Published in final edited form as:

Behav Brain Res. 2014 March 1; 260: 119–130. doi:10.1016/j.bbr.2013.11.043.

Central effects of ethanol interact with endogenous mu opioid activity to control isolation-induced analgesia in maternally separated infant rats

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Abstract

Endogenous opioid activity plays an important role in ethanol consumption and reinforcement in infant rats. Opioid systems are also involved in mediation and regulation of stress responses. Social isolation is a stressful experience for preweanling rats and changes the effects of ethanol through opioid-dependent mechanisms. The present study assessed effects of intracisternal (i.c.) administration of a selective mu-opioid antagonist (CTOP) and i.p. administration of a nonspecific opioid antagonist (naloxone) on voluntary intake and behavior in socially isolated 12-day-old (P12) pups treated with 0.5 g/kg ethanol. Voluntary intake of 0.1% saccharin or water, locomotion, rearing activity, paw licking and grooming were assessed during short-term isolation from littermates (STSI; 8-min duration). Thermal nociceptive reactivity was measured before and after this intake test, with normalized differences between pre- and post-test latencies of paw withdrawal from a hot plate (49°C) used as an index of isolation-induced analgesia (IIA).

Results indicated several effects of social isolation and ethanol mediated through the mu-opioid system. Effects of low dose ethanol (0.5 g/kg) and voluntary consumption of saccharin interacted with endogenous mu-opioid activity associated with STSI. Blockade of mu-opioid receptors on saccharin consumption and paw licking-grooming affected intoxicated animals. Low dose ethanol and ingestion of saccharin blunted effects of CTOP on rearing behavior and nociceptive reactivity. Central injections of CTOP stimulated paw licking and grooming dependent on ethanol dose and type of fluid ingested. Ethanol selectively increased saccharin intake during STSI in females, naloxone and CTOP blocked ethanol-mediated enhancement of saccharin intake. We suggest that enhancement of saccharin intake by ethanol during STSI is the product of synergism between isolation-induced mu- opioid activity that increases the pup's sensitivity to appetitive taste stimulation and the anxiolytic effects of 0.5 g/kg ethanol that decreases behaviors otherwise competing with independent ingestive activity.

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Keywords

ethanol; social isolation; stress; rats; infant; mu-opioid; CTOP; ingestive behavior

Introduction

Stress is an important factor mediating sensitivity to ethanol's effects as well as in the development of alcohol addiction (Aragon et al., 1990; Heilig and Koob, 2007; Koob, 2008; Uhart and Wand, 2009; Becker et al., 2011). Although the association between alcohol use and stressful events has been extensively investigated in adult humans (Brady and Sonne, 1999; Sayette, 1999; Uhart and Wand, 2009) and laboratory animals (Lynch et al., 1999; Blakley and Pohorecky, 2006; Boyce-Rustay et al., 2007; Chester et al., 2008; Pohorecky, 2008; Becker et al., 2011), the effects of stressors on development of preference for (or avoidance of) ethanol during ontogeny has not been as extensively studied. Several rodent and primate models of early stress-induced trauma have been used to analyze effects of early adverse experience on development of drug abuse, including preference for ethanol and heightened ethanol intake during infancy and adolescence (Sanchez et al., 2001; Andersen and Teicher, 2009; Pautassi et al., 2009; Wiedenmayer, 2009; Varlinskaya et al., 2010; Becker et al., 2011).

Social isolation represents a natural stressful experience for the preweanling rat. In infant rats exposure to ethanol can aggravate or ameliorate reactions to social isolation, with ethanol's effects dependent on dose of ethanol, duration of social isolation, age of the animal and type of behavioral activity expressed during isolation (Arias et al., 2010; Kozlov et al., 2012b; Pautassi et al., 2012b). Recently we found that in 12-day old rat pups the effects of 0.5 g/kg ethanol on ingestion of a palatable fluid emerged only in pups separated from their littermates for 8 minutes (short-term social isolation, STSI) (Kozlov et al., 2012b). Low doses of ethanol ameliorated isolation-induced analgesia, but ethanol-mediated effects were age-dependent suggesting an association between developmental changes in stress reactivity and sensitivity to ethanol.

A substantial body of literature demonstrates involvement of endogenous opioid activity in ethanol consumption and reinforcement in adult as well as in infant rats (Herz, 1997; Matsuzawa et al., 1999; Cichelli and Lewis, 2002; Zhang and Kelley, 2002; Nizhnikov et al., 2006; Arias et al., 2009b; Nizhnikov et al., 2009; Sperling et al., 2010; Trigo et al., 2010; Pautassi et al., 2012a). Several lines of neuropharmacological and behavioral evidence also support the involvement of endogenous opioid systems in the immediate response to stress as well as in development of adaptation to stressful events (Girardot and Holloway, 1984; Aragon et al., 1990; Yamada and Nabeshima, 1995; Hebb et al., 2005; Land et al., 2008; Butler and Finn, 2009; Bowers et al., 2012). During early ontogeny endogenous opioid activity has been suggested to affect some behavioral activities associated with social isolation in infant rats (Kehoe and Blass, 1986a; Kehoe and Boylan, 1994; Carden et al., 1996). Developmental studies have, however, revealed a complex mode of opioid involvement in the infant's reaction to social isolation, novelty, and threat (Kehoe and Blass, 1986b; Kavaliers and Innes, 1988; Winslow and Insel, 1991; Kehoe and Boylan, 1994; Nazarian et al., 1999; Shair et al., 2005).

