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CENTRAL REINFORCING EFFECTS OF ETHANOL ARE BLOCKED BY CATALASE INHIBITION

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Abstract

Recent studies have systematically indicated that newborn rats are highly sensitive to ethanol's positive reinforcing effects. Central administrations of ethanol (25–200 mg %) associated with an olfactory conditioned stimulus (CS) promote subsequent conditioned approach to the CS as evaluated through the newborn's response to a surrogate nipple scented with the CS. It has been shown that ethanol's first metabolite, acetaldehyde, exerts significant reinforcing effects in the central nervous system. A significant amount of acetaldehyde is derived from ethanol metabolism via the catalase system. In newborn rats catalase levels are particularly high in several brain structures. The present study tested the effect of catalase inhibition on central ethanol reinforcement. In the first experiment, pups experienced lemon odor either paired or unpaired with intracisternal (i.c.) administrations of 100 mg% ethanol. Half of the animals corresponding to each learning condition were pretreated with i.c. administrations of either physiological saline or a catalase inhibitor (sodium-azide). Catalase inhibition completely suppressed ethanol reinforcement in paired groups without affecting responsiveness to the CS during conditioning or responding by unpaired control groups. A second experiment tested whether these effects were specific to ethanol reinforcement or due instead to general impairment in learning and expression capabilities. Central administration of an endogenous kappa opioid receptor agonist (dynorphin A-13) was employed as an alternative source of reinforcement. Inhibition of the catalase system had no effect on the reinforcing properties of dynorphin. The present results support the hypothesis that ethanol metabolism regulated by the catalase system plays a critical role in determination of ethanol reinforcement in newborn rats.

Keywords

Ethanol; Newborn; Rat; Catalase; Acetaldehyde; Dynorphin; Reinforcement

Introduction

Recent studies indicate that heightened affinity for ethanol in terms of intake and sensitivity to its reinforcing properties is observed during early postnatal stages in development. Infant rat pups (12 days-old) will consume large quantities of 10 – 15% v/v ethanol from the floor of a heated chamber without the need of initiation procedures (Truxell and Spear, 2004). Slightly older animals (15 days-old) markedly prefer a texture paired with a conditioned stimulus (CS) which was originally associated with the early post-absorptive effects of ethanol. In other words, different ethanol doses have been observed to act as positive primary reinforcers when

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utilizing second order conditioning paradigms in the infant rat (Molina et al., 2006; Pautassi et al., submitted). Further evidence of ethanol's reinforcing properties are derived from a recent series of experiments where the drug was utilized to counteract an aversive memory (Pautassi et al., 2006 Pautassi et al., in press).

Ethanol intake and its reinforcing properties in newborn rat pups have been investigated using a surrogate nipple technique. Neonates readily ingest ethanol from a surrogate nipple in the same manner as they do saccharin or milk. When the drug is delivered through this device newborns attach for significantly longer periods of time than when the nipple provides water (Cheslock et al., 2001; Petrov et al., 2001; Varlinskaya et al., 1999). Furthermore, unlike heterogenous adult rats which require long periods of initiation to ethanol (Samson and Grant, 1990) or stress in conjunction with ethanol to exhibit drug-mediated reinforcing effects (Matsuzawa et al., 1998, 1999), neonates rapidly acquire and express conditioning to stimuli mediated through ethanol's unconditioned properties. For example, intraoral infusions of a low dose ethanol solution (US) paired with lemon odor (CS) transfer the drug's appetitive properties to the olfactory CS, which later evokes enhanced responding to a non-nutritive nipple (Cheslock et al., 2001; Petrov et al., 2001; Varlinskaya et al., 1999; Nizhnikov et al., 2006a).

These techniques, however, involve ethanol's orosensory properties, leaving unanswered whether the reinforcing effects of the drug are derived from its chemosensory or postabsorptive properties. To minimize ethanol's chemosensory components and hence to better understand the drug's potential pharmacological reinforcing properties Petrov et al, 2003 tested the reinforcing effects of ethanol injected intraperitoneal (i.p.). They discovered that newborn rat pups given low doses of ethanol (0.25 g/kg) delivered i.p. associated with ingestion of water from a surrogate nipple subsequently increases responding to a surrogate nipple providing water. Reinforcement from i.p. ethanol paired with other CSs has been found in various studies (Nizhnikov et al., 2006a and b). Interestingly, prenatal ethanol exposure increases the range of i.p. ethanol doses newborns find reinforcing (Nizhnikov et al., 2006b).

