RACC
Revista Argentina de Ciencias del Comportamiento

Revista Argentina de Ciencias del Comportamiento (RACC)

The Central Reinforcing Properties of Ethanol Are Mediated by Endogenous Opioid Systems: Effects of Mu and Kappa Opioid Antagonists

Michael E. Nizhnikov[*1], Elena I. Varlinskaya[1] & Norman E. Spear[1]

[1]Center for Development and Behavioral Neuroscience / Department of Psychology, Binghamton University – SUNY

Articulo Original

Resumen: Los sistemas de opiáceos endógenos están implicados en los efectos reforzantes del alcohol y pueden desempeñar un papel sustancial en modular los efectos reforzantes centrales del etanol en la temprana ontogenia. El presente estudio indagó esta posibilidad a través de un paradigma de condicionamiento olfatorio en el que la administración central de etanol sirvió como estímulo incondicionado (EI). En el experimento 1, los neonatos de rata fueron pre-tratados con antagonistas selectivos para los receptores mu o kappa (CTOP y nor-BNI, respectivamente) y luego entrenados en condicionamiento olfatorio. El experimento 2 evaluó la capacidad de un antagonista kappa de corta duración (GNTI) en alterar la capacidad reforzante del etanol. El experimento 3 investigó si el bloqueo farmacológico de los receptores opiáceos podía causar una alteración inespecífica del condicionamiento olfatorio. Para ello se empleó como EI la administración intraoral de quinina. La administración central de los antagonistas opiáceos mu y kappa bloqueó los efectos reforzantes del etanol. La efectividad de GNTI fue similar a nor-BNI. Es poco probable que los efectos de antagonistas opiáceos sobre el reforzamiento al etanol sean explicados por una disrupción general de todos los tipos de condicionamiento; desde el momento en que CTOP no afecto el reforzamiento aversivo inducido por la infusión intraoral de quinina. Estos resultados respaldan la hipótesis de que las propiedades reforzantes del etanol en el neonato de rata están mediadas por la actividad endógena de los receptores opiáceos mu y kappa.

Palabras claves:
Infantil, rata, los receptores opiáceos, etanol, acondicionado

Abstract: Endogenous opioid systems are implicated in the reinforcing effects of ethanol and may play a substantial role in modulating the central reinforcing effects of ethanol early in ontogeny. This possibility was explored in the present study through the use of an olfactory conditioning paradigm with centrally administered ethanol serving as an unconditioned stimulus (US). In Experiment 1, newborn rat pups were treated with either a selective mu antagonist CTOP or kappa selective antagonist nor-BNI prior to olfactory conditioning. Experiment 2 tested the effectiveness of an alternative, shorter-duration kappa opioid antagonist GNTI in altering ethanol reinforcement. Experiment 3 investigated whether the effectiveness of pharmacological blockade of opioid receptors was due to the disruption of learning per se using an olfactory aversive conditioning paradigm with intraoral quinine serving as a US. Central administration of either mu or kappa opioid antagonists prior to conditioning disrupted the reinforcing effects of ethanol in newborn rats. The kappa opioid antagonist GNTI was as effective as nor-BNI. These effects of opioid antagonists on ethanol reinforcement are unlikely to be due to a disruption of all types of conditioning, since CTOP did not affect aversive reinforcement to intraoral infusions of quinine. The present results support the hypothesis that in newborn rats, the reinforcing properties of ethanol are mediated by the endogenous activity at mu and kappa opioid receptors.

Key Words:
Infant, Rat, Opioid Receptor, Ethanol, Conditioning

1. Introduction

Endogenous opioid systems are known to mediate both ethanol intake and its reinforcing properties (Froehlich, 1993; Gianoulakis, 1996; Herz, 1997; Ulm et al., 1995). There is ample evidence showing that both selective (mu and delta) and general, nonselective, opioid antagonists reduce ethanol intake in adult laboratory animals (Davidson & Amit, 1997; DeWitte,

1984; Froehlich et al., 1990; Hubbell et al., 1986; Hyytiä, 1993; Hyytiä & Kiianmaa, 2001; Krishnan-Sarin et al., 1995a, b; Marfaing-Jallat et al., 1983; Stromberg et al., 1998). This effect is seen across a variety of species, selected or unselected lines and experimental conditions.

The release of endogenous ligands for mu (beta-

 Send correspondence to: Ph. Michael E. Nizhnikov
 Phone: (607) 777-3338 / Fax: (607) 777-2677
 E-mail: mnizhnikov@binghamton.edu

endorphin) and delta (enkephalins) opioid receptors due to ethanol exposure has been seen in brain regions associated with reward and reinforcement. Specifically, ethanol-induced release of beta-endorphin in the hypothalamus, nucleus accumbens, and ventral tegmental area (Boyadjieva & Sarkar, 1997; De Waele et al., 1992; De Waele & Gianoulakis, 1993; Olive et al., 2001; Rasmussen et al., 1998) and subsequent interaction of this endogenous ligand with mu opioid receptors located within the mesolimbic reward system may be viewed as a central event underlying the euphoric, positively reinforcing effects of ethanol.

In contrast to the mu and delta opioid systems, kappa opioid receptors and their endogenous ligands (dynorphins) have been shown to mediate the aversive properties of ethanol in adult laboratory rodents. Generally speaking, ethanol intake is attenuated by kappa opioid agonists and enhanced by antagonists in adult rats (Lindholm et al., 2001; Mitchell et al., 2005, but also see Nestby et al., 1999). Specifically, dynorphin, an endogenous ligand for kappa opioid receptors (Chavkin et al., 1982), was shown to reduce ethanol drinking preference in adults, and a selective kappa receptor agonist U50,488H effectively attenuated ethanol-induced conditioned place preference (Sandi et al., 1988; Matsuzawa et al., 1999). The above mentioned effect is not likely due to the kappa agonist substituting for ethanol's euphoric effect since kappa agonists have been shown to cause aversions in adult rats (Shippenberg & Herz, 1987; Fanselow et al., 1989; Mucha & Herz 1985; Barr et al., 1994). Furthermore, there are major differences in kappa receptor distribution between many preferring and non-preferring strains of rats and mice (i.e. Jamensky & Gianoulakis, 1997; Fadda et al., 1999).

The investigation of opioid involvement in ethanol reinforcement for infant rats has successfully utilized the surrogate nipple technique. Newborn pups ingest ethanol from a surrogate nipple in the same manner as saccharin or milk and attach for significantly longer periods of time than when the nipple provides only water (Cheslock et al., 2001; Petrov et al., 2001; Varlinskaya et al., 1999). Infant rats are responsive to ethanol's appetitive reinforcing properties without the initiation procedures or pre-exposure required in adults (Nizhnikov et al., 2006; Cheslock et al., 2001; Petrov et al., 2001). For example, when a lemon odor (conditioned stimulus; CS) is associated with intraoral infusions of ethanol (unconditioned stimulus; US) the appetitive properties of ethanol are transferred to the lemon odor CS. When tested one hour later on a lemon-scented nipple providing only water, paired subjects

attached for significantly longer periods of time than their unpaired counterparts (Cheslock et al., 2001). Blockade of either mu or kappa opioid receptors by the selective antagonist nor-BNI (kappa) or CTOP (mu) completely eliminated the reinforcing effects of ethanol (Nizhnikov et al., 2006). These findings suggest that for infants, unlike adults, both the mu and kappa opioid systems are critical in mediating ethanol's appetitive reinforcing properties.