Recently we found that exposure to saccharin ameliorated, to some extent, the stress reaction to social isolation (Kozlov et al., 2012b). Given opioid involvement in modulation of palatability and the hedonic effects of sweet taste (Doyle et al., 1993; Berridge, 2000; Ferraro et al., 2002), we suggest an interaction between opioid-mediated changes in saccharin intake and expression of isolation-induced analgesia that also seems dependent on

mu-opioid activity. Consequently, some behavioral effects of CTOP and ethanol on pups exposed to a palatable fluid during STSI could represent a product of competition between an ingestive reaction to appetitive taste stimulation and aversive (or anxiety) response to isolation. To dissect “pure” effects of blockade of mu opioid receptors (MOR) on stress reactivity from those coupled with opioid-mediated effects in ingestive behavior we exposed animals to two different fluids during STSI, water or 0.1% saccharin. Because intraoral delivery of water is neutral or even aversive for infant rats (Pautassi et al., 2009), we expected that under water exposure pups would express a relatively “pure” stress reaction to STSI compared to more complex appetitive-aversive behavior during exposure to saccharin.

Although in adult rats transport of CTOP through the blood brain barrier is low (Van Dorpe et al., 2010), relatively high cerebrospinal –fluid-plasma concentration ratios for macromolecules in cerebrospinal fluid and blood plasma have been demonstrated in the developing brain (Birge et al., 1974; Habgood et al., 1993; Saunders et al., 2008). This suggests that small molecules like CTOP would likely penetrate the brain of the preweanling rat. In our previous study we administered CTOP systemically because this route of administration seemed to require less stressful intervention than intracerebral injection and so would be less likely to interfere with the habituation process during LTISI.

There were two main aims for the present set of Experiments. 1) Given that our previous work has shown peripheral (intraperitoneal injection) CTOP mediates intake of saccharin and analgesia caused by social isolation, the question arose whether entral administration of CTOP would result in similar effects. The answer would allow a more informed consideration of the site of action for these opioid effects. 2) to delineate the role of endogenous mu-opioid activity in ethanol-mediated modulation of intake of a palatable fluid (0.1% saccharin) and STSI induced analgesia.

2. Materials and methods

2.1. Subjects

392 12-day-old Sprague-Dawley rats born and reared in the vivarium at the Center for Development and Behavioral Neuroscience, Binghamton University, were used in the study. The subjects per treatment were $n = 10$. However 8 subjects did not meet criteria (as listed below) and were removed from the study. Pregnant female rats were housed individually in plastic maternity cages (47 cm long x 20 cm high x 36 cm wide) containing pine shavings as bedding material. They were checked for birth daily between 8.00 and 10.00 a.m., and the day of birth was considered as postnatal day zero (P0). Litters were culled to 10 pups, with an equal sex ratio whenever possible. All animals were housed in a temperature controlled (22°C) vivarium maintained on a 12/12-hr light/dark illumination cycle (lights on at 7.00 a.m.), with ad libitum access to food (Purina Rat Chow, Lowell, MA) and water. In all respects, maintenance and treatment of the animals were in accord with guidelines for animal care established by the National Institutes of Health, using protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

2.2. Apparatus and procedures

After being removed from the dam, pups were cannulated and kept together with littermates ($n = 9-10$ per group) in warmed (30–32°C) holding boxes for 4.5–5 hours until testing. The holding boxes (size 18 x 18 cm) were filled with a mixture (1:1) of fresh and home cage shavings. The experimental protocol of this study included a single period of short-term isolation (STSI) from siblings during which animals were exposed to infused fluids (0.1% saccharin or water). STSI started after 4.5–5.0 hours of separation from the dam and lasted 8 minutes. This interval of isolation was selected based on our previous findings that it is

roughly the minimum time of isolation needed for conventional tests that assess behavioral change due to ethanol. Pups were exposed to fluids through intraoral cheek cannula. The intraoral cannula, consisting of polyethylene tubing (PE10) with flanged tip, was inserted into the anterior portion of the mouth through the cheek 2–3 mm caudal to the mystacial pad as described elsewhere (Kozlov et al., 2008). The cannulation procedure was accomplished within 8–12 s with no bleeding. The intake test (combined with STSI) occurred in a plastic circular arena with transparent walls (diameter 12 cm, wall height 8 cm). Absorbent paper covered the plastic floor of the arena. The floor of the test arena was heated to 31°C and this temperature was maintained ($\pm 0.2^\circ\text{C}$) throughout the experiment using a temperature controller (FHC, Inc. Bowdoinham, ME), ambient room temperature was kept at 22–24°C. Between tests the wall of arena was cleaned using Kimtech wipes (Kimberly-Clark, Roswell, GA) soaked in warm water and the floor as well as walls was dried between trials with a stream of fresh air from a pressurized can. If urination or fluid spills appeared on the arena floor, the paper was changed or the whole arena was replaced.

During the intake test, fluids (0.1% saccharin and water) were freely accessible through the intraoral cannula and intake of these fluids was measured using a custom-made flow tracker. The fluid flow tracking technique was described in detail elsewhere (Kozlov et al., 2008; Kozlov et al., 2012b). This technique used a photocell system that tracked light through a translucent tube in terms of the border between filled and unfilled parts of the tube. An output signal from a photo sensor controlled a stepper motor that rotated the wheel and moved the tube through the photocell assembly to track the fluid level in the tubing. When the pup ingested fluid from the cannula, the fluid moved down the tube. The number of steps the motor performed pulling the tube through the photocell assembly in order to locate the fluid edge against the light beam was directly proportional to the amount of fluid consumed. The resolution of this measurement was about $0.20\ \mu\text{l}/\text{step}$. The data was sampled at a rate of 10 Hz were transferred to a personal computer via a RS232 port.

