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Mu-opioid blockade reduces ethanol effects on intake and behavior of the infant rat during short-term but not long-term social isolation

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Abstract

Numerous findings in adult and infant rats have shown that the endogenous opioid system is involved in control of ethanol consumption and its reinforcing effects. Opioid systems are also involved in reactivity to social isolation with several factors (age, duration, and type of isolation) affecting this modulation. The present study investigated the effects of a selective mu – opioid antagonist CTOP (0, 0.1, 0.5 mg/kg), ethanol (0, 0.5 g/kg), and the interaction of the two drugs on the behavioral consequences of two types of social isolation given to preweanling rats: 1) short-term social isolation from littermates (STSI, duration 8 minutes) and 2) relatively long-term (5 hours) isolation (LTSI) from the dam and littermates. Voluntary intake of saccharin, locomotion, rearing activity, paw licking, and grooming were assessed during an 8 – min. intake test. Thermal nociceptive reactivity was also measured before and after the testing session with normalized differences in pre- and post-test latencies of paw withdrawal from a hot plate (49 °C) used as an index of isolation-induced analgesia (IIA).

The results indicate that pharmacological blockade of mu-opioid receptors by CTOP substantially attenuated ethanol's anxiolytic effects on the developing rat's reactions to social isolation. Some of these stress-attenuating effects of CTOP were observed only in animals exposed to short-term isolation (STSI) but not in pups isolated for 5 hours (LTSI). Ethanol selectively increased saccharin intake during STSI in females and CTOP blocked this effect. Ethanol decreased the magnitude of analgesia associated with STSI but had no effect on pain reactivity during LTSI. CTOP by itself did not affect IIA or saccharin intake in sober animals. The findings of the present experiments indicate that the anxiolytic effects of 0.5 g/kg ethanol on pups exposed to STSI are modulated by endogenous opioid activity.

Keywords

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

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1. Introduction

Stress affects the reinforcing properties of ethanol, patterns of ethanol drinking, and contributes to the development of alcohol addiction (for reviews, see: Phillips et al., 1997; Heilig and Koob, 2007; Pautassi et al., 2010; Becker et al., 2011). From an ontogenetic perspective the relationship between alcohol use and stress is quite complex and still not well understood despite extensive research efforts. The effects of ethanol exposure in adult or adolescent rats often depend on the strength and type of stress as well as dose of ethanol (Parker and Radow, 1974; Lynch et al., 1999; Brunell and Spear, 2005; Boyce-Rustay et al., 2007; Chester et al., 2008; Sacharczuk et al., 2008; Sperling et al., 2010; Varlinskaya et al., 2010). Early life stress associated with maternal separation and exposure to novelty has also been shown to modulate the pharmacological effects of ethanol. For example, Arias et al., (Arias et al., 2009a; Arias et al., 2010) have recently found that on postnatal day 15 (P15), 2.5 g/kg ethanol induced locomotor activation in rat pups separated from the dam but not in those grouped with littermates or kept in their home cage. Our recent study demonstrated that effects of 0.5 g/kg ethanol on ingestion of palatable fluids by preweanlings emerged only in pups exposed to short-term social isolation (STSI) (Kozlov et al., 2012). Alternatively, ethanol can ameliorate isolation-induced “distress” behavior. The calming effect of low doses of ethanol on socially isolated animals has been recently demonstrated in preweanling rats (Pautassi et al., 2007). Importantly, the sensitivity of preweanling rats to stress dramatically changes during the late phase of the stress hypo-responsive period (P8–P12) (Suchecki et al., 1995; Vázquez and Levine, 2005). Given these changes and the dependence of ethanol’s effects on the pup’s reactivity to isolation stress can affect ethanol acceptance and reinforcement during early ontogeny.

Numerous experimental findings in adult animals and infant rats have implicated the endogenous opioid system in the control of ethanol consumption and its reinforcing effects (Herz, 1997; Cichelli and Lewis, 2002; Nizhnikov et al., 2006; Nizhnikov et al., 2009; Pautassi et al., 2009; Trigo et al., 2010). Considerable molecular, neuropharmacological, and behavioral data support the hypothesis that opioid activity is involved in the immediate response to stress as well as adaptation to repeated stressful stimulation or chronic stress (Terman et al., 1986; Lightman and Young, 1987; Mogil et al., 1996; Drolet et al., 2001; Hebb et al., 2005; Ribeiro et al., 2005). Opioid activity has been suggested to affect some behavioral activities induced by social isolation in the infant rat (Kehoe and Blass, 1986; Carden et al., 1996); however, developmental studies of opioid regulation of reactions to social stress have produced conflicting results (Winslow and Insel, 1991; Kehoe and Boylan, 1994; Wiedenmayer and Barr, 2000; Shair et al., 2005). Generally, opioid involvement in reactions to social isolation can vary depending on age, duration of isolation, number of isolation episodes, as well as expression of hormonal responses and activity of other neurotransmitter systems. Moreover, activities at various families of opioid receptors make patterns of opioid involvement in response to early social isolation complex and diverse. Several lines of evidence suggest that mu-, kappa- and delta- opioid receptors are implicated in different aspects of the stress response. Specifically, stress-induced analgesia seems to be mediated by delta and mu- opioid activity while the dysphonic experience of stress is associated with the kappa opioid system (Carden et al., 1994; Kehoe and Boylan, 1994; Yamada and Nabeshima, 1995; Land et al., 2008; Wee and Koob, 2010). Contrasting roles of mu – and kappa – opioid activities in the motivational effects of ethanol have been demonstrated recently in infant rats isolated from their dam and littermates (Nizhnikov et al., 2009; Pautassi et al., 2012a).