In a subsequent set of experiments ethanol doses ranging between 25–400 mg% were directly administered into the newborn's cisterna magna, to further dissociate ethanol's pharmacological and chemosensory effects. These doses were selected from previous studies in which central administration of ethanol was found to exert positive reinforcing effects in both outbred and genetically selected adult rats (McBride et al., 1999; Rodd et al., 2003, 2004a, 2004b; Gatto et al., 1994). In newborns, doses ranging between 25 and 200 mg% were found to act as an appetitive unconditioned stimulus when utilizing a classical olfactory conditioning paradigm. With this intracisternal (IC) mode of ethanol administration it is highly unlikely that ethanol's unconditioned properties are derived from its chemosensory attributes or its caloric value. Taken together these results indicate the newborn pup is highly sensitive to ethanol's reinforcing properties and that early responding to the drug is plastic and can be changed with experience.

The mechanisms of ethanol reinforcement during early ontogeny are not yet well understood. However, recent studies indicate that such reinforcing effects seem to be partially mediated by the endogenous opiate system. Both kappa and mu opioid antagonists block the reinforcing effects of ethanol in 3-hour-old rat pups (Nizhnikov et al., 2006a) and naloxone appears to inhibit the formation of an ethanol-mediated conditioned preference in near term fetuses (Chotro and Arias, 2003). The present study tests the hypothesis that metabolic products of ethanol such as acetaldehyde also might mediate ethanol reinforcement.

In adult animals, acetaldehyde, a metabolite produced from the oxidation of ethanol, has also been implicated in ethanol's reinforcing effects. Peripherally this compound seems to have mainly aversive effects (Escarabajal et al., 2003; Quertemont, 2004; Quintanilla and Tampier,

2003). However, central administration of this bioactive compound appears to exert positive reinforcing effects. Outbred rats will self administer 2 and 5% acetaldehyde directly into the cerebral ventricles while pairing an odor with a wide range of acetaldehyde doses induces an olfactory conditioned preference (Brown et al., 1979; Quetremont and De Witte, 2001). Central inactivation of acetaldehyde has also been observed to decrease voluntary ethanol consumption in heterogenous rats (Font et al., 2006a and b). Although acetaldehyde derived from the metabolism of ethanol in the liver does not easily penetrate the blood brain barrier, the anti-oxidant catalase has been shown to metabolize alcohol into acetaldehyde in brain (Hamby-Mason et al., 1997; Aragon et al., 1992; Hunt, 1996). These and other studies suggest that acetaldehyde formation in the central nervous system is involved in ethanol's reinforcing capabilities and probably in the development of addictive processes (McBride et al, 2002).

In the neonatal rat the ability to metabolize ethanol is limited, perhaps due to the young animal's lack of alcohol dehydrogenase (Kelly et al., 1987; Lad et al 1984; Raiha et al., 1967; Sjoblom et al., 1978). At this age the function of converting ethanol into acetaldehyde is handled primarily by central catalase. Catalase brain levels are very high in several brain structures of the neonatal rat, allowing for substantial central metabolism of ethanol into acetaldehyde (Maestro and McDonald 1987). The central catalase system has been implicated in a variety of ethanol's effects in adult rats and mice. Catalase inhibition has been shown to decrease voluntary ethanol consumption, block ethanol's locomotor effects, mediate ethanol induced conditioned taste aversions, and lessen ethanol's anxiolytic properties (Koechinling and Amit, 1994; Aragon and Amit 1992; Tampier et al., 1994; Amit and Aragon, 1988, Aragon et al., 1985; Sanchis-Segura et al., 2005; Quertemont et al., 2003, 2005). Whether these effects are a direct result of acetaldehyde or of a condensate such as salsolinol is still to be fully answered, nevertheless it seems clear that acetaldehyde formation is a critical factor in ethanol's central motivational effects.

The current research was designed to assess catalase involvement in ethanol's reinforcing properties in newborn rats. The strategy used to investigate this phenomenon was derived from the studies conducted in this laboratory using central injections of ethanol as a US (Nizhnikov et al., 2006c) and sodium-azide central injections as a means of inhibiting catalase activity (Sanchis-Segura et al., 2005).