A hot plate Analgesia Meter (Model 39L, IITC Life Science, Woodland Hills, CA) was used to assess nociceptive thermal reactivity. Basically, tests consisted of placing a forepaw on the hot surface (temperature – 49.5°C) and measuring latency of paw withdrawal after contact. More specifically, the pup's right forelimb, two hind limbs, and trunk were gently yet firmly supported in the experimenter's right hand, allowing the animal's left forelimb to rest lightly on the hot plate surface. Establishing this contact triggered the electronic clock that was stopped with paw removal. Set-off time was 30 seconds. Prior to measurement of baseline nociceptive reactivity, pups were briefly checked for arousal level: they were placed on a paper towel and allowed to move for 5–10 sec. Subjects who were immobile or demonstrated baseline latency of paw withdrawal longer than 20 seconds were discarded from the experiment. Paw withdrawal latencies were measured 8 minutes before and immediately after the intake test. A two-trial measurement procedure (pre- and post-test) was adopted to assess changes in nociceptive reactivity caused by short-term interventions (isolation from peers, drug injections, type of fluid available) separately from long-term effects of maternal separation.

During the intake test behavior was monitored continuously using a CCD camera (Model AF-X8, Panasonic, Secaucus, NJ), positioned above the arena. The data acquisition module included a video overlay unit that provided simultaneous display of video images, flow counts, and markers of behavioral events. Video signal along with fluid flow counts, marks of behavioral events, and time marks produced by the flow tracker device were recorded on DVD (Liteon LVW-5001 recorder, Milpitas, CA) for subsequent offline processing. The video data from some experiments were also analyzed using video tracking software (Viewer, BIOBSERVE GmbH, Bonn, Germany).

For a full depiction of the procedural time-line see Figure 1.

2.3. Drugs administration

Pups were administered either saline or 0.5 g/kg ethanol intraperitoneal (i.p.) as a 12.6% (v/v) solution in physiological saline. Injections were delivered immediately after first measurement of hot plate analgesia and 6–7 minutes prior to the intake test.

CTOP was administered into the cisterna magna (intracisternally- i.c.) using a 30-gauge hypodermic needle attached to a length of transparent polyethylene tubing (PE-10). Successful placement of the needle into the cisterna magna was confirmed by the appearance of cerebrospinal fluid in the tubing. Doses of 1 or 10 μ g CTOP were diluted in physiological saline and infused in a standard volume of 1.5 μ l using a rotary micro pump (Kashinsky et al., 1990). Duration of infusion was 6 seconds and pups were held for additional 6–8 seconds after removal of the needle from insertion site. The doses of CTOP were selected according to data from our previous work that used intracisternal CTOP administrations (Nizhnikov et al., 2006). The nonspecific opioid antagonist naloxone (Sigma-Aldrich, St Louis, MO) was injected in a dose 4 mg/kg i.p., delivered as 0.4 mg/ml solution in a volume 0.1 ml/g of animal weight. This relatively high dose of naloxone (4 mg /kg) was used to provide blockade of both mu- and kappa-opioid receptors. CTOP was infused 15–16 minutes prior to the intake test and naloxone was injected 25 minutes before this test. After drug administration all animals were returned to their holding conditions according to the given experimental protocol.

2.4. Data analysis

To avoid confounding between litter and treatment effects, no more than two subjects per litter (one male and one female) were assigned to the same treatment group. The order of testing for the different treatment groups was counterbalanced across the experiment.

Intake of fluids (0.1% saccharin or water) during intake test was expressed as per-minute mean rate of flow through the intraoral cannula. Behavioral activity was scored manually by experimenters blind to treatment condition. Behavioral scores were recorded using a flow tracking device and were stored in one file with flow data. Randomly selected video records and data files obtained after processing with video tracking software were compared with corresponding samples of behavioral scores to verify accuracy of manual scoring. The behavioral analysis included the following categories of behavioral activities: locomotion and exploration combined; rearing activity - wall climbing and head lifting combined; paw licking and grooming combined. Grooming was mostly represented by forepaw movements aimed to the face. Frequently this activity started as forepaw licking and then was transformed to “face wiping”. Cheek scratching that sometimes followed grooming was scored as grooming if duration of uninterrupted scratching series did not exceed 10 seconds or the animal returned to grooming activity after scratching. If the animal demonstrated uninterrupted excessive scratching for 30 seconds or longer it was discarded from the analysis.

Nociceptive reactivity was assessed using two indexes: 1) pretest (baseline) latency of paw withdrawal from hot plate; 2) normalized difference between pre- and post- test latency (LT) of paw withdrawal expressed as maximum possible effect: $MPE = (posttest\ LT - pretest\ LT) / (set-off\ time - pretest\ LT)$. Positive values of normalized difference of paw withdrawal latency indicated isolation induced analgesia (IIA). We use the expressions “positive difference of paw withdrawal latency” and “magnitude of IIA” as interchangeable terms.

