety of experimental conditions.

Ethanol has been shown to stimulate the release of endogenous ligands for mu and delta opioid receptors (beta-endorphin, enkephalins) in distinct brain regions associated with reward and reinforcement (De Waele & Gianoulakis, 1993; Olive, Koenig, Nannini, & Hodge, 2001; Rasmussen et al., 1998). Ethanol-induced release of beta-endorphin in the hypothalamus, nucleus accumbens, and ventral tegmental area (De Waele & Gianoulakis, 1993; Olive et al., 2001; Rasmussen et al., 1998) and interaction of this endogenous ligand with mu opioid receptors in the mesolimbic reward system seem critical for the euphoric, positively reinforcing effects of ethanol. Furthermore, findings from clinical trials demonstrate that non-selective opioid antagonists are effective in reducing ethanol consumption in alcoholics (see Oswald & Wand, 2004 for references and review).

In contrast to mu opioid receptors and their endogenous ligands, the dynorphin/kappa opioid receptor system has been implicated in mediating ethanol's aversive properties in adulthood. Several studies have investigated the effects of kappa pharmacological manipulations on ethanol intake and reinforcement in adult animals. In general, selective kappa agonists have been shown to attenuate ethanol intake in adult rats while antagonists increase it (Lindholm, Werme, Brene, & Franck, 2001; Mitchell, Liang, & Fields, 2005, but also see Nestby et al., 1999). Dynorphin, the endogenous ligand for kappa opioid receptors (Chavkin, James, & Goldstein, 1982), reduces ethanol preference in adults, and a selective kappa receptor agonist, U50, 488H, effectively attenuated ethanol-induced place preference (Matsuzawa, Suzuki, Misawa, & Nagase, 1999; Sandi, Borrell, & Guaza, 1988). Another important point to consider is that enhanced ethanol-induced dopamine (DA) response in the nucleus accumbens has been reported following pharmacological blockade or genetic deletion of kappa opioid receptors (Zapata & Shippenberg, 2006). This indicates that endogenous activity at kappa opioid receptors counteracts activation of the mesolimbic DA system induced by ethanol and thereby diminishes the reinforcing effects of acute ethanol in adults (Shippenberg, Zapata, & Chefer, 2007).

Unlike adult responding, however, newborns seem to require activity at kappa opioid receptors in order to find ethanol rewarding (Nizhnikov, Molina, et al., 2006; Nizhnikov, Varlinskaya, et al., 2006). The effects of pharmacological blockade of these receptors on ethanol reinforcement were assessed using a surrogate nipple technique in 3-hr-old newborn rat pups. Blockade of kappa opioid receptors by a selective antagonist, nor-binaltorphimine, completely eliminated the reinforcing effects of ethanol (Nizhnikov, Molina, et al., 2006; Nizhnikov, Varlinskaya, et al., 2006) without affecting conditioning to an aversive stimulus. This finding indicates that the kappa opioid system is critical for mediating ethanol's appetitive reinforcing properties very early in ontogeny.

Taken together the data indicate an ontogenetic shift in the motivational properties of the kappa opioid system and may be one reason for the disparity in responding to alcohol and other reinforcers across ontogeny. One of our goals in this set of studies was to test the positive or aversive motivational properties of kappa opioid agonists at the two different ages employed in this set of studies. More specifically, we wanted to know whether the effects of kappa opioid agonists on ethanol reinforcement later in ontogeny are similar to those during very early infancy.

Another possible explanation for the ontogenetic difference in responding to ethanol could be differential metabolism of the drug across these young ages. For example, Kelly, Bonthius, and West (1987) found that adolescent rats eliminate ethanol from the blood at a much faster rate than infants. Silveri and Spear (2000) also found that 16 day-old rats metabolize ethanol at a much slower rate than older subjects (PD26 – PD56). In more general terms, older animals metabolize ethanol faster than their young counterparts (Hollstedt, Olsson, & Rydberg, 1980; Hollstedt & Rydberg, 1970; Walker & Ehlers, 2009). Therefore, any examination of differences in the reinforcing properties of ethanol across age must take this into account.

The goal of the current experiments was to assess ontogenetic differences in responding to ethanol during infancy. Experiment 1 examined differences in conditioned taste aversion across age when several high doses of ethanol were employed as the US. Conversely, Experiment 2 tested differences in responding to lower doses of ethanol across early infancy. Experiment 3 replicated the results obtained from Experiment 2 since they were so unexpected. Changes in responding to activation of the kappa opioid system across early infancy were tested in Experiment 4. Finally, age-related differences in blood ethanol content after i.p. injections of 2.0 and 0.25 g/kg ethanol were explored in Experiment 5.