Materials and Methods

Subjects

Three-to four-hour old cesarean delivered rat pups were used as experimental subjects. For breeding, 1 male and 1 female Sprague-Dawley rat (Taconic, Germantown, NY) were housed together in a wire mesh-hanging cage. The paper tray under the cage was checked daily for plugs and the day a plug was found was considered embryonic day zero (E0). Upon discovery of the plug the female was removed from the cage and housed with another pregnant female in a standard plastic maternity cage until E19, when they were separated and placed into individual cages. All animals were housed in a temperature-controlled (22°C) vivarium maintained on a 12-hr light/dark cycle (lights on at 0700) with ad libitum access to food (Purina Rat Chow, Lowell, MA) and water. Near expected term (E21), pups were delivered by cesarean section. Under brief isoflurane (Baxter Healthcare Corp, Deerfield IL) anesthesia (Chamber from VetEquip, Pleasanton, CA), the pregnant female was sacrificed via rapid cervical dislocation. A midline incision was then made through the abdominal wall to expose the uterus. Immediately after delivery, extra-embryonic membranes were removed, and the umbilical cord ligated and severed. Pups were delivered from each female and placed in a plastic container (12 cm long × 12 cm wide × 6 cm high) lined with moist paper towels. The temperature at the bottom of the plastic container was maintained at 35.0° C using a heating pad. The entire procedure (from cervical dislocation to placement of the last pup in the container) was

completed within 7 – 9 min. Pups were gently stimulated to promote independent respiration. They were then transferred to an incubator maintained at $34.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ with 90% humidity, where they remained for 3 – 3.5 hours until the beginning of the experimental session.

From the time of birth and throughout the experiment, neonates were maintained in temperature- and humidity-controlled environments with conditions that simulate the natural nest. At all times, rats used in these experiments were maintained and treated in accordance with guidelines for animal care established by the National Institutes of Health (1986). The Institutional Animal Care and Use Committee approved all of the procedures used in this study.

Central Drug Administration

Ethanol (100 mg%), physiological saline, sodium-azide (1 $\mu\text{g}/\mu\text{l}$) and dynorphin (1 $\mu\text{g}/\mu\text{l}$) were employed in the experiments that compose the present study. All these substances were directly administered into the cisterna magna (IC) (volume: 1 μl) using a 30ga hypodermic needle attached to transparent polyethylene tubing (PE-10, Clay Adams, Parsippany, NJ). In both experiments, at the beginning of conditioning sessions subjects were centrally injected with either saline or sodium-azide and placed into the conditioning chamber for 10 min. Following the 10 min delay either ethanol (Experiment 1) or dynorphin (Experiment 2) was injected IC and the subject was once again placed in the conditioning chamber to acclimate. The needle was inserted under visual guidance into the foramen magnum between the occipital bone and the first cervical vertebra (Petrov et al., 1998; Varlinskaya et al., 1996). Successful placement of the needle into the target site was confirmed by the appearance of cerebrospinal fluid in the tubing. All solutions were injected within 5 – 8 s. A micrometer syringe (Gilmont Instruments, Barrington, IL) driven by a rotary microsyringe pump served to deliver all fluids. (see Cheslock et al., 2000). This volume of infusion does not seem to cause any discomfort or distress and is not excessive for a newborn rat pup. All pups employed in this study weighed 5 – 6 grams. An observer, blind to the contents of the syringe delivered the injections and tested the animals.

Conditioning Procedure

Experimental subjects were exposed to lemon odor explicitly paired or unpaired with ethanol (Experiment 1) or dynorphin (Experiment 2) IC injections. Paired subjects received a central injection of either sodium-azide or saline and were then placed in a small plastic weigh boat (approximately 5 cm long $\times$ 5 cm wide warmed to $35^{\circ}\text{C} \pm 0.5^{\circ}$) for 10 minutes to allow the drug to take effect. Following the 10-minute delay another injection of either ethanol (Experiment 1) or dynorphin (Experiment 2) was administered and the subject was placed back into the weigh boat for 3-min. Immediately after this acclimation period, lemon odor (CS) was presented for a 5-min duration using a cotton applicator scented with 0.1cc of lemon oil (LorAnn Oils, Lansing MI). The unpaired subjects were also injected with either sodium-azide or saline and placed in a small plastic weigh boat for 10 minutes. Following this delay they were exposed to a cue tip injected with 0.1cc of lemon oil for 5 min. Following odor presentation the pup was left alone for 3 min. and then given an IC injection of either ethanol or dynorphin. Prior studies have indicated that at this age pups do not exhibit conditioning when the odor CS and different USs are temporally separated by an interval equivalent to or higher than 120 s (Cheslock et al., 2003). Figure 1 illustrates the basic procedures employed for both Paired (Figure 1a) and Unpaired (Figure 1b) groups. The conditioning session was video-recorded for future analyses of motor activity. Overall movements of each individual subject during CS presentation was analyzed via a 2-way ANOVA where the between factors were drug treatment (sodium-azide or saline) $\times$ conditioning treatment (paired versus unpaired).