Because previous experiments (Kozlov et al., 2009, 2012b) indicated that effects of ethanol in P12 rat were sex-dependent, gender was included in all experimental designs. With three

doses of i.c. CTOP and two doses of i.p. naloxone, the ANOVA was represented by four between-group factors. These were ethanol dose (saline; 0.5 g/kg), drug treatment (i.c. saline ; 1; 10 μ g i.c. CTOP; i.p. saline ; 4 mg/kg i.p. naloxone), fluid (0.1% saccharin; water), and sex (males, females). Data were processed using Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA) or Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA, USA). All behavioral scores were converted to percent of time spent in particular activity and were arc-sin transformed before being submitted to ANOVA. When ANOVA revealed significant main effects or interactions, planned comparisons were performed. Planned comparisons included assessment of means of all dose groups against a saline control or / and pair-wise comparisons within each level of drug treatment. For multiple comparisons the alpha level was corrected using the Bonferroni adjustment. A mixed between-within ANOVA with successive one-min time intervals treated as a repeated measure was employed for temporal analysis of flow intake. Whenever a significant effect of repeated measures was found or significant effects of within-between interaction were revealed, a linear regression analysis (Graph Pad Prism 5.0, Graph Pad Software, Inc., La Jolla, CA 92037 USA) was applied to estimate fitting parameters for time series and compare them between treatments. For estimation of effect size we also computed Cohen's d , defined as difference between the means, ($M_1 - M_2$), divided by pooled standard deviation. Unless otherwise specified population estimates are presented in the form of mean $\pm$ S.E.M.

Lastly, it is critical to note that the same animal was used for all measures described. That is, intake, locomotor activity, grooming, etc. were all recorded for each animal and analyzed using ANOVA. Furthermore, all animals were isolated from their dams for 4.5 – 5 hours.

3. Results

3.1. Intake

A 2(sex) x 2(ethanol dose) x 2(fluid) x 5(drug treatment) ANOVA revealed significant main effects of fluid, $F(1, 352)=70.12$, $p<0.001$, sex, $F(1, 352)=5.65$, $p=0.018$, and drug treatment, $F(4, 352)=2.71$, $p=0.03$, as well as fluid x drug treatment, $F(4, 352)=2.96$, $p=0.02$, and sex x ethanol dose x fluid x drug treatment, $F(4, 352)=2.82$, $p=0.025$ interactions.

Ethanol (0.5 g/kg) administration increased intake of saccharin but did not change water intake in subjects not treated with opioid antagonists (Fig. 2). However, as we have found before (Kozlov et al., 2009), the effect of ethanol was significant only in females given no CTOP (CTOP zero dose), $p=0.001$, Cohen's effect size $d=1.34$. No increase of intake was observed in ethanol-intoxicated males either injected with i.p. saline or infused with i.c. saline. Planned comparisons indicated that i.c. administration of 1 and 10 μ g CTOP as well as i.p. injection of 4 mg/kg naloxone significantly decreased the rate of saccharin consumption in females treated with 0.5 g/kg ethanol in comparison to their saline counterparts, $p<0.001$, $d=1.77$, $p=0.001$, $d=1.59$, and $p<0.001$, $d=1.86$, respectively (Fig. 2).

3.2. Locomotor activity

An ANOVA for locomotion indicated significant main effects of fluid, $F(1, 352)=7.11$, $p=0.008$, drug treatment, $F(4, 352)=3.13$, $p=0.015$, and a fluid x drug treatment, $F(4, 352)=4.73$, $p=0.001$ and sex x drug treatment, $F(4, 352)=2.87$, $p=0.023$ interaction. Intracisternal administration of 10 μ g CTOP significantly decreased time spent in locomotion in comparison to values observed after i.c. infusion of saline, $p=0.014$ (Fig. 3). Effects of 1 μ g CTOP as well as injection of 4 mg/kg naloxone were not significant. Moreover, the changes of time spent in locomotion after 10 μ g CTOP were found significant in males, $p<0.001$, but not in females. The sex effect was due to higher levels of locomotion in saccharin-exposed males given i.c. saline infusion compared to their female counterparts,

$p=0.002$, Cohen's effect size $d=1.02$, while males and females demonstrated comparable times spent in locomotion after 10 μg CTOP (Fig. 3). High motor activation of saccharin-exposed males infused with i.c. saline is responsible for the significant fluid x drug treatment interaction, because the difference between animals infused with 10 μg CTOP and their saline counterparts reached significance only during exposure to saccharin, $p<0.01$, Cohen's effect size $d=0.65$, but not to water, and because saccharin-exposed males given i.c. saline demonstrated higher motor activation than their water counterparts, $p<0.01$, Cohen's $d=0.81$.

3.3. Rearing activity

ANOVA comparing rearing activity revealed significant main effects of fluid, $F(1, 352)=8.39$, $p=0.004$, drug treatment, $F(4, 352)=6.59$, $p<0.001$, and a significant fluid x drug treatment, $F(4, 352)=6.06$, $p<0.001$ interaction. Control subject of both sexes, injected with saline, spent more time in rearing activity during exposure to water than during exposure to saccharin, $p<0.01$, Cohen's effect size $d=0.72$ (Fig. 4).

Effects of opioid antagonists differed between exposure to saccharin, when pups exhibited relatively low rearing activity, and exposure to water, when elevated rearing activity was observed. While rearing activity in pups exposed to saccharin was not affected by i.c. infusion of 10 μg CTOP, the same dose decreased time spent in rearing during exposure to water, $p=0.003$, Cohen's $d=0.83$, in rats not given ethanol (Fig. 4, upper left). No significant effect of 4 mg/kg naloxone was found in water-exposed animals while rearing activity increased, after naloxone, in animals ingesting saccharin that exhibited low rearing activity under saline treatment ($6.55 \pm 1.47\%$). However, the significance of the naloxone-induced increase was at marginal level $p=0.08$ (Fig. 4, right column) and seemed to be driven by ethanol exposed subjects of both sexes.