Several studies also support involvement of endogenous opioid systems in control of intake of palatable fluids and ethanol as well as conditioning to ethanol in newborn and fetal rats (Smotherman and Robinson, 1992; Petrov et al., 2000; Nizhnikov et al., 2006). It has been

shown that activity at mu- as well as kappa – opioid receptors is required for the expression of appetitive conditioning to ethanol in newborn rats (Nizhnikov et al., 2006; Petrov et al., 2006). However, the effects of pharmacological modulation of opioid activity on behavior and reinforcement from various appetitive stimuli (including ethanol) differ in animals exposed to social isolation and those tested in the suckling context (Kehoe and Blass, 1986; Blass et al., 1990; Barr et al., 1994; Petrov et al., 2000; Petrov et al., 2006; Pautassi et al., 2012a; Pautassi et al., 2012b). It should be noted that methodologically, it is difficult to compare data obtained in socially non-deprived rats tested in a nursing context and in maternally- isolated pups. The behavioral and ingestive activities exhibited by animals attached to a dam or surrogate nipple are totally different from behavior and feeding reactions in maternally deprived subjects (Hall, 1990; Kozlov et al., 2006; Kozlov et al., 2008).

Few studies have compared opioid modulation of the infant rat's reactivity to ethanol across various conditions of social isolation or analyzed multiple indices of complex behavioral reaction to isolation. The interaction between voluntary intake of ethanol and behavioral reaction to social isolation is important to study to further our understanding of developmental variations of ethanol acceptance. Therefore the present study assessed the difference in opioid-mediated effects of ethanol injections between two conditions of social isolation. Specifically, we compared mu-opioid mediation of ethanol (0.5 g/kg) effects on motor activity, and ingestive behavior of water or saccharin for two distinct isolation conditions: 1) short-term social isolation from littermates (STSI, duration 8 minutes; Experiment 1); or 2) relatively long-term (5 hours) isolation (LTSI) from the dam and littermates (Experiment 2).

Specifically, the present study assessed effects of systemic, intraperitoneal (i.p.) administration of the selective mu opioid antagonist CTOP (D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂), on voluntary saccharin or water intake and associated behavior in twelve-day-old (P12) pups treated with 0.5 g/kg ethanol. Although in the adult rat transport of CTOP through the blood brain barrier (BBB) is low (Van Dorpe et al., 2010), relatively high cerebrospinal fluid - plasma concentration ratios for macromolecules have been demonstrated in the developing brain (Birge et al., 1974; Saunders et al., 2008) indicating that CTOP can pass the BBB in the preweanling rat. However, with the current set of experiments it is impossible to know whether the effects of CTOP seen would be due to central or peripheral action of the mu opioid antagonist. While this is an interesting question it is outside the purview of this manuscript. We chose to administer CTOP systemically since this route of administration seemed to represent a less stressful intervention than intracerebral injection in the neonatal rat and, therefore, did not interfere with habituation processes during LTSI. There is no way to obtain a "no-stress" isolation condition for maternally separated preweanling rats (since maternal separation itself is stressful). We therefore thought it important to induce as little baseline stress as possible, other than the isolation, to allow accurate comparison of the effects of ethanol and CTOP on STSI and LTSI. The effects of central administration of selective mu-opioid antagonists on ethanol's effects during short-term stress (STSI) were tested in a separate study that employed direct intracerebral (intracisternal) administration of CTOP (Kozlov et al., 2012, in preparation).

The primary focus of the present experiments was analysis of voluntary intake of water or 0.1% saccharin (Kozlov et al., 2008) and nociceptive reactivity. Our main hypothesis was that ethanol administration would ameliorate the effects of isolation on nociception and fluid intake and that CTOP (mu antagonist) would block ethanol's effects. We employed a technique of continuous fluid accessibility through an intraoral cannula (Kozlov et al., 2003; Kozlov et al., 2009), which models early independent ingestive behavior (Hall and Bryan, 1980). We have also recorded several non-ingestive behavioral activities to assess changes

in the pup's reactivity to isolation after administration of opioid antagonists and ethanol. Several studies demonstrated that measurement of nociceptive reactivity is a relevant tool for assessment of fear and anxiety associated with reaction to stress or novelty (Kehoe and Blass, 1986; Kavaliers and Innes, 1988; Butler and Finn, 2009; Cornelio and Nunes-De-Souza, 2009). Measurement of nociceptive reactivity was previously employed in preweanling rats for the assessment of emotional reactivity to social isolation (Kehoe and Blass, 1986; Blass, 1997) as well as for analysis of ontogenetic development of fear and defensive reactions in infant rats (Takahashi et al., 1991).