Method

Subjects

Six-hundred and eighty eight Sprague-Dawley rat pups (ages PD4 or PD12), representative of 85 litters, were employed in Experiments 1a through 5 (Experiment 1a: 128; Experiment 1b: 52; Experiment 2a: 128; Experiment 2b: 60; Experiment 3: 30; Experiment 4: 122; Experiment 5: 160). The animals were either 4 or 12 days old at the start of the experimental procedures. All litters were born and reared at the Center for Developmental Psychobiology (Binghamton University, USA). Births were examined daily and the day of parturition was considered as postnatal day 0 (PD 0). Pups were housed with the dam in standard maternity cages with free access to water and food. The colony was kept at 22–24 °C and a 12-h light–dark cycle was used with light onset occurring at 700 AM. To control for litter effects, no more than one male and one female from the same litter were assigned to a given group. Sex exerted neither significant main effects nor significant interactions with the remaining factors in all experiments. Therefore, data were collapsed across this variable. All procedures were in compliance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1986) and were also approved by the Institutional Animal Care and Use Committee within an AAALAC-accredited facility.

Intraoral cannulation

Experiments 1 through 4 required cannulation for direct intra-oral infusion. A piece of PE-10 polyethylene tubing (Clay Adams, Parsippany, NJ), 3 cm in length, was flanged at one end using a heat source. The non-flanged end of the tubing was tightly fit onto one end of a sharpened and curved stainless-steel wire (0.285 mm diameter, approximately 4 cm in length). The wire was then inserted gently midline through the cheek, with the flanged tip resting in an anterior position of the inside portion of the cheek (Pautassi et al., 2002; Spear, Spetch, Kirstein, & Kuhn, 1989). This cannulation procedure was accomplished within 2–5 s with no bleeding. These positions were chosen to maximize contact with taste receptors and to allow voluntary ingestion of the fluid (a more posterior position leads to uncontrolled swallowing in very young rat pups). The free end of the cannula was connected to PE-50 polyethylene tubing, which was in turn connected to a computer-controlled rotary microsyringe pump. The size and schedule of the infusions was computer controlled. Cannulation was performed 3 h prior to fluid infusion.

Infant taste conditioning procedure

The experimental design was similar to the one used by Broadbent, Muccino, and Cunningham (2002). Experimental subjects were separated from the dam, cannulated and placed in pairs in a maternity tub lined with pine shavings and warmed to approximately 35° ± 0.5 °C. The subjects were left to acclimate in the holding tub for 3 h. Following the acclimation period subjects were voided and weighed. The average weight of all subjects was calculated and used as a bench mark for volume of intra-oral infusion of saccharin during conditioning. Each subject's cannula was connected to a length of PE-50 tubing which in turn was connected to a 10 ml syringe which was placed into a computerized rotary pump. Subjects were then be placed into a plexiglass container divided into 8 sections measuring 6′ × 12′. The bottoms of these containers were lined with cotton and slightly heated (cotton at 26–27 °C). Subjects then received an intra-oral infusion of 2.5% of their body weight of 0.1% saccharin solution over a 10-min period. Immediately following intra-oral infusion pups were disconnected from the tubing, weighed, and injected intraperitoneally (i.p.) with ethanol (Experiment 1a: 0.0, 1.0 1.5, or 2.0 g/kg; Experiment 2a: 0.0, 0.15, 0.25, or 0.5 g/kg; Experiment 3: 0.0, 0.25, or 2.0 g/kg) or a kappa agonist (U62066; Experiment 4: 0.0, 1.0, 5.0, or 10.0 mg/kg). Subjects in Experiments 1b and 2b were not all injected at this point but divided into 2 groups (Paired or Unpaired). Half the subjects received i.p. injections of ethanol immediately following flavor exposure (Experiment 1b: 0.0 or 2.0 g/kg; Experiment 2b: 0.0 or 0.25 g/kg; paired subjects) while the other half did not receive injections. All pups were placed back into the warmed holding chambers with same group con-specific partners for 90 min. Following this delay the unpaired pups were i.p injected with the appropriate dose of ethanol. Pups were then returned to the warmed holding chamber for 2 h to recover and all pups where then returned to the dam overnight. The use of unpaired controls in Experiments 1b and 2b was aimed at addressing several caveats, such as the possibility of non-specific changes (i.e., sensitization, habituation) resulting from the mere exposure to the US or the CS. For instance, there could have been ethanol hangover effects or lingering effects of ethanol at test (Spiers & Fusco, 1992) which interfered with the expression of ethanol-induced taste conditioning.

The kappa agonist was chosen for its ease at crossing the blood–brain barrier and the doses of U62066 were selected after considering dosages of kappa agonists used in several previous works (e.g., Barr, Wang, & Carden, 1994; Piercey & Einspahr, 1989). The effects of higher doses of the drug were tested in preliminary, pilot studies. Several adverse effects were observed, which lead to the discontinuation of these doses in the experiments here presented.

Ethanol was administered as a 12.6% (v/v) solution for doses of 1.0 g/kg or higher and as a 6.3% (v/v) solution for the lower doses mixed in physiological saline. These are relatively low concentrations that induce little (if any) tissue irritation at the site of injection. U62066 solution concentrations varied with administration dose and injection volume was kept constant (0.0 mg/kg: 1.0 mg/ml; 5.0 mg/kg: 10.0 mg/kg). The kappa agonist was dissolved in physiological saline. Control subjects were injected with physiological saline isovolumetric to the highest dose of ethanol administered for Experiments 1–3 and in the same volume as U62066 in Experiment 4.