3.4. Paw licking and grooming

ANOVA for paw licking-grooming revealed significant main effects of fluid, $F(1, 352)=30.44$, $p<0.001$ and drug treatment, $F(4, 352)=12.59$, $p<0.001$, as well as a significant fluid x drug treatment interaction, $F(4, 352)=3.32$, $p<0.011$. All significant effects were seen in pups given i.c. infusion of 1 μg and 10 μg CTOP; injection of 4 mg/kg naloxone had no effect on this behavior (Fig. 5). When CTOP was not injected the time spent in paw licking – grooming did not differ between exposures to saccharin and water, or between ethanol-intoxicated and sober pups. The effect size of CTOP treatment varied as a function of fluid and dose of CTOP. The most powerful effect was produced with 10 μg CTOP in water-exposed animals, $p<0.001$, Cohen's $d=1.87$, with 1 μg CTOP being less effective, $p=0.0018$, Cohen's $d=1.06$ (Fig. 5, left column). The efficacy of both CTOP doses was reversed when pups were exposed to saccharin: 1 μg CTOP stimulated paw licking-grooming more effectively than 10 μg CTOP, $p<0.001$, Cohen's $d=0.84$, and $p<0.01$, Cohen's $d=0.66$, correspondingly (Fig. 5, right column). Ingestion of saccharin attenuated the stimulatory effect of 10 μg CTOP on paw licking-grooming in comparison to water exposure, $p<0.001$, but not with the lower CTOP dose, 1 μg CTOP, $p=0.299$. Note that 10 μg CTOP produced stronger stimulation of paw licking-grooming after 0.5 g/kg ethanol than after saline (water and saccharin data pooled), Cohen's $d=1.54$ and 1.15, correspondingly.

3.5. Nociceptive reactivity

Pups not treated with ethanol or opioid antagonists demonstrated positive values of normalized difference between post- and pre- isolation latencies of paw withdrawal from hot plate (scored as MPE) that significantly differed from zero for saccharin- and water-exposed pups, $t(19)=2.38$, $p=0.028$ and $t(19)=6.67$, $p<0.001$, respectively (Fig. 6). The significant increase above zero in normalized latency after STSI indicates isolation induced analgesia

(IIA). Opioid antagonists affected the magnitude of IIA, but this differed as function of sex, fluid ingested and ethanol dose. ANOVA revealed significant main effects of fluid, $F(1,352)=4.21$, $p=0.041$ and ethanol dose, $F(1,352)=7.64$, $p=0.006$, and two significant interactions: drug treatment x fluid, $F(4,352)=3.78$, $p=0.005$ and drug treatment x sex, $F(4,352)=2.67$, $p=0.032$.

Among control pups not treated with opioid antagonists, ingestion of saccharin during STSI ameliorated the analgesic response. Normalized latency of paw withdrawal decreased in pups exposed to saccharin compared to those exposed to water (i.p. and i.c. saline combined), $p=0.027$, Cohen's $d=0.57$, Fig. 6. Injection of 0.5 g/kg ethanol also decreased the magnitude of IIA in pups not given opioid antagonists, $p<0.01$, Cohen's $d=0.63$. In pups ingesting saccharin, IIA slightly, but not significantly, increased after 10 μ g CTOP, $p=0.062$, Cohen's $d=0.46$, a marginal effect at best. Injection of 4 mg/kg naloxone did not affect analgesic response in pups ingesting saccharin, but significantly decreased normalized latency of paw withdrawal in water-exposed animals, $p=0.014$, Cohen's $d=0.72$ (Fig. 6). The effect of CTOP occurred only among pups not given ethanol: a significant decrease of IIA after 10 μ g CTOP was observed in non-intoxicated water-exposed subjects, males and females pooled, $p<0.01$ Cohen's $d=1.15$.

In short, opioid blockade reduced IIA and all treatments that, theoretically, resulted in anxiolysis (ethanol administration or saccharin exposure during testing) also decreased IIA. However, blockade of opioid function by 10 μ g CTOP reduced the anxiolytic effectiveness of saccharin, partly restoring IIA (marginal effect).

4. Discussion

The present study demonstrated that pharmacological blockade of mu-opioid activity with centrally injected CTOP significantly attenuated effects of low doses of ethanol (0.5 g/kg) on intake of saccharin in females and changed the expression of several behavioral reactions, most importantly isolation induced analgesia (IIA). In general, the present experiments replicated the data obtained in our previous study that employed the same behavioral paradigm (STSI) (Kozlov et al., 2013) but used systemic administration of CTOP. This is especially important given that in adults CTOP has very low permeability through the BBB slowly [$K_{in} (\mu\text{L}/(\text{g} \times \text{min})) = 0.40 \pm 0.55$] (Van Dorpe et al., 2010). However it does transfer across eventually. Thus, it seems that in preweanling rats blockade of mu-opioid receptors in brain was substantial even with systemic injection of the selective mu-antagonist CTOP (Kozlov et al., 2012a). However, the analysis of multiple components of behavioral reactions to STSI as well as assessment of voluntary intake demonstrates a rather complex pattern of effects of blockade of MOR and its interaction with ethanol that is difficult to explain if only changes of mu- opioid activity were responsible for the effects revealed.

The magnitude (effect size) and direction of changes after blockade of MOR differed for each behavioral component measured. Pharmacological blockade of MOR differentially affected intake and behavioral reaction to STSI depending on ethanol dose and fluid exposure. Administration of 0.5 g/kg ethanol in combination with ingestion of saccharin blunted or even reversed the effects of CTOP on rearing behavior and nociceptive reactivity. In contrast, the effects of MOR blockade on saccharin consumption and paw licking-grooming were most pronounced in intoxicated animals. Male and female 12 day-old pups differed in sensitivity to blockade of mu-opioid activity as well as in their reactivity to 0.5 g/kg ethanol and consumption of saccharin.