After conditioning subjects were placed back into the incubator. After a 1-hr retention interval newborns were presented with a surrogate nipple providing water in the presence of the lemon odor for 10 min.

Surrogate Nipple Procedure

A surrogate nipple was cast from latex rubber (AMACO rubber latex, Indianapolis, IN) and shaped into a conical form to measure 12 mm in length with 1 mm diameter at the rounded tip and 2.5 mm diameter at the base. A circular piece of vinyl, measuring 5 mm in diameter, was positioned 6mm from the tip of the nipple to provide a point of contact for the pup's snout during oral grasping of the surrogate nipple. The base of the surrogate nipple was attached to the end of an angled dental probe to facilitate presentation by the experimenter (Petrov et al., 1997). A length of PE 10 tubing extended through the length of the surrogate nipple and ended flush with the rounded tip of the nipple.

The pup actively extracted water during suckling due to the application of slight negative pressure on the nipple (Petrov et al., 1997). This was accomplished by the creation of a 1 mm diameter hole through the wall of the syringe that permitted the movement of water through the tubing. The syringe was placed in a holder in the test chamber and positioned to be level with the experimental subject's snout. In this position, the hole in the wall of the syringe was on the upper surface of the body of the syringe and in front of the piston. The PE 10 tubing and the syringe, when filled with a fluid, represented an open hydraulic system with two ends: the end of the PE 10 tubing located at the tip of the nipple and the hole located on the upper surface of the body of the syringe. The small diameter of the tubing, along with the surface tension of fluids, prevented its spontaneous effusion from the end located at the tip of the nipple. Slight negative pressure, produced by the pup while attached to the nipple, was necessary and sufficient to extract water from the surrogate nipple. The open hydraulic system employed in this study is virtually identical to the vacuum-free bottle procedure, which has been proven to be an effective tool in human neonatal nurseries (Lau and Schanler, 2000).

Pups were exposed to a nipple providing water on a mirrored surface (5 cm long $\times$ 5 cm wide) placed in a transparent glove box (63 cm long $\times$ 50cm wide $\times$ 25 cm high). The mirrored surface was maintained at $35.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and the inside of the glove box at $28.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ by two temperature controllers (Model 40-90-8B, Frederick Haer, Inc., Brunswick, ME). Exposure to the surrogate nipple involved gentle contact between the tip of the nipple and the oral area of the test subject. No attempt was made to force the tip of the nipple into the mouth of the pup. The subject was completely free to grasp and capture the nipple or disengage from it (Petrov et al., 1997). The nature of the procedure excluded any compulsion, since an attempt to force the nipple into the oral cavity aside from active grasping and voluntary oral capture evokes vigorous nipple rejection, and inevitably and rapidly results in choking and asphyxiation of the experimental subject.

During nipple presentation, in order to facilitate nipple exposure and minimize individual differences in gross body movement, each rat pup was strapped and buckled into a "vest" made from ultra-thin, elastic rubber (Petrov et al., 2001). The vest was designed to hold the pup in a semi-supine posture. This simulated the natural position of neonatal rats suckling at the maternal nipple (Eilam and Smotherman, 1998). It also prevented pups from righting (Pellis, et al., 1991) in the beginning of the testing procedure. The vest did not otherwise restrict the pup's spontaneous motor activity -- the subsequent oral grasping of the nipple in the supine posture involved active suppression of righting (Eilam and Smotherman, 1998) -- and with our previous studies to date has not produced any apparent discomfort or special distress.

Testing Procedure

Motor activity of each rat during the test was videotaped. The subject was illuminated with cool light from a fiber-optic light source (Scientific Instruments, Inc., Skokie, IL). For ease of scoring, real time was directly recorded onto the videotape (EZ Reader II, Telcom Research TCG 550, Burlington, Ontario). Playback of the videotape records permitted detailed analyses of oral grasp responses and attachment behavior. The oral grasp response was determined as occurring when an active movement of the head toward the surrogate nipple, which resulted in the tip of the nipple entering the oral cavity and the mouth closing around the nipple, was detected. Attachment to the nipple was confirmed by periodic (every 15s) gentle attempts to withdraw the nipple from the pup. Attachment was regarded as sustained if the pup resisted withdrawal of the nipple. The pup's active release of the nipple was considered to be a disengagement from the nipple. Before exposure to a surrogate nipple providing water the anogenital region of the pup was stroked gently with cotton to stimulate urination and defecation. Immediately prior to the nipple test pups were weighed to the nearest 0.01 g and placed into the test container. At the end of the test session, pups were removed from the testing container, dried with Kimwipes, and again weighed to the nearest 0.01 g. Percentage body weight gain was used as an indirect measure of fluid intake. Time attached, mean grasp duration, and body weight gain were scored and analyzed using separate 2 (drug treatment) $\times$ 2 (conditioning treatment) ANOVAs.