Immediately following i.p. injections, the cheek cannula was removed and the subjects were placed back into the holding tub for 2. Following this period subjects were placed back with their respective dams overnight. The following day this procedure was repeated but without the injection of ethanol following intake testing.

Blood ethanol concentrations

Infant rats used for measurement of blood ethanol levels (BELs) were naïve to any previous treatment and ethanol. In the morning rat pups were removed from the dams and placed in pairs in a maternity tub lined with pine shavings and warmed from the bottom to approximately 35° ± 0.5 °C for 3 h.

Following the 3 h waiting period (equated to subjects undergoing conditioning) rat pups were i.p. injected with ethanol (Experiment 5: 2.0 g/kg or 0.25 g/kg ethanol). Trunk blood was collected at several time points for analysis (5, 30, 60, 90, or 120 min after i.p. injection). Trunk blood (2 ml samples) was obtained through decapitation, employing a heparinized capillary tube, and centrifuged at high speed (15 min/3000 rpm; Micro-Haematocrit Centrifuge, Hawksley and Sons LTD, Sussex, England). The vials containing the plasma phase were stored at −15 °C for later

analysis. Blood ethanol levels were measured with an AM5 Alcohol Analyzer (Analox Instruments, Lunenburg, MA). Blood ethanol level values were expressed as milligrams of ethanol per deciliter of body fluid (mg/dl = mg%).

Research design and data analysis

Experiments 1a and 2a consisted of 4 (ethanol dose) groups. Experiments 1b and 2b consisted of a 2 (condition) × 2 (ethanol dose) design while Experiment 3 consisted of 3 (ethanol dose) groups. Experiment 4 used 4 (kappa agonist dose) groups. Finally, Experiment consisted of 5 (time of blood withdrawal) groups. All experiments, except Experiments 1b, 2b and 3, included both PD4 and PD12 rat pups. Age, however, was not used as a factor in the statistical analyses since there are significant baseline differences in the ingestive capabilities of the pups across these specific ages. These differences have been consistently found in our lab and emerged in the present set of experiments as well. The younger pups (PD4) consistently drank approximately 1.2% of their body weight while the PD12 subjects drank around 1.9% of their body weight when presented with the saccharin solution. Furthermore, the experimental design necessitated the use of different litters for the two ages since using the same litter would have meant exposing some pups that would be conditioned at P12 to siblings that had already undergone all procedures on PD4 through PD5. Therefore direct statistical comparisons between ages were not made.

The dependent variables were saccharin intake (percent body weight gain, %BWG; Experiments 1 and 2) or blood ethanol levels (Experiments 3 and 4). Percent body weight gain was calculated as follows [((Body weight after fluid infusion − body weight before infusion)/(body weight before infusion))*100]. These dependent variables were analyzed through either factorial or one-way Analyses of Variance (ANOVAs). Significant main effects or interactions were further examined through pair-wise post-hoc comparisons for Experiments 1a and 2a (Fisher's Least Mean Significant tests, alpha level set at 0.05). When conducting multiple comparisons, the alpha value of post-hoc tests was lowered by the Bonferoni correction to avoid spurious positives. Planned comparisons were used for Experiments 1b and 2b since we had a-priori hypothesis that the paired subjects would significantly differ in responding from controls.

In order to avoid overrepresentation of litters within each specific group, no more than 2 animals per litter (one male and one female) were assigned to each particular treatment. Sex was considered as a factor in the present and following experiments. Yet, since this variable failed to exert significant main effects or interact with the remaining factors in all experiments, data were collapsed across this variable.

Data analysis and results

Experiment 1a: Percent body weight gain after test was analyzed using a one-way ANOVA for each age (PD4 and PD12) separately. The between group factors were dose (0.0, 1.0, 1.5, or 2.0 g/kg ethanol). The ANOVA yielded a significant main effect of dose for PD12 subjects $F(3, 75) = 3.37$, $p < 0.05$. Conversely, no significant differences were found at PD4 $F(3, 60) = 1.08$, $p > 0.05$. Post-Hoc analysis revealed that PD12 pups injected with 2.0 g/kg ethanol following saccharin infusions ingested significantly less saccharin during testing than all other groups except the 1.5 g/kg group. None of the other groups differed from each other. No differences in ingestion were found in the PD4 rat pups during testing. These results are depicted in Fig. 1A and B.

Experiment 1b: Results from Experiment 1a indicated that PD12 subjects decreased their intake of saccharin 24 h after receiving a pairing of a high dose of ethanol and saccharin. Although this result suggest aversive conditioning, it is possible that lingering, unspecific effects of ethanol are responsible for this decrease in saccharin intake. In order to ascertain whether the decrease in intake was non-specific or associative in nature a control experiment using PD12 subjects and unpaired groups (i.e., given unrelated exposure to ethanol and saccharin) was performed.

An ANOVA analyzing percent body weight gain showed significant main effects of solution (saline or ethanol) and condition (paired or unpaired) [$F(1, 48) = 27.57$, $p < 0.0001$; $F(1, 48) = 8.86$, $p < 0.01$, respectively] as well as a significant solution × condition interaction $F(1, 48) = 4.83$, $p < 0.05$. Planned comparisons revealed that ethanol paired subjects ingested significantly less saccharin at test than all other groups, a result indicative of ethanol-mediated aversion. These results are depicted in Fig. 2.