One major concern when interpreting these results is associated with the use of nor-BNI. Although a potent kappa opioid antagonist, this compounds is extremely long lasting. Several studies have shown that the kappa opioid antagonist effects of nor-BNI can last for weeks (Jewett et al., 1997, 1995; Broadbear et al., 1994, Picker et al., 1996). Therefore, testing in the surrogate nipple paradigm described above probably occurred with nor-BNI still acting upon kappa opioid receptors in the infant rat. Another concern stems from the fact that several studies have found that nor-BNI clearly diminishes the nociceptive effects of mu opioid agonists, especially during the first few hours post administration (Endoh et al., 1992; Wettstein & Grouhel, 1996). Although nor-BNI clearly blocks kappa opioid receptors in newborn rats (Petrov et al., 2006), the use of a more specific and shorter lasting kappa opioid antagonist may lead to better understanding of the mechanisms involved in ethanol reinforcement at this young age.

One possibility that could not be discounted in the "surrogate nipple" studies described above is that the orosensory properties of ethanol could be the driving force behind the appetitive reinforcement observed. Direct injections of ethanol (25-400 mg%) into the cisterna magna allowed for the pharmacological and chemosensory effects of ethanol to be separated. Using the olfactory conditioning paradigm described above but with central injections of ethanol serving as a US, it was found that a range of ethanol between 25 and 200 mg% was positively reinforcing (Nizhnikov et al., 2006). This clearly indicates that the pharmacological properties of the drug acting on central mechanisms are at least partly responsible for the reinforcing effects of ethanol. However, the mechanisms behind this effect have yet to be fully uncovered.

The current set of experiments was conducted to explore the possible roles of endogenous opioid systems in the reinforcing properties of centrally administered ethanol. The effects of a selective mu opioid antagonist CTOP and kappa opioid antagonist nor-BNI on ethanol's appetitive reinforcing properties using an olfactory conditioning paradigm in newborn rats were

tested in Experiment 1. In Experiment 2, a novel, short-lasting and more specific kappa antagonist (GNTI) was tested for its effectiveness at this age. Experiment 3 was designed to assess possible roles of endogenous activity at mu opioid receptors in aversive learning. This experiment investigated whether selective blockade of mu opioid receptors by CTOP was effective in attenuating aversive conditioning with quinine used as a US. Quinine is a bitter substance which has been shown to produce aversions in rat pups of this age (Nizhnikov et al., 2002).

2. Materials and Methods

2.1. 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, Pleasenton, 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 x 12 cm wide x 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 minutes. Pups were gently stimulated to promote independent respiration. They were then transferred to an incubator maintained at 34.5° C $\pm$ 0.5° 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.

2.2. Central Drug Administration

Ethanol (100 mg%), physiological saline, mu antagonist CTOP (1.0 μg), kappa antagonist nor-BNI (1.0 μg) or GNTI (0.0174, 0.174 or 0.348 μg) were used in the present experiments. The doses for GNTI were chosen on the basis of those used in the Jewett et al. (2001) study. The dose of ethanol was chosen because it was the most effective dose at inducing appetitive reinforcement in neonatal rat pups (Nizhnikov et al., 2006). All experimental solutions were injected directly into the cisterna magna (IC) in a volume of 1μl using a 30 gauge hypodermic needle attached to transparent polyethylene tubing (PE-10, Clay Adams, Parsippany, NJ). In all three experiments, at the beginning of conditioning sessions subjects were centrally injected with either saline, CTOP, nor-BNI or GNTI and placed into the conditioning chamber for 10 minutes. Following the 10-minute delay, ethanol (Experiment 1 and 2) was injected IC and the subject was once again placed in the conditioning chamber to acclimate for 3 minutes. Following the 3-minute delay conditioning to lemon odor was performed (see Conditioning Procedure section). 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 seconds. A micrometer syringe (Gilmont Instruments, Barrington, IL) driven by a rotary microsyringe pump served to deliver all fluids. (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 (Nizhnikov et al., 2006; Petrov et al., 1998, 2006; Varlinskaya et al., 1996). All pups employed in this study weighed 5 – 6 g. An experimenter, blind to the contents of the syringe, delivered the injections and tested the animals.

2.3. Cannulation Procedure

In Experiment 3, pups were implanted with a cannula as described in Pautassi et al. (2002). Specifically, two hours after delivery a 30-35 mm section of PE-10 polyethylene tubing with one end flanged was inserted through the subject's cheek with the flanged end resting on the interior of the cheek. The

pup was then placed back into the incubator for 1 hour to recover from the procedure.

2.4. 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 6 mm 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 loose end of the tubing was then attached to a needle and syringe which had a small hole on one side. When the syringe was placed at the level of the subjects' snout with the hole on top, the surrogate nipple attached to the syringe represented an open hydraulic system. The pup was able to withdraw fluid from the surrogate nipple by the application of slight negative pressure.

During testing for ethanol reinforcement, pups were exposed to a nipple providing water on a mirrored surface (5 cm long x 5 cm wide) placed in a transparent glove box (63 cm long x 50cm wide x 25 cm high). The mirrored surface was maintained at $35.5\,^{\circ}C \pm 0.5\,^{\circ}C$ and the inside of the glove box at $28.0\,^{\circ}C \pm 1.0\,^{\circ}C$ 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).

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.

2.5. Conditioning Procedure

Rat pups were exposed to lemon odor explicitly paired or unpaired with IC injections of ethanol (Experiment 1 and 2) or intraoral infusions of quinine (Experiment 3; 0.1% w/v). For Experiments 1 and 2, subjects in the paired groups received a central injection of CTOP, nor-BNI, GNTI or saline and were then placed in a small plastic weigh boat (approximately 5 cm long x 5 cm wide warmed to $35°C \pm 0.5\,°$) for 10 minutes to allow the drug to take effect. Following the 10-minute delay central injection of ethanol (Experiment 1 and 2) was administered, and the subject was placed back into the weigh boat for 3 minutes. Immediately after this acclimation period, lemon odor (CS) was presented for a 5-min period using a cotton applicator scented with 0.1cc of lemon oil (LorAnn Oils, Lansing MI). The unpaired subjects were also injected with one of the antagonist solutions or saline and placed in a small plastic weigh boat for 10 minutes. Following this delay they were exposed to a Q-tip infused with 0.1cc of lemon oil for 5 minutes. Following odor presentation the pup was left alone for 3 minutes and then given an IC injection of ethanol. The 3-minute delay between odor presentation and ethanol injection has previously been shown to be sufficient at this age to preclude trace or backwards conditioning (Cheslock et al., 2004). Figure 1 illustrates the basic procedures employed for both paired and unpaired groups in Experiments 1 and 2.