One finding of interest in this study was opioid involvement in isolation induced analgesia. This effect seems to be mediated by the opioid system since administration of 10 μ g CTOP

(mu opioid antagonist) reduced the analgesic response to isolation. Therefore, it seems that the analgesic response is, at least partly, due to activation of the central mu opioid system. Furthermore, IIA can also be ameliorated by both ethanol and saccharin (sweet taste) as seen in figure 6 (Kozlov et al., 2012). The ameliorating effects of saccharin on IIA also seem to involve activation of the mu opioid system since prior administration of 10 μ g CTOP partly restored IIA.

The pattern of effects of opioid antagonists on nociceptive reactivity can be explained via an interaction between the effects of 0.5 g/kg ethanol or saccharin and mu-opioid activity. Attenuation of IIA by ethanol or saccharin was observed in experimental groups with high values of IIA: saline-water-exposed pups not treated with antagonist (Fig. 6, upper left). In other words, the latency to withdraw the paw from the hot plate was much lower in animals that did not receive an opioid antagonist if they were exposed to either ethanol or saccharin compared with water controls. Alternatively, no effects of opioid antagonists or slight increases in normalized latency of nociceptive reaction were observed in pups exposed to saccharin or 0.5 g/kg ethanol during STSI.

The low efficacy of CTOP in animals exposed to saccharin and ethanol can be explained either by a decrease of endogenous mu-opioid activity as result of these treatments (i.e., ethanol and saccharin) or by a floor effect related to low values of behavioral variables in animals not treated with CTOP. Indeed, the amplitude of IIA after exposure to 0.5 g/kg ethanol and 0.1% saccharin was close to zero. However, several factors argue against the small effect of CTOP infusions on IIA being due to a floor effect. First, subjects can become more sensitive to pain, in which case IIA would be expressed in values lower than zero. Second, mean values of relative time spent in rearing significantly below 10% have been observed in other conditions (socially non-deprived animals; - (Kozlov et al., 2012b)) Thus, from a technical point of view, we were capable of revealing still lower values of nociceptive reactivity than those shown in Figures 6. The fact that such changes were not detected in ethanol- and saccharin-exposed animals suggests that the low dose of ethanol and ingestion of saccharin blocked development of opioid-dependent IIA.

It seems likely that anxiolytic and anti-aversive effects of 0.5 g/kg ethanol and saccharin were mediated through a non-opioid mechanism. If mu-opioids were responsible for the effects of 0.5 g/kg ethanol on IIA and rearing activity, CTOP should have reversed the changes caused by ethanol. Although the 10 μ g dose of CTOP partly attenuated changes caused by 0.5 g/kg ethanol in IIA the interpretation of this result is complicated by the fact that opioid antagonism alone attenuates IIA.

Identification of neurochemical mechanisms of low-dose-ethanol's anxiolytic effects is clearly complicated by the diversity of neurotransmitter and neuromodulator systems engaged by ethanol (Cowen et al., 2004; Breese et al., 2006; Heilig and Koob, 2007; Wee and Koob, 2010). Among multiple brain systems involved in the anxiolytic and fear ameliorating effects of ethanol, GABA neurotransmission is prominent (Koob, 2004; Enoch, 2008), particularly GABA circuits in the amygdala. In adult and developing rats the amygdala is implicated in control of fear and anxiety-like behavior as well as in aversive conditioning (Pandey et al., 2005; Phelps and LeDoux, 2005; Pape and Pare, 2010; Bach et al., 2011). Ethanol-sensitive GABA systems in the amygdala have been shown to help regulate several conditioned and unconditioned activities dependent on alcohol (McBride, 2002; Gremel and Cunningham, 2008; Roberto et al., 2010). The pattern of amygdala connectivity exhibits dramatic developmental changes during the second and third weeks of postnatal life (Bouwmeester et al., 2002). Remarkably, the functional emergence of amygdala corresponds to the end of the stress hyporesponsive period, when corticosterone (CORT) response to short-lasting stressors is attenuated (Suchecki et al., 1995; Vázquez et

al., 2005). When stress sensitivity of the rat increases during the third postnatal week, GABA circuits, among other neuronal networks in the amygdala, are sequentially incorporated into control of anxiety and fear (Moriceau et al., 2004; Thompson et al., 2008) and thus IIA. Interestingly, this amygdaloid maturation coincides temporally with emergence of anxiolytic effects of low doses of ethanol. Whereas administration of 0.5 and 1.0 g/kg ethanol ameliorated the development of IIA in P12 rats, the same doses of ethanol did not block IIA in P9 rats (Kozlov et al., 2012b). We cannot rule out the possibility that developmental changes in internal and external connectivity of the amygdala (Moriceau et al., 2010) enable ethanol to exert anxiolytic and fear-suppressive action on the socially isolated P12 rat.

The most consistent effect observed after intracerebral or systemic administration of CTOP and also after i.p. injection of naloxone was a significant reduction in total intake of saccharin in ethanol-treated female (but not male) pups exposed to STSI. Numerous findings have implicated mu-opioid activity in evaluation of orosensory properties of palatable solutions (Doyle et al., 1993; Berridge, 2000; Ferraro et al., 2002). A substantial body of literature demonstrates that stimulation of MOR in ventral striatum increases the hedonic value of food and fluids and enhanced intake of sweet solutions, including saccharin (Ragnauth et al., 2000; Zhang and Kelley, 2002; Katsuura and Taha, 2010). In contrast, the nonselective opioid antagonist, naltrexone, has been shown to decrease saccharin intake in socially isolated preweanling rats (Kehoe and Sakurai, 1991). Thus, the reduction of saccharin intake after blockade of mu-opioid activity was an expected effect. However, opioid modulation of palatability as a single cause of intake changes cannot explain other findings we observed after CTOP infusions and naloxone injections. Specifically, 1) opioid antagonists selectively blocked ethanol-mediated increases of saccharin intake only in female pups and did not affect saccharin intake in sober pups of either sex and 2) as our recent study (Kozlov et al., 2012b) demonstrated, blockade of opioid activity did not affect saccharin intake in pups socially isolated for 5 hours. We propose that the whole pattern of effects of opioid antagonists on voluntary intake of saccharin is due to an interaction among an opioid-dependent reaction to STSI, non-opioid anxiolytic effects of 0.5 g/kg ethanol, and a time-dependent natural reinforcement mechanism.