Experiment 2a: An ANOVA analyzing percent body weight gain was performed for each age (PD4 and PD12) separately. The between group factors was dose (0.0, 0.15, 0.25, or 0.5 g/kg ethanol). The ANOVA did not find any significant main effect on intake at test for the PD12 rat pups $F(3, 60) = 0.89$, $p > 0.05$. However, the ANOVA analyzing body weight gain in PD4 pups showed a significant main effect of dose $F(3, 60) = 4.28$, $P < 0.01$. Rat pups injected with any of the ethanol doses (0.15, 0.25, 0.5 g/kg) paired with saccharin on PD4 had greater intake of saccharin than either the untreated or saline controls, which did not differ from each other. These results are depicted in Fig. 3A and B.

Experiment 2b: Results from Experiment 2a indicated that PD4 subjects increased their intake of saccharin 24 h after receiving

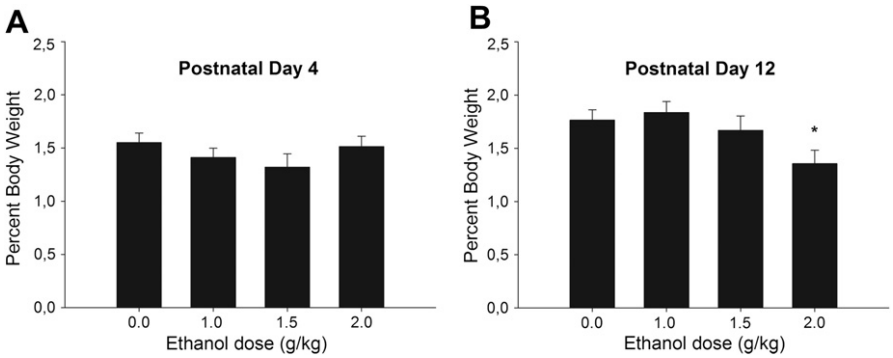

Fig. 1. Intake of a 0.1% saccharin solution depicted as percent body weight gain over a 10 min test for both PD4 (Fig. 1A) and PD12 (Fig. 1B) rat pups. Twenty-four hours prior to testing rat pups were exposed to a saccharin solution and immediately after injected i.p. with one of 4 ethanol doses (0.0–2.0 g/kg). Bars represent mean values; vertical lines depict the standard error of the mean. Asterisk (*) indicates a significant difference from saline controls.

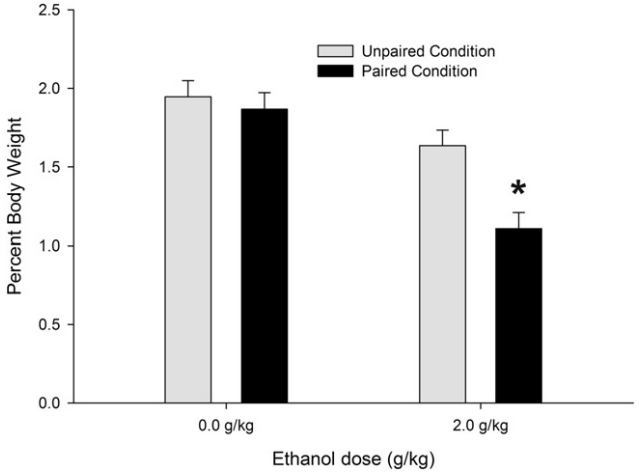

Fig. 2. Intake of a 0.1% saccharin solution depicted as percent body weight gain over a 10 min test for PD12 rat pups. Twenty-four hours prior to testing rat pups were exposed to a saccharin solution and injected i.p. with ethanol (2.0 g/kg) either immediately following (paired) or 90 min after (unpaired) saccharin infusions. Bars represent mean values; vertical lines depict the standard error of the mean. Asterisk (*) indicates a significant difference from saline controls.

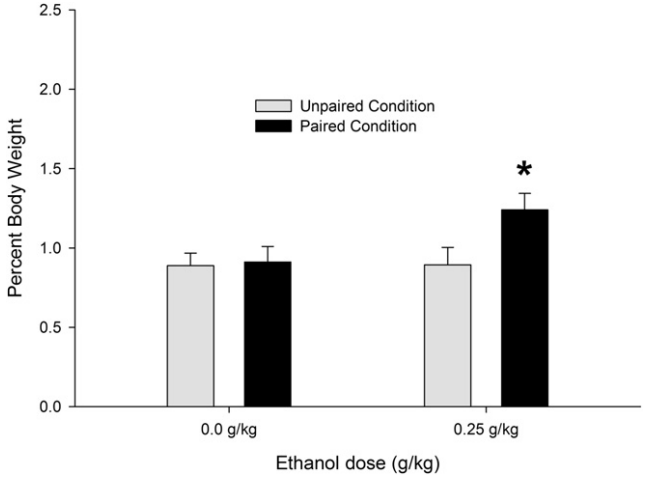

Fig. 4. Intake of a 0.1% saccharin solution depicted as percent body weight gain over a 10 min test for PD4 rat pups. Twenty-four hours prior to testing rat pups were exposed to a saccharin solution and injected i.p. with ethanol (0.25 g/kg) either immediately following (paired) or 90 min after (unpaired) saccharin infusions. Bars represent mean values; vertical lines depict the standard error of the mean. Asterisk (*) indicates a significant difference from saline controls.

a pairing of a low ethanol dose and saccharin. Akin to Experiment 1b this study employed a more conservative, unpaired control condition to address the possibility this pattern of responding obeying to unspecific effects of ethanol or associative learning.