In Experiment 3, test subjects were randomly assigned to one of four groups described by conditioning contingency (paired, explicitly unpaired) and central injection (CTOP, saline). Pups in the paired group were given central injections of either CTOP or saline, placed in the same container as used in Experiment 1 and 2, left undisturbed for 10 minutes, and then exposed to an olfactory CS (0.1 cc of lemon oil on a cotton Q-tip) for 5 minutes. Simultaneous with odor presentation, five intraoral infusions of quinine (0.1% w/v) were given (5 µl each, one infusion per minute). Pups in the unpaired condition were also given central injections of CTOP or saline and placed in the conditioning container for 13 minutes. Following the acclimation period pups were exposed to the CS (lemon odor) for 5 minutes, left undisturbed for another 5 minutes, and then given US infusions over the next 5 minutes. Following conditioning treatment, pups were returned to the incubator. Cannulae were gently removed 10 minutes later, and 1 hour following

conditioning pups were tested for response to a lemon-scented nipple providing water (see Figure 4). After conditioning subjects were placed back into the incubator. After a 1-hour retention interval newborns were presented with a surrogate nipple providing water in the presence of the lemon odor for 10 minutes.
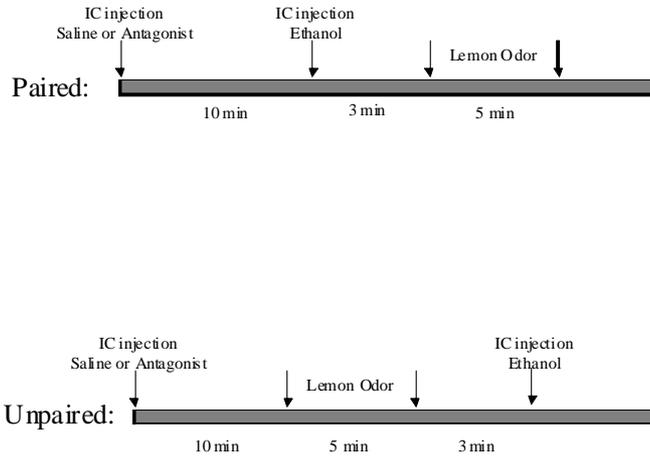

Figure 1: Olfactory IC conditioning: Design for paired and unpaired procedures used in Experiments 1 and 2. IC = into cisterna magna.

2.6.   Testing Procedure

Behavior 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). Detailed analysis of oral grasp responses and attachment behavior was scored via video playback. An oral grasp response was scored when the pup displayed an active movement of the head toward the surrogate nipple and grasped the nipple in its mouth. Attachment to the nipple was confirmed by periodic (every 15 seconds) 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.

2.7.   Experimental Design and Data Analysis:

To eliminate confounding of litter with treatment effects, no more than one male and one female 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 in the surrogate nipple paradigm falls between 3 and 6 hours after birth (Smotherman, et al., 1997), so conditioning procedures

began no earlier than 3 hours after delivery and testing was completed within 6 hours after cesarean section. Within litters, order of testing for the different treatment groups was counterbalanced.

The dependent variables under analysis comprised measurements of oral grasping of the nipple. Specifically, the measures consisted of total time spent on the nipple (sum of the duration of all grasps) and mean grasp duration (total time divided by number of grasps). These variables describe the appetitive response to the surrogate nipple. These behaviors 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 probability of Type I error set at 0.05.

3.   Experiment 1: Effects of mu and kappa antagonists on central ethanol reinforcement.

Central administration of ethanol (100 mg%) has been shown to be reinforcing in 3-hour old rat pups using an olfactory conditioning paradigm (Nizhnikov et al., 2006). Furthermore, the reinforcing properties of orally delivered or intraperitoneally administered ethanol are clearly mediated by the opioid systems (Nizhnikov et al 2006). However, it is not clear if the opioid mechanisms responsible for the reinforcing properties of peripheral delivery of the drug are the same as of centrally administered ethanol. Therefore, Experiment 1 was designed to test the hypothesis that the endogenous opioid systems play a substantial role in the reinforcing properties of centrally delivered ethanol.

3.1.   Methods

A total of 48 pups from 8 cesarean deliveries were tested in Experiment 1. The subjects were assigned to one of six groups: Saline/Paired, Saline/Unpaired, CTOP/Paired, CTOP/Unpaired, nor-BNI/Paired, nor-BNI/Unpaired defined by a 3 (drug treatment) x 2 (conditioning treatment) factorial design, with 8 animals placed into each experimental group. Experimental subjects were conditioned as described in the Methods section (see Figure 1) and placed back into the incubator for 1 hour. Following the 1-hour delay subjects were tested on a lemon-scented nipple for 10 minutes (see Materials and Methods). Total time spent on the nipple providing water, and mean grasp duration were analyzed using separate 3 (drug treatment) x 2 (conditioning treatment) ANOVAs.

3.2.   Results

The reinforcing properties of centrally administered ethanol were blocked by either kappa (nor-BNI) or mu (CTOP) opioid receptor antagonists. These results were

clearly seen in both the total time attached and mean grasp duration measures. Analysis of total time attached revealed a significant drug by conditioning interaction, $F(2, 42) = 6.05$, $p < 0.01$ (see Figure 2a), with subjects in the Saline/Paired group attaching for longer periods of time than all other groups. Rat pups injected with either CTOP or nor-BNI prior to ethanol administration showed responding similar to that of unpaired controls. The ANOVA analyzing mean grasp duration also revealed a significant drug by conditioning interaction, $F(2, 42) = 7.77$, $p < 0.01$ (see Figure 2b), with subjects pre-treated with opioid receptor antagonists showing responding at the levels of unpaired controls and the Saline/Paired group attaching for significantly longer bouts than all other groups.
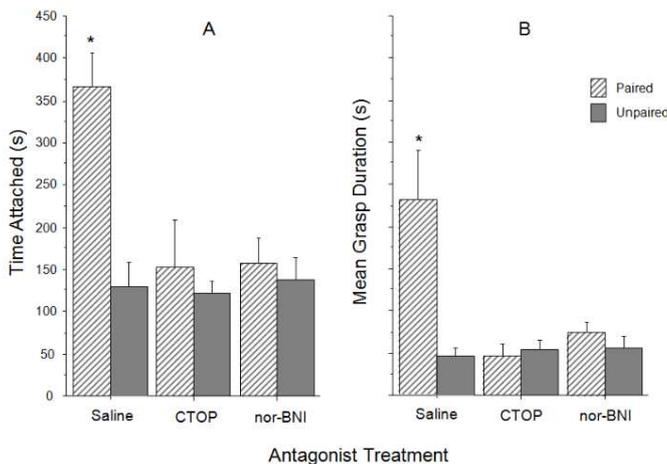

Figure 2: Total time attached (A) and mean grasp duration (B) on a surrogate nipple providing water in the presence of lemon odor. One hour prior to testing subjects were injected IC with saline, 1μg of CTOP or 1μg of nor-BNI and then exposed to lemon odor either explicitly paired or unpaired with central 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.