Experimental Design and Data Analysis

The design for each experiment was a 2 (drug treatment) $\times$ 2 (conditioning treatment) between subject factorial. To eliminate confounding of litter with treatment effects, no more than one subject from a given litter was assigned to the same treatment condition (Holson and Pearce, 1992). Each condition included an equal number of male and female subjects. Previous findings showed that the optimal time window for pre-exposure and test on the empty nipple falls between 3 and 6 hr after birth (Smotherman, et al., 1997), so conditioning procedures began no earlier than 3 hr after delivery and testing was completed within 6 hr after cesarean section. Within litters, order of testing for the different treatment groups was counterbalanced.

The dependent variables under analysis were oral grasping of the nipple and fluid intake. The suckling response was delineated accordingly into two components consisting of: (1) measures of latency (duration to first grasp), total time spent on the nipple (sum of the duration of all grasps) and mean grasp duration (total time divided by number of grasps); and (2) percent body weight gain (measure of fluid intake). The former may be viewed, roughly, as appetitive measures and the latter as consummatory. As previously stated these behavioral scores as well as gross motor activity during the conditioning session were compared using separate between-groups analysis of variance (ANOVA) procedures. The loci of significant interactions was further analyzed using Fisher's least mean significant difference tests with a probability of Type I error set at 0.05.

Experiment 1: Effects of Catalase Inhibition on Central Ethanol

Reinforcement It has been shown that central administration of ethanol (25–100 mg%) is reinforcing in newborn rats in terms of an olfactory conditioning paradigm (Nizhnikov et al., 2006). These effects may be mediated via the brain catalase system which transforms ethanol into acetaldehyde (Mason et al., 1997), a metabolite shown to have centrally mediated positive reinforcing effects (Arizzi et al., 2003; Brown et al., 1979; Correa et al., 2003; Smith et al., 1984; Rodd et al., 2002, 2003, 2005). When focusing on the ontogeny of the brain catalase system, it appears that this antioxidant agent is particularly high in several brain structures of the newborn rat (see Maestro and McDonald, 1987). In adult rats catalase activity has been shown to mediate ethanol's appetitive reinforcement properties as well as voluntary intake of the drug, and appears to reduce the magnitude of ethanol-mediated conditioned taste aversions

(Aragon et al., 1985, 1992a, 1992b; Smith et al., 1997; Amit and Aragon, 1988). Taking the above considerations into account, the following experiment was conducted to test the hypothesis that central catalase activity can modulate ethanol's reinforcing effects in neonatal rat pups.

Method A total of 32 pups from 10 cesarean section deliveries were tested in Experiment 1. The subjects were assigned to one of four treatment conditions (Saline/Unpaired, Saline/Paired, AZ/Unpaired, AZ/Paired; $n=8$ per group) defined by a factorial design which took into account central drug injection and conditioning status. Experimental subjects were conditioned according to the methods described above (see Figure 1) and placed back into the incubator for one hour. Following the one hour delay subjects were voided and weighed, placed into the testing chamber and presented with a surrogate nipple providing water in the presence of lemon odor for 10 minutes. Motor activity during lemon presentation in the conditioning session, latency to grasp the nipple, total time spent on the nipple providing water, mean grasp duration, and percent body weight gain during test were analyzed using separate $2 \text{ (drug)} \times 2 \text{ (conditioning)}$ ANOVAs.

Results Ethanol reinforcement and its elimination by sodium-azide was shown in the analysis of total time attached to the nipple, which revealed a significant drug by conditioning interaction $F(1,28)=4.28$, $p < 0.05$ (see Figure 2). Paired pups receiving saline injections exhibited significantly longer times of attachment when compared with the remaining groups. The latter groups did not differ from each other. In other words, treatment with AZ reduced total time attached in pups that also experienced the explicit contingency between lemon odor and central ethanol injection. This group did not differ from unpaired controls. The ANOVA for mean grasp duration revealed a similar significant drug by conditioning interaction $F(1, 28)=4.39$, $p < 0.05$ (see Figure 2). Ethanol reinforcement and its elimination by sodium-azide was shown in that paired neonates pretreated with saline exhibited significantly longer attachment bouts than any of the remaining groups, which included control groups for conditioning as well as paired groups pretreated with sodium-azide. Sodium-azide completely inhibited ethanol's reinforcing effects.