The ANOVA comparing intake across groups did not result in significant main effects or a significant interaction. Although the interaction was tending toward significance $F(1, 56) = 2.76$, $p = 0.106$. Planned comparisons using ANOVA to analyze percent body weight gain between the paired ethanol group and all other groups showed significant differences in saccharin intake at test. Specifically, PD4 subjects which experienced low-dose ethanol immediately following saccharin exposure drank significantly more saccharin at test than the paired saline, unpaired saline and unpaired ethanol groups [$F(1, 28) = 5.33$, $p < 0.04$; $F(1, 28) = 7.34$, $p < 0.02$; $F(1, 28) = 5.29$, $p < 0.04$, respectively]. More specifically, the paired ethanol group drank significantly more saccharin at test than any other group. Saccharin intake scores are presented in Fig. 4.

Experiment 3: In Experiment 2 we found that PD4 rat pups exhibited increased intake of saccharin flavor after it had been paired with low doses of ethanol. Very few previous studies have found increased acceptance of a flavor paired with ethanol at any age, none in such young animals. Although some findings have shown that ethanol at this age is reinforcing, the response measure was increased attachment to a surrogate nipple following conditioning (Nizhnikov, Molina, et al., 2006; Nizhnikov, Varlinskaya, et al., 2006). To our knowledge this is the first set of experiments showing conditioned acceptance of a flavor with ethanol as the unconditioned stimulus at this age, so it was important to replicate this finding. Experiment 3 tested both a dose of ethanol (0.25 g/kg) previously shown to be appetitively reinforcing in very young rats and the highest dose used in Experiment 1 (2.0 g/kg) as the US. All other procedures were identical to Experiments 1 and 2 for the younger rat pups.

The ANOVA analyzing percent body weight gain at test showed a main effect of treatment $F(2, 27) = 3.64$, $p < 0.05$. Post-hoc analysis revealed that those pups receiving pairings of 0.25 g/kg ethanol and saccharin drank more saccharin at test (mean = 1.67, se = 0.11) than either of the other groups (saline: mean = 1.13, se = 0.18; 2.0 g/kg: mean = 1.16, se = 0.17), which did not differ from each other.

Experiment 4: As discussed earlier there is an apparent shift in kappa opioid function through ontogeny. This may be one of the factors responsible for differential responding to the motivational

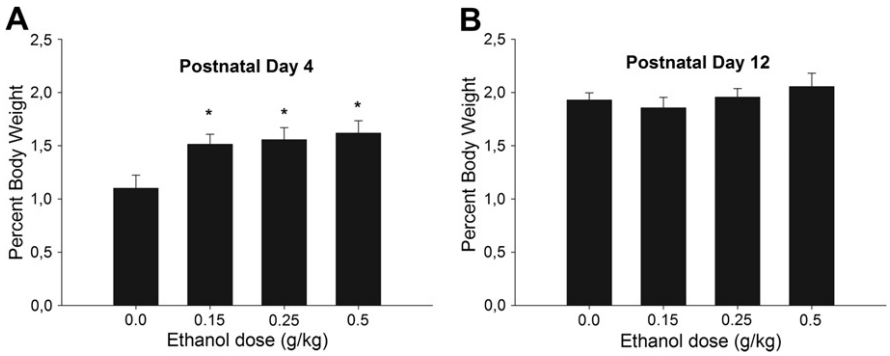

Fig. 3. Intake of a 0.1% saccharin solution depicted as percent body weight gain over a 10 min test for both PD4 (Fig. 2A) and PD12 (Fig. 2B) rat pups. Twenty-four hours prior to testing rat pups were exposed to a saccharin solution and immediately following injected i.p. with one of 4 ethanol doses (0.0–0.5 g/kg). Bars represent mean values; vertical lines depict the standard error of the mean. Asterisk (*) indicates a significant difference from saline controls.

properties of ethanol. The current experiment explored differences in the motivational effects associated with the activation of the kappa opioid system in 4 and 12 day-old rat pups.

An ANOVA analyzing percent body weight gain was performed for each age (PD4 and PD12) separately. The between group factors were kappa agonist dose (0.0, 1.0, 5.0, or 10.0 mg/kg; U62066). The ANOVA did not find any significant main effect of dose on intake at test for the PD12 rat pups $F(3, 58) = 1.49$, $p > 0.05$. However, the ANOVA analyzing body weight gain in PD4 pups showed a significant main effect of dose $F(3, 56) = 5.12$, $P < 0.01$. Post-hoc analysis revealed that pups which received the highest kappa agonist dose (10.0 mg/kg) immediately following saccharin infusions developed a significant preference for saccharin compared to controls, which did not differ from each other (see Fig. 5A and B).