These results are in agreement with those reported by Nizhnikov and colleagues (2006) showing that pre-treatment with mu or kappa antagonists disrupts the reinforcing properties of ethanol administered either intraorally or intraperitoneally. The present data expand on these results by demonstrating that mu and kappa opioid antagonists effectively disrupt the ethanol's direct central reinforcing effects.

It is important to note that the unpaired groups in the current design also experienced the effects of centrally injected opioid receptor antagonist (CTOP, nor-BNI) or saline paired with lemon odor. Effects of these treatments provided, in essence, a measure of the motivational properties of the antagonists themselves.

Since the unpaired groups pre-treated with the antagonists did not differ in their responding to the nipple from saline controls, it can be concluded that, at this age and dose, neither CTOP nor nor-BNI has any inherently aversive or appetitive motivational properties.

4. Experiment 2: Effects of GNTI, a potent alternative kappa antagonist, on central ethanol reinforcement.

The results of Experiment 1 clearly indicate that administration of either a mu or kappa opioid antagonist prior to ethanol injection disrupts its central reinforcing effects. One problem with the interpretation of results derived from tests of nor-BNI is the long lasting effects of this kappa antagonist (Jewett et al., 1997, 1995; Broadbear et al., 1994, Picker et al., 1996) and its possible effects on mu opioid receptors (Endoh et al., 1992; Wettstein & Grouhel, 1996). A relatively new kappa opioid antagonist, GNTI dihydrochloride, has a much shorter half-life and higher potency than nor-BNI. Specifically, effects of GNTI are gone 24 hours post-administration, and GNTI more potently inhibits U50,488 (kappa opioid agonist) induced feeding than nor-BNI (Jewett et al., 2001). The goal Experiment 2 was to test whether GNTI was as effective as nor-BNI in reducing the reinforcing properties of central ethanol injections.

4.1. Methods

A total of 48 pups from 9 cesarean deliveries were tested in Experiment 2. Animals were assigned to one of the 8 groups defined by a 4 (GNTI dose: 0, 0.0174, 0.174, or 0.348 μg) x 2 (conditioning treatment: paired, unpaired) experimental design, with 6 animals placed into each group. Conditioning and testing procedures were identical to those used in Experiment 1. Total time spent on the nipple providing water and mean grasp duration were analyzed using separate 4 (GNTI dose) x 2 (conditioning treatment) ANOVAs.

4.2. Results

The reinforcing properties of centrally delivered ethanol were blocked by GNTI. The ANOVA analyzing total time attached revealed a significant GNTI dose by conditioning treatment interaction, $F(3, 40) = 9.48$, $p < 0.001$ (see Figure 3a), with subjects in the paired condition pre-treated with saline or the lowest dose of GNTI attaching for significantly longer periods of time than all other groups, which did not differ from each other or the Saline/Unpaired controls. The ANOVA analyzing mean grasp duration also revealed a significant GNTI dose by conditioning treatment interaction, $F(3, 40) = 5.90$, $p < 0.01$ (see Figure 3b).

Subjects pre-treated with the 2 highest doses of GNTI (0.174 and 0.348 µg) showed responding at the levels of unpaired controls while the paired groups pre-treated with saline or the lowest dose of GNTI attached for significantly longer bouts than all other groups but did not differ from each other.
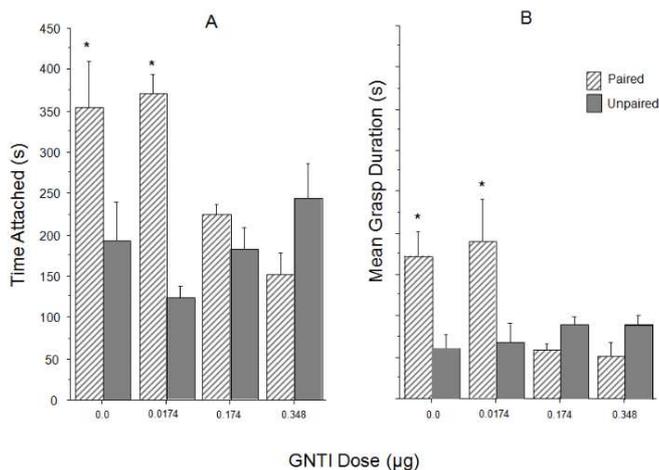

Figure 3: Total time attached (A) and mean grasp duration (B) on a surrogate nipple providing water in the presence of lemon odor. One hour prior to testing subjects were injected IC with one of the 4 doses of GNTI (0, 0.0174, 0.174 or 0.348 µg) and then exposed to lemon odor either explicitly paired 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 control and both the paired and unpaired GNTI 0.174 and GNTI 0.348 µg groups.

The results of Experiment 2 suggest that GNTI is as effective as nor-BNI at disrupting the reinforcing properties of centrally administered ethanol at the higher doses employed. More specifically, the results show that the kappa opioid system is intimately involved in the reinforcing effects of centrally delivered ethanol. Furthermore, the effects of GNTI were dose-dependent, with the lowest dose of GNTI being ineffective at reducing the reinforcing properties of ethanol and both higher doses effectively blocking these reinforcing properties of ethanol.

As in Experiment 1, the rats in Experiment 2 experienced the effects of a centrally injected opioid receptor antagonist in conjunction with lemon odor. Once again the unpaired groups receiving GNTI did not differ from saline controls in their responding to the surrogate nipple. Therefore, is seems that these doses of GNTI do not exert inherent motivational effects on newborn rats.

5. *Experiment 3: Effects of CTOP on aversive conditioning.*

Experiments 1 and 2 indicate that the appetitive reinforcing properties of ethanol can be affected by pre-treatment with either a mu or kappa opioid antagonist. However, since previous work has shown that both CTOP and nor-BNI also reduce the reinforcing properties of orally delivered saccharin (Nizhnikov et al., 2006), it is possible that antagonism of these systems disrupts all forms of learning in the infant rat.

Aversive conditioning has been established in infant rats in the past using the surrogate nipple technique (Nizhnikov et al., 2002). Specifically, pairings of lemon odor and intraoral infusions of quinine (0.1% w/v) result in rejection of a surrogate nipple 1 hour later. This aversive conditioning is strong enough to disrupt responding even to a surrogate nipple providing milk (Nizhnikov et al., 2002). The goal of Experiment 3, therefore, was to ascertain whether administration of CTOP would reduce or eliminate the aversive reinforcing properties of quinine using an olfactory conditioning paradigm.