The ANOVA utilized to process overall motor activity when pups were stimulated with the lemon CS during conditioning did not show significant main effects attributable to drug or conditioning treatment. The interaction between these factors was also not significant. Duration of motor activity for each particular group was as follows: Saline/Unpaired, 55.1 ± 18.2 s; Saline/Paired: 35.9 ± 7.8 s; AZ/Unpaired, 36.1 ± 10.4 s and AZ/Paired, 39.9 ± 5.4 s (values represent means $\pm$ standard errors).

At test, latency to grasp the surrogate nipple did not differ significantly as a function of drug dose or conditioning treatment or the interaction between these factors (Saline/Unpaired: 119.0 ± 15.8 s; Saline/Paired: 92.6 ± 14.3 s; AZ/Unpaired: 124.5 ± 24.8 s and AZ/Paired: 90.4 ± 19.1 s). Pups corresponding to the different treatments did not differ in terms of percent body weight gain as a function of drug or conditioning (Saline/Unpaired: 1.48 ± 0.14 %; Saline/Paired: 1.69 ± 0.15 %; AZ/Unpaired: 1.19 ± 0.18 % and AZ/Paired, 1.55 ± 0.39 %).

The results of the present experiment suggest that the brain catalase system is critical in terms of regulating ethanol's central reinforcing properties in newborn rat pups. Subjects in the Saline/Paired group exhibited robust conditioned responses in terms of time attached and mean grasp duration. Pups that also experienced the contingency between lemon and ethanol's central effects but were pretreated with sodium-azide (AZ/Paired) did not differ from unpaired controls in either measure. Neither of the unpaired groups (Saline/Unpaired or AZ/Unpaired) differed from each other. This suggests that catalase inhibition alone had no particular effect

on future responding to the surrogate nipple at test. Furthermore, it appeared that sodium-azide treatment had no effect upon motor activity when pups were stimulated with lemon odor during conditioning or when evaluating latency to grasp the nipple at test, which suggests that neither perception of the CS or the pup's motor capabilities changed as a function of drug treatment. As a whole these results suggest that the integrity of the brain catalase system is a critical component in ethanol's central reinforcing properties in the newborn rat.

Experiment 2

Effects of Catalase Inhibition on Central Dynorphin A(1–13) Reinforcement

The results of Experiment 1 suggest that inhibition of catalase activity disrupts the central reinforcing effects of ethanol. It is not clear, however, whether this effect is specific to ethanol or reflects general disruption of associative learning processes mediated by positive reinforcement. Recently it has been shown that central injections of dynorphin A(1–13) are positively reinforcing in neonatal rats in terms of an olfactory conditioning strategy similar to that employed in Experiment 1 (Petrov et al., 2006). In order to investigate whether central injections of sodium-azide exert specific effects upon ethanol reinforcement or instead disrupt appetitive conditioning generally, dynorphin A(1–13) rather than ethanol was used as the US in Experiment 2.

Method

A total of 32 pups from 10 cesarean section deliveries were tested in Experiment 1. The subjects were assigned to one of four treatment conditions (n=8 per group) based on central drug injection and conditioning status (Saline/Unpaired, Saline/Paired, AZ/Unpaired, AZ/Paired). Experimental subjects were conditioned according to the methods previously described (see Figure 1). The only difference between this experiment and the previous one is that dynorphin, rather than ethanol, served as the unconditioned stimulus. It is important to note that dynorphin was administered intracisternally in the same way as ethanol in Experiment 1. At test, latency to grasp the nipple, total time spent on the nipple providing water, mean grasp duration, and percent body weight gain served as dependent variables. Two-way ANOVAs (drug $\times$ conditioning) were utilized to analyze the data.

Results

Analyses of total time attached revealed a significant main effect of conditioning $F(1,28) = 17.65$, $p < 0.001$ (see Figure 3). Paired groups attached to the surrogate nipple scented with lemon for a significantly longer period than unpaired groups. There was no effect of sodium-azide treatment on attachment duration. The ANOVA for mean grasp duration also revealed a significant main effect of conditioning $F(1, 28) = 17.64$, $p < 0.001$ (see Figure 3) with the paired subjects exhibiting longer attachment bouts than their unpaired controls. The significant conditioning with dynorphin as the US was unaffected by sodium-azide.