Experiment 5: One possible reason for differences in ethanol's reinforcing properties across age could be differential levels of ethanol in blood following conditioning. An ANOVA analyzing BELs for PD4 and PD12 rat pups receiving the 0.25 g/kg showed no significant differences between groups. Conversely, the ANOVA analyzing subjects receiving 2.0 g/kg injection yielded a main effect of age $F(1, 70) = 32.78$, $p < 0.001$. Post-hoc analysis showed that the PD4 pups had a slightly ($\approx$20 mg%), but significantly, lower BEL (see Fig. 6A and B). This is unlikely due to faster metabolism of the drug since previous data has clearly shown that older animals process ethanol at a much faster rate than younger subjects. The most likely factor contributing to this effect is a byproduct of the procedure itself. Since PD4 subjects are so small the injection volumes are also small and any leakage following injection will have a major effect on the amount of ethanol received. It has been our observation that the injections for the PD12 subjects have almost no leaking while the PD4 subjects show significant leakage for subjects receiving the larger volume of injection (2.0 g/kg).

Discussion

The present study assessed changes in responding to the motivational properties of ethanol through the use of taste conditioning during the first or second week of life in the rat (i.e., PD4 or PD12). Changes across these ages in responding to activation of the kappa opioid system were also tested. The overall findings indicate that responding to ethanol's motivational properties change across early infancy. While older infants (PD12) readily express a conditioned taste aversion to saccharin when it is paired with a relatively high dose of ethanol (2.0 g/kg) younger rat pups (PD4) do not. On the other hand, PD12 rats do not show conditioned responding to a flavor paired with lower doses of ethanol (0.15, 0.25, 0.5 g/kg) while younger pups (PD4) show a clear preference for this flavor. Ontogenetic differences in responding were also seen in conditioning to specific doses of a kappa agonist (10.0 mg/kg: U62066). Mirroring what had been found when using low-dose ethanol as the unconditioned stimulus, PD4 rat pups found kappa opioid activation appetitively reinforcing, while PD12 rat pups did not.

These results suggest significant changes in the perception of ethanol's motivational consequences from PD 4 to PD 12. The difference in responding to the high doses of ethanol, however, may be attributable to differences in blood ethanol content since younger pups had lower levels of ethanol in blood. Although the actual gap between the ages at every time point measured was only around 20 mg%, statistically this was significant. This explanation, however, cannot be applied to the age-related differences found when using the lower doses of ethanol. Blood ethanol levels after administration of low-dose ethanol did not diverge significantly at any time point measured. Another explanation for the lack of appetitive conditioning in the older (PD12) pups could be that their intake is close to maximum offered in the intake test. The fluid delivered across the 10 min test is equivalent to 2.5% of the litter's average body weight. The older subjects drank around 2.0% of their body weight, or 80% of the fluid delivered. It is thus possible that the lack of conditioning in older pups reflects a ceiling effect.

It should be noted that the present set of Experiments is heavily grounded in the assumption that a decrease in the acceptance of a flavor paired with ethanol reflects an aversive acquired valance. Although this is the mainstream view on this phenomenon apposing points of view exist. For example, Grigson (1997) has suggested that decreases in intake of a solution following conditioning with drugs of abuse may be the result of a successive contrast effect. That is, the animal may reject thee CS because it pales in comparison with the upcoming, highly appetitive pharmacological US. It is possible to view the results with the 2.0 g/kg dose in this light. However, a large body of evidence, using a variety of species and methods (for example conditioned place preference and taste reactivity) has shown that, in adults, this dose of ethanol is clearly aversive. Moreover Arias et al. (2010) showed that two-week old rats exhibited similar taste avoidance when stimulated with a CS that predicted 2.0 g/kg ethanol or the prototypical aversive, emetic agent lithium chloride. Furthermore, when exposed to the CS pups emitted orofacial aversive reactions, which were indistinguishable between the ethanol or lithium chloride paired CS. This suggests that decreases in intake due to a flavor being paired with this dose of ethanol at this age are due to aversive effects of ethanol, most likely nausea.

This is not the first report of differences in responding to ethanol during early ontogeny. Infant rat pups are known to have high levels of immediate acceptance of ethanol during the first two weeks of postnatal life when tested in the context of either suckling behavior (Varlinskaya, Petrov, Cheslock, & Spear, 1999) or independent,

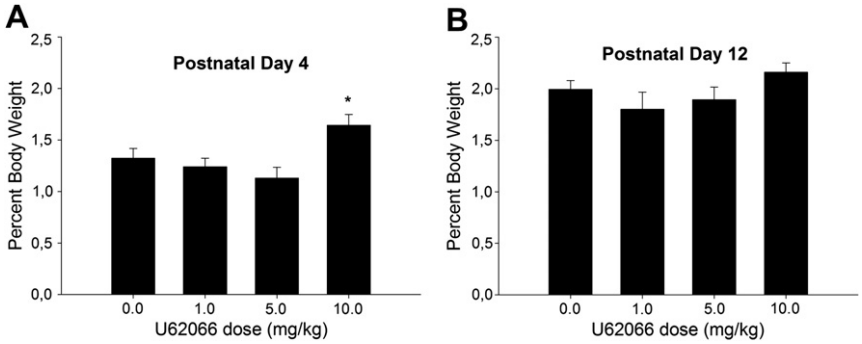

Fig. 5. Intake of a 0.1% saccharin solution depicted as percent body weight gain over a 10 min test for PD4 (Fig. 4A) and PD12 (Fig. 4B) rat pups. Twenty-four hours prior to testing rat pups were exposed to a saccharin solution and immediately following infusion injected i.p. with one of 4 doses of a kappa opioid agonist (U62,066: 0.0, 1.0, 5.0, 10.0 mg/kg). Bars represent mean values; vertical lines depict the standard error of the mean. Asterisk (*) indicates a significant difference from saline controls.