5.1. Methods

A total of 32 pups from 8 cesarean section deliveries were tested in Experiment 3. The subjects were assigned to one of four groups defined by a 2 (drug treatment: saline, CTOP) x 2 (conditioning treatment: paired, unpaired) factorial design, with 8 animals placed into each experimental group. Subjects were conditioned as described in the Material and Methods section (see Figure 4) and placed back into the incubator for 1 hour. Following the 1-hour delay subjects were tested on a lemon-scented surrogate nipple for 10 minutes. Total time spent on the nipple and mean grasp duration were analyzed using separate 2 (drug) x 2 (conditioning) ANOVAs.
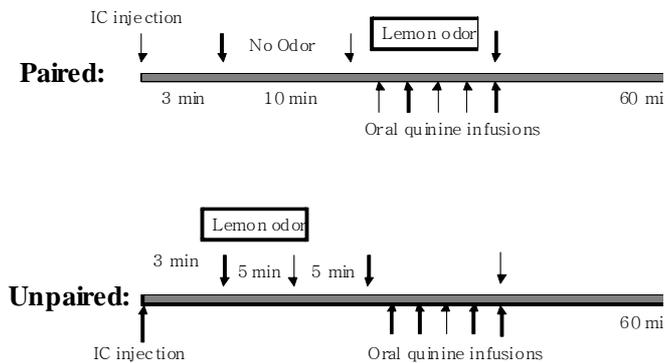

Figure 4: Classical olfactory conditioning: design for paired and unpaired procedures used in Experiments 1 and 2. IC = into cisterna magna

5.2. Results

Aversive conditioning with quinine as the US was not affected by CTOP. The ANOVA analyzing total

time attached revealed only a significant main effect of conditioning treatment, $F(1, 28) = 16.94$, $p < 0.001$ (see Figure 5a), with subjects in the paired groups attaching for significantly shorter periods of time than the unpaired controls. The ANOVA analyzing mean grasp duration also revealed only a significant main effect of conditioning treatment, $F(1, 28) = 9.47$, $p < 0.01$ (see Figure 5b). Similarly, paired subjects exhibited significantly shorter bouts of attachment than their unpaired counterparts.

The results of Experiment 3 demonstrate that blockade of mu opioid receptors has no effects on aversive conditioning to an exteroceptive stimulus (quinine). The aversion conditioned to lemon odor paired with quinine infusions was not affected by pre-treatment with CTOP, with rat pups in the paired groups significantly reducing responding to a lemon-scented surrogate nipple relative to their unpaired counterparts.
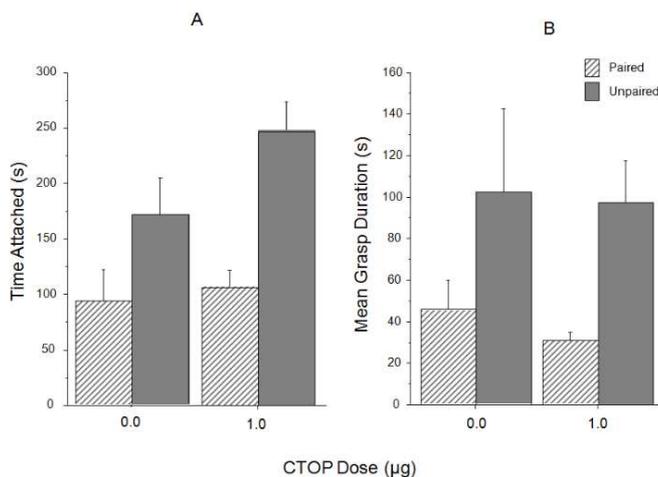

Figure 5: Total time attached (A) and mean grasp duration (B) on a surrogate nipple providing water in the presence of lemon odor. One hour prior to testing subjects were injected IC with either saline or 1.0 μg CTOP and then exposed to lemon odor while receiving intraoral infusions of 0.1% quinine (paired) or exposed to lemon odor 5 min prior to intraoral quinine infusions (unpaired) in Experiment 3. Bars represent mean values; vertical lines depict the standard error of the means. There was a significant main effect of conditioning. Unpaired subjects attached for both longer periods of time and longer bouts than paired rat pups which indicates an aversion.

6. *Discussion*

The results of the current set of experiments confirm that both the mu and kappa opioid systems play a substantial role in the reinforcing properties of centrally delivered ethanol. Administration of either a mu or kappa opioid antagonist prior to central injections of 100 mg% ethanol completely eliminated the reinforcing effects of the drug. Furthermore, the alternative kappa opioid antagonist GNTI was as effective as nor-BNI and CTOP at disrupting ethanol's positive motivation properties. This effect can not be accounted for by a complete disruption of learning by pharmacological blockade of opioid receptors, since aversive conditioning to quinine was not affected by CTOP.

This is not the first time that the central reinforcing properties of ethanol have been explored using a rat model of conditioning. Several lines of research have clearly shown that centrally injected ethanol exerts positive reinforcing effects. Newborn rat pups injected with 25 – 200 mg% ethanol into the cisterna magna in the presence of lemon odor find that odor positively reinforcing when tested one hour later (Nizhnikov et al., 2006). Studies using intracranial self-administration of ethanol have also shown that several rat strains will actively work for infusions of ethanol (25 – 200 mg%) into the posterior ventral tegmental area (Gatto et al., 1994; Rodd et al., 2000, 2004a & b). These results demonstrate that ethanol reinforcement acts centrally to exert appetitive effects.

Previous research has shown that the endogenous opioid systems are involved in the reinforcing properties of ethanol in both adult and infant rats. For example, both general and selective opioid antagonists have been shown to disrupt ethanol's appetitive motivational properties in adult rats (Kuzmin et al., 2003; Matsuzawa et al., 2000, 1999, 1998). Similar results are seen in infant rats as well. When newborn rat pups were given i.p. injections of low ethanol doses (0.25 g/kg) in the presence of lemon odor, they later increased their responding to a lemon-scented surrogate nipple compared to unpaired controls. Pre-treatment with either CTOP (mu antagonist) or nor-BNI (kappa antagonist) eliminated this effect (Nizhnikov et al., 2006). In general, these findings suggest that the reinforcing properties of ethanol are dependent on endogenous activity at mu and kappa opioid receptors. The results of Experiment 1 indicate that either mu or kappa opioid antagonists also disrupt the reinforcing properties of centrally delivered ethanol in newborn rats.

One of the goals of the current set of experiments was to assess the effectiveness of an alternative kappa opioid antagonist in lieu of nor-BNI. One of the major problems with using nor-BNI is the extremely long lasting effects (weeks) as well as its seeming affinity for mu opioid receptors soon after administration (Jewett et al., 1997, 1995; Broadbear et al., 1994, Picker et al., 1996; Wettstein & Grouhel, 1996). In contrast, work with GNTI has shown that it is both more specific for

kappa opioid receptors and its action is shorter lasting than that of nor-BNI. GNTI is significantly more effective in reducing kappa opioid agonist-induced feeding in rats than nor-BNI and this effect is gone after only 24 hours (Jewett et al., 2001). The shorter lasting effects of GNTI make it especially attractive in tests for the pharmacological mechanisms of ethanol's reinforcing properties. The results of Experiment 2 demonstrate that GNTI is as effective as nor-BNI in disrupting the reinforcing properties of ethanol in infant rats. It seems advantageous to use GNTI in future studies involving kappa opioid manipulations.