Latency to grasp the surrogate nipple did not differ significantly as a function of drug dose, conditioning treatment or the interaction between these factors. Latencies were very similar to those observed when using ethanol rather than dynorphin as a US (Saline/Unpaired: 111.0 $\pm$ 18.9 s; Saline/Paired: 85.6 $\pm$ 15.1 s; AZ/Unpaired: 119.9 $\pm$ 21.3 s and AZ/Paired: 100.4 $\pm$ 25.3 s). Percent body weight gain also did not differ as a function of drug or conditioning (Saline/Unpaired: 1.37 $\pm$ 0.16 %; Saline/Paired: 1.19 $\pm$ 0.19 %; AZ/Unpaired: 1.37 $\pm$ 0.21 % and AZ/Paired: 1.42 $\pm$ 0.20 %).

The results of Experiment 2 clearly indicate that central injections of sodium-azide do not disrupt all learning processes involving positive appetitive reinforcement. Inhibition of the brain catalase system had no effect upon the reinforcing properties of dynorphin, an

endogenous peptide that activates the kappa opioid system and was clearly an effective US in this test of neonates. The results obtained with dynorphin are in agreement with our previous findings concerning its centrally reinforcing effects in newborn rat pups (Petrov et al., 2006).

Discussion

The present results confirm previous findings indicative of ethanol's reinforcing effects in the newborn rat (Cheslock et al., 2001; Nizhnikov et al. 2006; Petrov et al., 2001; 2003; Varlinskaya, 1999) as well as the positive hedonic valence of centrally administered dynorphin (Petrov et al., 2006). Most importantly, this study indicates that catalase function is critical in terms of mediating ethanol's central reinforcing properties. Inhibition of the brain catalase system via central administration of sodium-azide completely blocked the expression of appetitive conditioned olfactory responses mediated by ethanol. This effect appeared not to be attributable to sensorimotor disruptions originated through inhibition of this antioxidant enzyme (Experiment 1). The results of Experiment 2 indicated that sodium-azide administration had no effects upon reinforcement by dynorphin, implying that its effect was specific to ethanol. It could be argued that differential effects of sodium-azide upon ethanol and dynorphin reinforcement are dependent upon the magnitude of the corresponding conditioned responses under analysis. Explicit comparisons across experiments indicate that this is probably not the case. Levels of conditioned attachment in paired/saline groups were very similar when using ethanol or dynorphin as USs. Hence, it appears that the effects of catalase inhibition were specific to ethanol without causing a general detrimental effect upon the capability of the newborn organism to perceive an olfactory CS or establish associations between this stimulus and an alternative reinforcer such as the kappa opioid agonist employed in Experiment 2.

Central acetaldehyde derived from ethanol metabolism in the brain, via the catalase system, can represent a critical element in the determination of ethanol's central reinforcing properties. Several bodies of experimental evidence seem to support this. First, a growing body of literature supports the notion that the first metabolite of ethanol, acetaldehyde, exerts both positive and negative (anxiolytic) reinforcing effects when directly administered into the lateral ventricles (Arizzi et al., 2003; Brown et al., 1979; Correa et al., 2003, Smith et al., 1984), the ventral tegmental area (Rodd et al., 2002, 2005) and the nucleus accumbens shell (Rodd et al., 2003). In other words it is possible that acetaldehyde is reinforcing simply in its own right (positive reinforcement) or that it removes an unpleasant stimulus, in this case stress and is rewarding in this fashion (negative reinforcement). The last two structures mentioned have been proposed as critical brain regions involved in ethanol reinforcement (Koob, 1992; McBride et al., 1999). Inactivation of acetaldehyde has also been shown to block positive reinforcing effects of ethanol as assessed through conditioned place preferences (Font et al., 2006). Second, drugs that exert inhibitory effects upon the catalase metabolic pathway reduce ethanol intake (Aragon and Amit, 1992) and exacerbate the capability of relatively low ethanol doses to act as aversive unconditioned stimuli using a taste aversion paradigm (Quertemont et al., 2003).

The conversion of ethanol into acetaldehyde in the rat brain seems to be strikingly elevated during late gestation and early stages of postnatal development (Devi et al., 1993; Gill et al., 1992; Hamby-Mason et al., 1997). In this respect, catalase concentrations in various brain structures of the newborn rat (cerebellum, striatum, cerebral hemispheres and brain stem) are approximately 8 times higher than those observed in the adult organism (see Maestro and McDonald, 1987). The levels of catalase in the brain progressively decrease during the ontogeny of the rat, in much the same pattern as ethanol intake (Truxell, Molina and Spear, 2007). Interestingly, while heightened affinity for ethanol ingestion and marked sensitivity to the drug's reinforcing effects are often found early in ontogeny, similar findings are far less frequent in the mature organism, particularly when studying genetically heterozygous animals

(i.e. neither selected for ethanol preference patterns or sensitivity to ethanol reinforcement; see e.g. Molina et al., 2007a see e.g. Molina et al., 2007b; Spear and Molina, 2005). This apparent association between catalase, affinity for ethanol and ontogenetic development leads to the hypothesis that this antioxidant agent mediates the observed changes in ethanol acceptance and reinforcement across ontogeny.