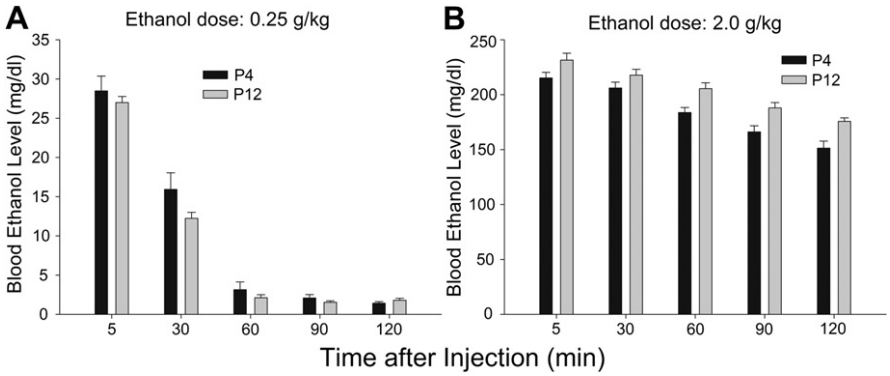

Fig. 6. Blood ethanol levels for PD4 (dark bars) and PD12 (grey bars) rat pups following an i.p. injection of 0.25 g/kg (Fig. 5A) or 2.0 g/kg (Fig. 5B) ethanol. Figures depict BELs following a temporal delay of 5–120 min. Bars represent mean values; vertical lines depict the standard error of the mean.

adult-like feeding (Sanders & Spear, 2007; Truxell & Spear, 2004; Truxell, Molina, & Spear, 2007), with ethanol intake during early ontogeny differing as a function of ethanol concentration and age. Neonatal rats readily ingest 5% ethanol from a surrogate nipple (Varlinskaya et al., 1999). Intake of 15% ethanol from the floor of a heated chamber reaches its peak around P12 and decreases thereafter, with absolute ethanol intake in inexperienced P12 animals exposed to ethanol for only 15 min being about 2.0 g/kg (Truxell & Spear, 2004). Greater intake has been found in rats which are experienced with ethanol or are exposed to higher concentrations of ethanol (30% for example) on the floor (Sanders & Spear, 2007). On the other hand, adult rats from lines not selected for ethanol preference will not consume significant amounts of ethanol unless given extensive initiation procedures. Without these acclimation procedures ethanol intake progressively decreases as the concentration of ethanol surpasses 6% (Boyle, Smith, Spiyak, & Amit, 1994; Kiefer & Dopp, 1989; Wayner et al., 1972).

Conditioning to alcohol has also been show to be affected by age. Appetitive conditioning to ethanol is easily seen in newborn rat pups. For example, 3-hr old rat pups readily condition preferences for stimuli paired with intra-oral infusions of ethanol (Cheslock et al., 2001; Nizhnikov, Varlinskaya, et al., 2006; Petrov et al., 2003). Furthermore, both peripheral and central injections of low doses of ethanol are found rewarding by these newborn rat pups (Nizhnikov, Molina, et al., 2006; Nizhnikov, Varlinskaya, et al., 2006; Nizhnikov et al., 2007). As rat pups age they tend to exhibit aversions to stimuli paired with ethanol (Abate, Spear, & Molina, 2001; Arias & Chotro, 2006; Hunt, Molina, Spear, & Spear, 1990; Molina and Chotro, 1989; Pautassi et al., 2002, Pautassi, Ponce, & Molina, 2005; Pueta, Abate, Spear, & Molina, 2005). One clear example of this ontogenetic discontinuity can be found in the studies of Chotro and Arias (2007). These researchers found that 7-8 day-old rat pups exposed to ethanol exhibit a preference for ethanol three days later. On the other hand, exposure during a later time point (PD10–PD11) elicited an aversion to ethanol following the same delay.