One possible explanation for the results from Experiment 1 and 2 is that endogenous activity at opioid receptors plays a substantial role in learning per se. Previous work has shown that both CTOP and nor-BNI also disrupt the appetitive olfactory conditioning seen when saccharin was used as an US in infant rats (Nizhnikov et al., 2006). If mechanisms of learning are disrupted by opioid antagonists, this would indicate a developmental discontinuity, since in adults opioid antagonists tend to facilitate learning (Aloyo et al., 1993; Cerro & Borrell, 1990; Cicala et al., 1990; Hernandez et al., 1983; Schulteis & Martinez Jr., 1990; Westbrook et al., 1991). However, this would not be unprecedented since there is some experimental evidence suggesting that the kappa opioid system mediates appetitive reinforcement (Petrov et al., 2006) in early infancy, whereas pharmacological activation of the kappa opioid receptors is aversive in adulthood (Bals-Kubik et al., 1993, 1989; Bechara & Van Der Kooy, 1987; Mori et al., 2004; Mucha & Herz, 1985; Shippenberg & Herz, 1986).

The results of Experiment 3 suggest that learning per se is not disrupted by a mu opioid antagonist. Animals given an oral infusion of a bitter tastant (0.1% quinine) in the presence of lemon odor displayed a robust and significant aversion to that odor when tested one hour later. Pre-treatment with CTOP had no effect on olfactory aversive conditioning at this age. These results show that endogenous activity at mu opioid receptors does not play a sufficient role in aversive learning to at least exteroceptive cues early in ontogeny. It should be noted that the US's used in Experiments 1 and 2 are different from that used in Experiment 3. Specifically, IC injections of ethanol act directly on central receptors while quinine is a taste stimulus delivered orally. Nevertheless, the process of aversive learning is commonly accepted to involve central mechanisms. Therefore, it seems reasonable to at least suggest that learning altogether is not blocked.

It is possible that appetitive learning generally is disrupted by opioid antagonists. However, previous work has shown this is most likely not the case. Rat pups find kappa opioid agonists extremely appetitive. When a central injection of dynorphin A(1-13) is paired with lemon odor rats show a distinct preference for that odor an hour later compared to unpaired controls. Injection of CTOP prior to the conditioning session does not disrupt this appetitive learning (Petrov et al., 2006). These findings suggest that the activation of kappa opioid receptors was not prevented by CTOP and that not all forms of appetitive learning are disrupted by opioid antagonists. Therefore, it is unlikely that CTOP, nor-BNI, or GNTI disrupt all appetitive learning, but rather are specific to those stimuli invoking either mu or kappa opioid receptors in the conditioning process. It is likely that ethanol is an unconditioned stimulus that requires activation of mu and kappa opioid receptors in order to transfer its appetitive properties onto a CS (in this case lemon odor).

The actual mechanisms behind ethanol's reinforcing effects are still not fully explored. One possibility is that acetaldehyde, derived from the metabolism of ethanol by catalase in the brain, is the critical element that drives the drugs reinforcing properties. The inactivation of acetaldehyde has been shown to disrupt ethanol reinforcement (Font et al., 2006). Furthermore, disruption of ethanol metabolism via catalase blockers has also been shown to disrupt the reinforcing properties of ethanol as well as exacerbate its aversive effects in rats (Nizhnikov et al., 2007; Aragon and Amit, 1992; Quertemont et al., 2003). In turn acetaldehyde or one of its condensates (e.g. salsolinol) may activate the endogenous opioid system and the opioid antagonists employed in this study may be disrupting this cascade.

In conclusion, the present studies demonstrate that mu and kappa opioid antagonists disrupt central processing of ethanol reinforcement. Furthermore, GNTI is as effective as nor-BNI in disrupting ethanol's positive reinforcing effects and has special advantages for future studies of ethanol reinforcement. Finally, Experiment 3 showed that mu opioid antagonists do not disrupt all forms of learning in infant rats, since conditioned aversion was not affected by CTOP.

References

Aloyo VJ, Romano AG, & Harvey JA (1993). Evidence for an involvement of the μ-type of opioid receptor in the modulation of learning. *Neuroscience, 55*, 511-519.

Aragon, C. M., and Amit, Z. (1992). The effect of 3-amino-1,2,4-triazole on voluntary ethanol consumption: Evidence for catalse involvement in the mechanism of action. *Neuropharmacology. 31*, 709-712.

Bals-Kubik R, Ableitner A, Herz A, & Shippenberg TS (1993). Neuroanatomical sites mediating the motivational effects of opioids as mapped by the conditioned place preference paradigm in rats. *J Pharmacol Exp Ther., 264*, 489-495.

Bals-Kubik R, Herz A, & Shippenberg TS (1989). Evidence that the aversive effects of opioid antagonists and kappa-agonists are centrally mediated. *Psychopharmacology*, 98, 203-206.

Barr GA, Wang S & Carden, S (1994). Aversive properties of the k opioid agonist U50,488 in the week-old rat pup. *Psychophamacology, 113*, 422-428.

Bechara A, & Van der Kooy D, (1987). Kappa receptors mediate the peripheral aversive effects of opiates. *Pharmacol Biochem Behav., 28*, 227-233.

Boyadjieva NI, Sarkar DK (1997). Effects of ethanol on basal and prostaglandin E1-induced increases in beta-endorphin release and intracellular camp levels in hypothalamic cells. *Alcohol Clin Exp Res, 21*, 1005-1009.

Broadbear JH, Negus SS, Butelman ER, de Costa BR, Woods JH (1994). Differential effects of systemically administered nor-binal-trophimine (nor-BNI) on K-opioid agonists in the mouse writing assay. *Psychopharmacology, 115*, 311-319.

Cerro SD & Borrell J (1990). Dynorphin 1-17 can enhance or impair retention of an inhibitory avoidance response in rats. *Life Sci., 47*, 1453-1462.

Chavkin C, James IF, Goldstein A (1982). Dynorphin is a specific endogenous ligand of the κ opioid receptor. *Science, 215*, 413-415.

Cheslock SJ, Varlinskaya EI, Petrov ES, Silveri MM, Spear LP, Spear NE (2001). Ethanol as a reinforcer in the newborn's first suckling experience. *Alcohol Clin Exp Res., 25*, 391-402.

Cheslock SJ, Varlinskaya EI, Petrov ES, Spear NE (2000). Rapid and robust olfactory conditioning with milk before suckling experience: promotion of nipple attachment in the newborn rat. *Behav Neurosci, 114*, 484-495.

Cheslock SJ, Sanders SK, & Spear NE (2004). Learning during the newborn's first meal: special resistance to retroactive interference. *Dev Sci., 7*, 581- 598.

Cicala GA, Azorlosa JL, Estall LB, & Grant SJ (1990). Endogenous opioids interfere with Pavlovian second-order fear conditioning. *Psychological Science, 1*, 312-315.