It has been recently found (Kozlov et al., 2012b) that the rate of saccharin consumption in females receiving 0.5 g/kg ethanol is inversely correlated with locomotor reaction to STSI and magnitude of IIA. The inverse correlation between saccharin intake and locomotion seems to reflect competition between ingestion and motor activation to social isolation. Taking into account these findings and data on association between pain attenuation and anxiety-inducing stress (Butler and Finn, 2009; Cornelio and Nunes-De-Souza, 2009), we suggest that by reducing anxiety and motor activation, 0.5 g/kg ethanol increases the isolated female pup's sensitivity to appetitive taste stimulation and allows opioid-mediated enhancement of intake to occur. In other words, by decreasing time spent in rearing and locomotion and increasing the duration of ingestive activity, low doses of ethanol may "enable" mechanisms of opioid-mediated sensitization. Thus, it seems that synergistic interaction between stimulation of MOR associated with STSI and anxiolytic effects of a low dose of ethanol provided conditions for enhancement of intake of 0.1 % saccharin in female pups.

In contrast to CTOP the effects of naloxone on rearing and locomotion were negligible or low. The lack of effects of naloxone on locomotor activity contrasted with the findings of Arias et al (Arias et al., 2009a) who revealed reduction of locomotor activity with 2 mg/kg naloxone in preweanling rats in circumstances that resembled the STSI condition in the present experiments. However, Arias et al. (2009a) used a higher dose of ethanol (2.5 g/kg) to induce locomotor activation, and in the present study naloxone was administered in a

higher dose (4 mg/kg) than they used. Comparison of these two studies suggests that endogenous opioid activity plays a different role in effects of high and low doses of ethanol.

The stimulation of paw licking and grooming is a striking effect of selective blockade of MOR. Actually, augmentation of paw licking and grooming was the largest effect (effect size range: 0.86–1.87) among other effects induced by CTOP. This is puzzling because augmentation of grooming was specific to i.c. administration of CTOP and was not affected by naloxone. Previous work has shown that acute or chronic administration of morphine decreased grooming observed in a non-feeding context (Patti et al., 2005; Taraschenko et al., 2007), with grooming activity being reinstated by nonspecific or selective mu-opioid antagonists during withdrawal from opiate dependence (Hendrie, 1985; Madhavan et al., 2010). Systemic injections of nonspecific opioid antagonists are usually unable to elicit grooming without prior stimulation of mu-opioid activity (Hendrie, 1985). However, increased grooming activity in mice was reported after central infusion of the highly potent mu-opioid antagonist, CTAP (Kramer et al., 1989). Importantly, naloxone was effective in reinstating grooming-like activity (“facial wiping”) very early in ontogeny, in morphine-dependent perinatal rats (Jones and Barr, 2000). Although the rate of spontaneous grooming in infant rats (younger than P14) is low, the grooming-related reaction of facial wiping could be evoked in fetal or newborn rats via intraorally infused aversive tastants or by applying mechanical stimulation to the perioral whisker area (Browne et al., 1994; Robinson and Smotherman, 1994; Brumley and Robinson, 2004). The present findings with older (12-day old) rats are in agreement with these perinatal data that implicate involvement of endogenous opioid activity in modulation of the responsiveness of fetal and newborn rats to orofacial and taste stimulation.

In the present experiments fluids were available to P12 animals through an intraoral cheek cannula, a mode of fluid availability that could provide aversive orofacial stimulation. In particular, the cannula could irritate the skin when pups make grooming strokes. In the present experiments we observed that after extensive facial grooming, pups usually scratched their cheek. On the other hand, water can be aversive when delivered through an intraoral cannula (R. Pautassi, personal communication). It is possible that aversive stimulation associated with fluid extraction through the intraoral cannula elicited grooming whereas endogenous mu-opioid activity decreased the sensitivity of the orofacial receptive zones to this stimulation. Alternatively, increased responsiveness to aversive orofacial stimulation after CTOP infusions can be responsible for excessive grooming activity in P12 rats. We did not test CTOP against pretreatment with mu-opioid agonists, but considering data from perinatal rats (Smotherman et al., 1993; Petrov et al., 2000) and the strong effects of CTOP on intake and grooming activity in STSI conditions of the present experiments, we hypothesize that release of mu-opioids evoked by STSI increased the pup’s sensitivity to appetitive taste stimuli (saccharin) and decreased reactivity to aversive orofacial stimulation. We do not rule out the possibility that blockade of mu-opioid activity in the brainstem after i.c. administration of CTOP produced some local disbalance in activity of other neurotransmitters and neuropeptides that, in turn, could elicit grooming.