Recently, we reported that ethanol's reinforcing effects in neonates is modulated by the opioid system. Specifically, it was observed that blockade of mu and kappa opioid receptors completely eliminated appetitive reinforcement derived from intraperitoneal administrations of a low ethanol dose (0.25 g/kg; Nizhnikov et al., 2006). Prior research has demonstrated that the endogenous opioid system is intimately involved in the regulation of ethanol's positive reinforcing effects (e.g. Froelich, 1993; Herz, 1997; Koob, 1992; Li et al., 2001; McBride and Li, 1998). Salsolinol, a condensate of acetaldehyde and dopamine binds directly to mu and delta opioid receptors and also has high affinity for the D3 dopamine receptor when assessed through competitive binding assays (Lucchi et al., 1982; Airaksinen et al., 1984; Patsenko et al., 1987). Furthermore, a common interaction between ethanol's and salsolinol's reinforcing effects and stress can be seen in the work of Matsuzawa and colleagues. Ethanol conditioned place preference, in unselected rat strains, can be readily seen only when a mild stressor (shock) is applied in conjunction with the drug (Matsuzawa et al., 1998a and b). Similarly, conditioned place preference to i.p. administration of salsolinol is most profound when it is applied in conjunction with a mild stressor (shock) (Matsuzawa et al., 2000). This functional similarity (the need for a mild stressor) in achieving optimal reinforcement suggests that salsolinol may be one of the critical components in this type of reinforcement to ethanol. Perhaps, release of the corresponding neuromodulators (opiates) or neurotransmitters (dopamine) is actually modulated through salsolinol formation and is critical for the establishment of ethanol's appetitive post-absorptive effects (Koob, 1992; Lee et al., 2005; McBride and Li, 1998). Taking this into account it is possible that inhibition of the catalase system in the newborn animal reduces acetaldehyde levels, which in turn affect the formation of salsolinol and, hence, the recruitment of critical neurochemical systems modulating or otherwise determining ethanol's reinforcing effects. More in depth study of acetaldehyde levels due to catalase inhibition is needed to clarify whether lower levels of salsolinol contributes to the decrease in ethanol's reinforcing properties.

The absence of an effect of catalase inhibition on kappa opioid receptor function provides more than a control experiment to discount an impairing effect of sodium-azide on conditioning. Previous experiments have shown that a decrease in kappa activity induced by an antagonist (nor-BNI) can eliminate reinforcement from intraoral ethanol or saccharin (Nizhnikov et al 2006). The implication is that the decrease in ethanol reinforcement from sodium-azide in Experiment 1 was not due, at least, to disruption of kappa opioid receptor function.

The present study indicates that ethanol's central reinforcing effects are likely to be dependent upon the role of the catalase system in ethanol metabolism. This conclusion must be tempered slightly by the fact that only one dose of both ethanol and sodium-azide was used. The control conditions employed in these experiments, nevertheless, allow useful conclusions about the mechanisms involved. The conditioning procedure here employed permits exclusion of chemosensory and caloric properties of ethanol that might otherwise cloud the search for central mechanisms responsible for ethanol's motivational properties. The present findings also support a growing body of literature indicating that early experiences with ethanol can rapidly result in heightened drug seeking and consummatory behaviors (Molina et al., 2007; Spear and Molina, 2005).

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Figure 1.
Classical olfactory conditioning: design for paired and unpaired procedures used in Experiments 1 and 2. IC = intracisternal.

Figure 2.

Total time attached and mean grasp duration on a surrogate nipple providing water in the presence of lemon odor. One hour prior to testing subjects were IC injected with saline or 1 μ g of sodium-azide and then exposed to lemon odor either explicitly paired or unpaired with central injections of 100 mg% ethanol. Bars represent mean values; vertical lines depict the standard error of the mean. Asterisk (*) indicates a significant difference from all other groups.

Figure 3.

Total time attached and mean grasp duration on a surrogate nipple providing water in the presence of lemon odor. One hour prior to testing subjects were IC injected with saline or 1 μ g of sodium-azide and then exposed to lemon odor either explicitly paired or unpaired with central injections of dynorphin (A-13). Bars represent mean values; vertical lines depict the standard error of the mean. Asterisk (*) indicates a significant difference from corresponding unpaired controls.