Davidson D & Amit Z (1997). Naltrexone blocks acquisition of voluntary ethanol in take in rats. *Alcohol Clin Exp Res., 21*, 677-683.

De Waele JP, Gianoulakis C (1993) Effect of single and repeated exposure to ethanol on the hypothalamic ß-endorphin release by the C57BL/6 and DBA/2 strains of mice. *Neuroendocrinology, 57*, 700-709.

De Waele JP, Papachristou ND, Gianoulakis C (1992) The alcohol-preferring C57BL/6 mice present an enhanced sensitivity of the hypothalamic ß-endorphin system to ethanol than the alcohol-avoiding mice. *J Pharmacol Exp Ther, 261*, 788-794.

DeWitte P (1984). Naloxone reduces alcohol intake in a free choice procedure even when both drinking bottles contain saccharin sodium or quinine substances. *Neuropsychobiology, 12*, 73-77.

Eilam D & Smotherman WP (1998). How the neonatal rat gets to the nipple: common motor modules and their involvement in the expression of early motor behavior. *Dev Psychobio, 32*, 57-66.

Endoh T, Matsuura H, Tanaka C, Nagase H (1992). Nor-binaltrophimine: a potent and selective kappa-opioid receptor antagonist with long-lasting activity in vivo. *Arch Int Pharmacodyn Ther., 316*, 30-42.

Fadda P, Tronci S, Colombo G, Fratta W (1999) Differences in the opioid system in selected brain regions on alcohol-preferring and alcohol-nonpreferring rats. *Alcohol Clin Exp Res., 23*, 1296-1305.

Fanselow MS, Calcagnetti DJ & Helmstetter FJ (1989). Modulation of appetitively and aversively motivated behavior by the kappa opioid antagonist MR2266. *Beh. Neuro., 103*, 663-672.

Froehlich JC (1993). Interactions between alcohol and the endogenous opioid system. In Zakhari, S. (Ed*.), Alcohol and the endocrine system* (pp. 31-35). NIAAA Research Monograph 23, Bethesda, MD.

Froehlich JC, Harts J, Lumeng L & Li TK (1990). Naloxone attenuates voluntary ethanol intake in rats selectively bred for high ethanol preference. *Pharmacol Biochem Behav., 35*, 385-390.

Gatto GJ, McBride WJ, Murphy JM, Lumeng L & Li TK (1994). "Ethanol self-infusion into the ventral tegmental area by alcohol-preferring rats." *Alcohol, 11*, 557-564.

Gianoulakis C (1996). Implications of endogenous opioids and dopamine in alcoholism: Human and basic science studies. *Alcohol Alcohol., 31*, 33-42.

Hernandez LL & Powell DA (1983). Naloxone induces multiple effects on aversive Pavlovian conditioning in rabbits. *Behav Neurosci., 97*, 478-491.

Herz A (1997). Endogenous opioid systems and alcohol addiction. *Psychopharmacology, 129*, 99-111.

Holson RR, Pearce B (1992). Principals and pitfalls in the analysis of prenatal treatment effects in multiparous species. *Neurotoxicol Teratol, 14*, 221-228.

Hubbell CL, Czirr SA, Hunter GA, Beaman CM, LeCann NC & Reid LD (1986). Consumption of ethanol solution is potentiated by morphine and attenuated by naloxone persistently across repeated daily

administration. *Alcohol, 3*, 39-54.

Hyytiä P, Kiianmaa K (2001). Suppression of ethanol responding by centrally administered CTOP and naltrindole in AA and Wistar rats. *Alcohol Clin Exp Res, 25*, 33.

Hyytia P (1993). Involvement of mu opioid receptors in alcohol drinking by alcohol-preferring AA rats. *Pharmacol Biochem Behav.*, *45*, 697-701.

Jamensky NT, Gianoulakis C (1997). Content of dynorphins and kappa-opioid receptors in distinct brain regions of C57BL/6 and DBA/2 mice. *Alcohol Clin Exp Res., 21*, 1455-1464.

Jewett DC, Cleary J, Levine AS, Schaal DW, Thompson T (1995). Effects of neuropeptide Y, insuline, 2-deoxyglucose, and food deprivation on food-motivated behavior. *Psychopharmacology, 120*, 267-271.

Jewett DC, Grace MK, Jones RM, Billington CJ, Portoghese PS, Levine AS (2001). The kappa-opioid antagonist GNTI reduces U50,488-, DAMGO-, and deprivation-induced feeding, but not butorphanol- and neuropeptide Y-induced feeding in rats. *Brain Res.,* 909, 75-80.

Jewette DC, Grace M, Billington CJ, Levine AS (1997). Duration of the anorectic action of norbinaltrophimine on neuropeptide-Y induced feeding in rats. Soc. *Neurosci. Abstr.*, 23, 1346.

Krishnan-Sarin S, Jing SL, Kurtz DL, Zweifel M, Portoghese PS, Li TK & Froehlich JC (1995a). The delta opioid receptor antagonist naltrindole attenuates both alcohol and saccharin intake in rats selectively bred for alcohol preference. *Psychopharmacology, 120*, 177-185.

Krishnan-Sarin S, Portoghese PS, Li TK, & Froehlich JC (1995b). The delta$_2$-opioid receptor antagonist naltriben selectively attenuates alcohol intake in rats bred for alcohol preference. *Pharmacol Biochem Behav.*, *52*, 153-159.

Kuzmin A, Sandin J, Terenius L & Ogren SO (2003). Acquisition, expression, and reinstatement of ethanol-induced conditioned place preference in mice: Effects of opioid receptor-like 1 receptor agonists and naloxone. J *Pharmacol Exp Ther. 304*, 310-318.

Lindholm S, Werme M, Brene S, Franck J (2001) The selective kappa-opioid receptor agonist U50,488H attenuates voluntary ethanol intake in the rat. *Behav Brain Res., 120,* 137-146.

Marfaing-Jallat P, Miceli C, LeMegnen J (1983) Decrease in ethanol consumption by naloxone in naive and dependent rats. *Pharmacol Biochem Behav 18,* 537-539.

Matsuzawa S, Suzuki T, Misawa M, Nagase H (1999) Different roles of μ, δ- and κ-opioid receptors in ethanol-associated place preference in rats exposed to conditioned fear stress. *Europ J Pharmacol 368*, 9-16.

Matsuzawa S, Suzuki T, & Misawa M (2000). Involvement of mu-opioid receptor in the salsolinol-associated place preference in rats exposed to conditioned fear stress. *Alcohol Clin Exp Res., 24*, 366-372.

Matsuzawa S, Suzuki T, Misawa M, & Nagase H (1998). Involvement of mu- and delta-opioid receptors in the ethanol-associated place preference in rats exposed to foot shock stress. *Brain Res., 803*, 169-177.

Mitchell JM, Liang MT, Fields HL (2005) A single injection of the kappa opioid antagonist norbinaltorphimine increases ethanol consumption in rats. *Psychopharmacology 182*, 384-392.