Likewise, changes in responding to kappa opioid agonists differ across age. Very young rat pups (3-hr old) find activation of the kappa opioid system positively reinforcing (Petrov, Nizhnikov, Varlinskaya, & Spear, 2006) while adult animals form aversions to stimuli paired with kappa opioid agonists (Bals-Kubik, Ableitner, Herz, & Shippenberg, 1993; Bals-Kubik, Herz, & Shippenberg, 1989; Mucha & Herz, 1985; Shippenberg & Herz, 1986; Wood, Norris, Daniel, & Papini, 2008). Experiment 4 replicated this general phenomenon, but narrowed the ages at which this effect is seen. Specifically, for 4 day-old rat pups a moderate dose of a kappa opioid agonist was positively reinforcing, while 12 day-old pups did not respond to this dose. Furthermore, responding to activation of the kappa opioid system by the highest dose of U62 (10.0 mg/kg) seems to mirror that of low-dose ethanol. It is important to point out that for the experiments using U62 no unpaired controls were used. Therefore, while the results suggest appetitive reinforcement in PD4 subjects after kappa agonism, further controls are needed to remove any doubt of unspecific effects of the kappa agonist. These results also do not indicate that the kappa opioid system is involved in the change of ethanol's motivational properties across age. Rather, the similarity of responding to ethanol and a kappa opioid agonist in experiment 4 suggest a direction for future investigation.

As seen in Experiments 1–4, response to ethanol differs across age. Young rat pups seem to be more responsive to the appetitive properties of low doses of ethanol while older pups condition aversions to higher doses more readily. This phenomenon cannot be explained by an inability of such young pups to form aversions in general, given a number of studies that have demonstrated conditioned aversions during the 1st week of life (Gemberling & Domjan, 1982; Haroutunian & Campbell, 1979; Molina, Serwatka, & Spear, 1986; Nizhnikov, Petrov, & Spear, 2002) and also in rat fetuses (Smotherman, 1982).

Major differences in responding to aversive stimuli across age have been seen in the past. For example, 8 day-old infant rats develop a preference for an odor paired with a mild shock while 12 day-old pups develop an aversion for this same odor (Roth & Sullivan, 2001). The difference in responding across age to the mild shock seems to be mediated by both the opioid system and corticosterone release as well as changes in the development of the amygdala (Roth & Sullivan, 2003; Sullivan & Holman, 2010). This result is very similar to the one found in the current set of experiment, albeit using a different CS and US. Nevertheless, stimuli found aversive by older rat pups was not found so by younger subjects. It is possible that similar mechanisms are responsible for these observed differences.

One possible explanation for these differences in responding to ethanol across ontogeny is that there are major changes occurring in the neurochemistry of the central nervous system at these ages. The function of the kappa opioid system seems to change from mediating positive to aversive reinforcement as the rat ages (Barr et al., 1994; Petrov et al., 2006). Furthermore, neurotransmitter systems shown to be relevant to ethanol acceptance and reinforcement in adults change similarly, fluctuating regularly in their quantitative and qualitative characteristics (e.g., Herlenius & Lagercrantz, 2004). For instance, neurotransmitter systems implicated in ethanol reinforcement (e.g., Herz, 1997; Koob, Mason, De Witte, Littleton, & Siggins, 2002; Oswald & Wand, 2004) change drastically within 1–3 weeks after birth. Some, such as GABA-A, kappa receptors and 5HT receptors, seem to change in their

function as well as density or number, whereas others, such as mu and delta receptors of the opioid system and NMDA receptors of the glutamate system, change primarily in density or number, in some cases from negligible quantities to high numbers or densities within a few days (e.g., Herlenius & Lagercrantz, 2004; Spain, Roth, & Coscia, 1985). Differences in the ontogenetic details of opioid system development have been reported, depending of course on neuroanatomical location (e.g., Georges, Normand, Bloch, & Le Moine, 1998; for reviews, Herlenius & Lagercrantz, 2004; Leslie & Loughlin, 1992). Many other parameters clearly are relevant as well (e.g., the extent to which the increase in each of the receptor types is preceded by increases in their respective endogenous ligands), but this does illustrate the interesting ontogeny of neurochemical factors associated with theories of ethanol reinforcement.

The present set of experiments utilized the classical taste conditioning paradigm to test ethanol's and kappa opioid agonist's motivational properties during the first and second postnatal weeks. The rationale for the use of taste conditioning was that it provides a relatively uniform method of assessment over the development of the animal. A major complication for assessing reinforcement throughout early ontogeny is that most tests are not comparable for animals of differing ages. Such tests are needed for development of hypotheses about the relationship between neurochemical maturation and ethanol ingestion, and for eventual conversion of the correlational evidence into experimental evidence. Although it has been established that ethanol-mediated conditioned tactlile preference can occur for infant rats (Pautassi et al., 2011), to our knowledge these tests have not yet been used to make clear comparisons of age-related changes in the efficacy of ethanol reinforcement (not to be confused with intake).

In conclusion, this set of experiments suggests differences in the motivational properties of low and high doses of ethanol between PD4 and PD12 rat pups. For the older subjects high doses of ethanol were aversive while the younger rats did not readily condition an aversion to the same dose. On the other hand PD4 pups readily conditioned preferences to lower doses of ethanol while older pups do not find these doses reinforcing. The same pattern of results as the lower doses of ethanol was seen for the highest dose of the kappa opioid agonist. Younger subjects found a kappa opioid agonist appetitively reinforcing while the older subjects did not condition any responding to the agonist. The taste conditioning procedure employed here can be a useful tool to analyze the reinforcing properties of a variety of US's across age. In general this set of studies provides a clear experimental modal which can be used to explore ontogenetic differences in responding to ethanol and the mechanisms behind any disparity in motivational properties of the drug across ontogeny.

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