We also found that 0.5 g/kg ethanol aggravated CTOP-induced facial grooming. This is surprising given the rather sedative and/or anxiolytic effect of low doses of ethanol. However, increased frequency of behavioral reactions to skin stimulation (either grooming or skin scratching) is not unusual after exposure to ethanol (Allan and Isaacson, 1985; Fujii et al., 2009). Authors of a recent study that assessed not only central but peripheral routes of ethanol action ruled out the possibility of peripheral origin of ethanol-aggravated scratching (Fujii et al., 2009). It has been suggested that synergistic changes of GABAA and NMDA receptor activity (Fujii et al., 2009) as well as stimulation of neuropeptide links to the hypothalamus–pituitary–adrenal axis are responsible for ethanol-induced grooming and/or

scratching. Therefore, the increased grooming in animals given a mu antagonist on top of ethanol administration could be due to a synergistic effect of both making the area more sensitive and ethanol induced scratching. On the other hand, the decreased grooming seen in subjects receiving saccharin as well as ethanol and CTOP could be due to mu-opioid activity associated with STSI. This increase resulted in heightened sensitivity to appetitive taste stimuli, which increased saccharin intake and decreased reactivity to tactile (aversive) stimulation of orofacial region attenuating activities concurrent to feeding - grooming and paw licking.

The sex factor was involved in several significant effects and interactions revealed in the present study. In assessing the pattern of significant differences revealed in males and females after CTOP infusion, one might conclude that socially isolated females were more vulnerable to blockade of MOR than their counterparts in terms of consumption of sweet solution and nociceptive reactivity, whereas locomotor activity was more affected with CTOP among males than females. However, analysis of sex differences in control animals not treated with CTOP indicates that sex effects are consequences of sex differences in “baseline” reactivity to STSI rather than due to differential sensitivity of male and female pups to blockade of MOR. For example, CTOP and naloxone did not significantly reduce intake in males exposed to STSI because males did not exhibit ethanol-mediated enhancement of saccharin intake while females did. Similarly, although CTOP decreased motor activation to STSI only in males, the magnitude of this activation differed between control (saline) males and females. Finally, females not treated with CTOP demonstrated greater amelioration of IIA after 0.5 g/kg ethanol than males. Since sex differences appeared in animals not treated with the mu- opioid antagonist we suggest that male and female P12 rats are fundamentally different in their mode of responding to social isolation and this difference importantly determines effects of CTOP in these circumstances (Simpson and Kelly, 2012)..

In summary, analysis of behavioral reaction to STSI after intracisternal infusion of selective mu-opioid antagonist CTOP suggests that several effects of social isolation were mediated through activity of the mu-opioid system. Effects of low dose ethanol (0.5 g/kg) and voluntary consumption of the sweet fluid, saccharin interacted with endogenous mu- opioid activity in a rather complex way. Whereas effects of MOR blockade on saccharin consumption and paw licking- grooming were most expressed in intoxicated animals, the low dose of ethanol and ingestion of saccharin blunted effects of CTOP for rearing behavior and nociceptive reactivity (compared to sober animals). We suggest that enhancement of saccharin intake by ethanol during STSI is the product of synergism between isolation-induced mu- opioid activity that increases the pup’s sensitivity to appetitive taste stimulation and the anxiolytic effects of 0.5 g/kg ethanol that decreases behavioral reactions which otherwise compete with independent ingestive activity. This hypothetical model can be useful in explaining ontogenetic variations of ethanol acceptance in preweanling rats. However, further experiments are needed to identify specific mechanisms of interaction between isolation-induced reactions and pharmacological effects of ethanol on behavior and ingestive activity of the infant rat.

Acknowledgments

The research presented in this paper was supported by NIAAA grants (RO1AA015992, RO1AA013098 and 7P50AA017823) to Norman E. Spear

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Highlights

CTOP blocked ethanol enhancement of saccharin intake in socially isolated females.

CTOP affected intake and behavior to isolation depending on ethanol and fluid.

CTOP stimulated paw licking and grooming dependent on ethanol dose and fluid.

Fig. 1.
Time course of Experimental procedures beginning at separation from the dam to final nociceptive test

Fig. 2.

Effects of i.c. administration of selective mu-opioid antagonist CTOP (0; 1; 10 μg) and systemic (i.p.) administration of nonspecific opioid antagonist naloxone (0; 4 mg/kg) on intake of P12 pups exposed to water (left column) or 0.1% saccharin (right) during STSI and i.p. injected with saline (top row) or 0.5 g/kg ethanol (bottom row) before social isolation. Data are presented as mean $\pm$ S.E.M. Abbreviations: 0_ip, 0_ic -injection of saline i.p. or i.c. correspondingly; CTOP_1, CTOP_10, Nlx_4 –injection of 1, 10 μg CTOP i.c. and naloxone, 4 mg/kg (i.p.)

Fig. 3.

Effects of CTOP (i.c.) and naloxone (i.p.) on locomotor reaction to STSI in pups exposed to water (left column) or 0.1% saccharin (right) during STSI and injected with saline (top row) or 0.5 g/kg ethanol (bottom row) before social isolation.

Fig. 4.

Effects of administration of CTOP (i.c.) and naloxone (i.p.) on rearing activity in pups exposed during STSI to water (left column) or 0.1% saccharin (right) and injected with saline (top row) or 0.5 g/kg ethanol (bottom row) before social isolation.

Fig. 5.

Effects of administration of CTOP (i.c.) and naloxone (i.p.) on grooming-paw licking in pups exposed during STSI to water (left column) or 0.1% saccharin (right) and injected with saline (top row) or 0.5 g/kg ethanol (bottom row) before social isolation.

Fig. 6.

Effects of administration of CTOP (i.c.) and naloxone (i.p.) on analgesic response to STSI, scored as normalized difference between post- and pre- isolation latencies of paw withdrawal from hot plate, in pups exposed during STSI to water (left column) or 0.1% saccharin (right) and injected with saline (top row) or 0.5 g/kg ethanol (bottom row) before social isolation.