Mori T, Nomura M, Yoshizawa K, Nagase H, Narita M, & Suzuki T (2004). Differential properties between TRK-820 and U-50,488H on the discriminative stimulus effects in rats. *Life Scie., 75*, 2473-2482.

Mucha RF & Herz A (1985). Motivational properties of kappa and mu opioid receptor agonists studied with place and taste preference conditioning. *Psychopharmacology, 86*, 274-280.

National Institutes of Health (1986). Guide for the Care and Use of Laboratory Animals (DHEW Publication No. 86-23). US Government Printing Office, Washington, DC.

Nestby P, Schoffelmeer ANM, Homberg JR, Wardeh G, De Vries TJ, Mulder AH, Vanderschuren LJMJ (1999). Bremazocine reduces unrestricted free-choice ethanol self-administration in rats without affecting sucrose preference. *Psychopharmacology 142*, 309-317.

Nizhnikov ME, Varlinskaya, EI, Petrov, ES, and Spear, NE (2006). Reinforcing properties of ethanol in neonatal rats: Involvement of the opioid system. *Behav. Neurosci. 120,* 267-280.

Nizhnikov, M.E., Petrov, E.S., Varlinskaya, E.I., & Spear, N.E. (2002). Newborn first suckling experience: Taste differentiation and suckling plasticity. *Physiol Behav., 76,* 181-198.

Nizhnikov, M.E., Petrov, E.S. & Spear, N.E. (2002). Olfactory aversive conditioning in the newborn (3-hr-old) rat impairs later suckling for water and milk. *J Exp Psychol Anim Behav Process., 28*, 277-283.

Nizhnikov, M.E., Molina, J.C., & Spear, N.E. (2007). Central reinforcing effects of ethanol are blocked by catalase inhibition. *Alcohol, 41,* 525-534.

Olive MF, Koenig NH, Nannini MA, Hodge CW (2001). Stimulation of endorphin neurotransmission in the nucleus accumbens by ethanol, cocaine and amphetamine. *J Neurosci 21,* RC184 (1-5).

Pautassi RM, Godoy JC, Spear NE, Molina JC (2002). Early responsiveness to stimuli paired with different stages within the state of alcohol intoxication. *Alcohol Clin Exp Res., 26*, 644-654.

Pellis SM, Pellis VC, & Teitelbaum P (1991). Air righting without the cervical righting reflex in adult rats. *Beh Brain Res., 45,* 185-188.

Petrov ES, Nizhnikov ME, Varlinskaya EI, Spear NE (2006) Dynorphin A (1–13) and responsiveness of the newborn rat to a surrogate nipple: Immediate behavioral consequences and reinforcement effects in conditioning. *Beh Brain Res., 170*, 1-14.

Petrov ES, Varlinskaya EI, Spear NE (2001) Self-administration of ethanol and saccharin in newborn rats: Effects on suckling plasticity. *Behav Neurosci 115,* 1318-1331.

Petrov ES, Varlinskaya EI, & Smotherman WP (1997). The newborn rat ingests fluids through a surrogate nipple: a new technique for the study of early suckling behavior. *Physiol Behav., 62*, 1155-1158.

Petrov ES, Varlinskaya EI & Smotherman WP (1998). Endogenous opioids in the first suckling episode in the rat. *Dev Psychobiol., 33,* 175-183.

Picker MJ, Methewson C, Allen RM (1996). Opioids and rate of positively reinforced behavior. III. Antagonism by the long-lasting kappa antagonist norbinaltrophimine. *Behav. Pharmacol. 7,* 495-504.

Quertemont, E., Escarabajal, M.D., and De Witt, P. (2003). Role of catalase in ethanol-induced conditioned taste aversion: A study with 3-amino-1,2,4-triazole. *Drug. Alcohol. Depend. 70,* 77-83.

Rasmussen DD, Bryant CA, Boldt BM, Colasurdo EA, Levin N, Wilkinson CW (1998). Acute alcohol effects on opiomelanocortinergic regulation. *Alcohol Clin Exp Res 22,* 789-801.

Rodd ZA, Bell RL, Melendez RI, Kuc KA, Lumeng L, Li TK, Murphy JM & McBride WJ (2004b). Comparison of intracranial self-administration of ethanol within the posterior ventral tegmental area between alcohol-preferring and Wistar rats. *Alcohol Clin Exp Res., 28,* 1212-1219.

Rodd ZA, McKinzie DL, Crile RS, Murphy JM & McBride WJ (2000). Regional heterogeneity for the intracranial self-administration of ethanol within the ventral tegmental area of female Wistar rats. *Psychopharmacology 149,* 217-224.

Rodd ZA, Melendez RI, Bell RL, Kuc KA, Zhang Y, Murphy JM & McBride WJ (2004a). Intracranial self-administration of ethanol within the ventral tegmental area of male wistar rats: Evidence for involvement of dopamine neurons. *The J of Neurosci., 24*, 1050-2057.

Sandi C, Borrell J, Guaza C (1988). Kappa-opioid receptors and relapse-like drinking in long-term ethanol-experienced rats. *Psychopharmacology, 153*, 93-102.

Schulteis G & Martinez Jr JL (1990). ICI 174,864, a selective delta opioid antagonist, reverses the learning impairment produced by [leu]enkephalin. *Psychopharmacology, 100*, 102-109.

Shippenberg TS & Herz A (1986). Differential effects of mu and kappa opioid systems on motivational processes. *NIDA research monograph, 75*, 563-566.

Shippenberg TS & Herz A (1987). Motivational properties of opioids. *Pol. J. Pharmacol. Pharm., 39*, 577-583.

Smotherman WP, Petrov ES, Varlinskaya EI (1997) Experimental study of the first suckling episode: Rat pups ingest fluids through the surrogate nipple. *Behav Neurosci 111,* 1383-1394.

Ulm RR, Volpicelli JR & Volpicelli LA (1995). Opiates and alcohol self-administration in animals. *J. Clin. Psychiatry,* 56, 5-14.

Varlinskaya EI, Petrov ES, Cheslock SJ, Spear NE (1999). A new model of ethanol self-administration in newborn rats: Gender effects on ethanol ingestion through a surrogate nipple. *Alcohol Clin Exp Res 23*, 1368-1376.

Varlinskaya, E. I., Petrov, E. S., & Smotherman, W. P. (1996). Classical conditioning in the fetal rat: Reinforcing properties of dynorphin A (1-13). *Behav Neurosci., 110,* 154-167.

Westbrook RF, Greeley JD, Nabke CP, & Swinbourne AL (1991). Aversive conditioning in the rat: Effects of a benzodiazepine and of an opioid agonist and antagonist on conditioned hypoalgesia and fear. *J of Experi Psych., 17,* 219-230.

Wettstein JG, Grouhel A. (1996). Opioid antagonist profile of SC nor-binaltorphimine in the formalin paw assay. *Pharmacol. Biochem. Behav. 53,* 411-6.