In the present study we measured changes in thermal nociceptive reactivity after social isolation (isolation-induced analgesia, IIA) to assess anxiety and fear induced by social isolation. Assessment of IIA in the present study was also combined with analysis of several other indices of behavioral reaction to social isolation, including a measure of motor activation and exploration (locomotion), rearing activity as an index of aversive reactivity to isolation, and paw licking–grooming. Grooming has been used as a behavioral marker of stress in adults (van Erp et al., 1994; Kalueff and Tuohimaa, 2004) and infant rats (Goodwin and Barr, 2005). Grooming-like activity observed in fetal or infant rats to stimulation of orofacial areas is probably a manifestation of an aversive response to this stimulation (Browne et al., 1994; Brumley and Robinson, 2004). The set of behavioral variables used in the present study for assessment of effects of mu-opioid antagonist on the pup's reactivity to isolation was successfully employed in our previous work (Kozlov et al., 2012b) to characterize other behavioral patterns observed in infant rats during short-term, long-term isolation and in grouped pups separated from the dam.

2. Materials and methods

2.1. Subjects

Experimental subjects were 12-day-old Sprague-Dawley rats born in our colony at Binghamton University. A total of 252 rat pups were used in the study. Pregnant female rats were housed individually in plastic maternity cages (47 cm long × 20 cm high × 36 cm wide) containing wood 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 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

We employed two behavioral protocols of short-term and long-term social isolation (STSI and LTSI correspondingly). Under both protocols subjects went through an intake test during which fluids were freely available to pups through an intraoral cheek cannula. The intraoral cannula consisted of polyethylene tubing (PE10) with a flanged tip. The cannula was implanted through the cheek 2–3 mm caudal to the mystacial pad, as described in previous papers (Kozlov et al., 2009, 2012), and placed in an anterior position inside the mouth (Kozlov et al., 2009). The cannulation procedure was accomplished within 8–12 s with no bleeding.

The intake test 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.

In the STSI protocol (Experiment 1) pups were removed from the dam, cannulated, and kept together with littermates (n=8 per group) in warmed (30–32° C) holding boxes for 4.5–5 hours until testing. The holding boxes (size 18 × 18 cm) were filled with a mixture (1:1) of fresh and home cage shavings. During STSI, which started 4.5–5.0 hours after separation from the dam and lasted 8 minutes, pups were placed in the testing arena and allowed to voluntarily ingest 0.1% saccharin or water through their intraoral cannula. Because exposure to novelty was important in the STSI protocol we thoroughly eliminated all traces of odors that could be familiar for the pups. Between tests the walls of arena were cleaned using Kimtech wipers (Kimberly-Clark, Roswell, GA) soaked in warm water. The floors as well as walls were dried between trials with a stream of fresh air from a pressurized can of air. If urination or fluid spills appeared on the arena floor, the paper was changed or the whole arena was replaced.

In the LTSI protocol (Experiment 2) pups were separated from the dam, cannulated, isolated from siblings for 4.5 – 5.0 hr, then were placed in the testing arena and remained separated from their peers throughout the remainder of the experiment. The timing of treatment procedures, measurement of nociceptive reactivity, and duration of the intake test were the same as in the STSI protocol (Experiments 1).

During the intake test 0.1% saccharin was freely accessible through an intraoral cannula and intake of this fluid was measured using a custom-made flow tracker. The fluid flow tracking technique has been described in detail in a previous manuscript (Kozlov et al., 2009). In brief, a piece of translucent PE-90 tubing (Clay Adams, Parsippany, NJ), 90–100 cm long served as a reservoir for the fluid to be presented to the subject. This tubing was connected through a valve and adapter to the pups intraoral cannula. The outer end of PE – 90 tubing was attached to the wheel of the fluid tracker and remained open, resulting in an open hydraulic system. The fluid tracker used a photocell system that was aimed through the translucent tubing to detect the border between filled and unfilled parts of the tube. An output signal from the photo sensor controlled a stepper motor that rotated the wheel and moved the tube through the photocell assembly until the light beam “hit” the filled part of the tubing. When the experimental subject consumed the test fluid, 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 linearly proportional to the amount of fluid ingested. The resolution of flow measurement was about 0.2 µl/step. The data, sampled at a rate of 10 Hz, were transferred to a personal computer via an RS232 port.

A hot plate Analgesia Meter (Model 39L, IITC Life Science, Woodland Hills, CA) was used to assess nociceptive thermal reactivity. We ran a modification of the procedure used in adults for the infant rat (Kehoe and Blass, 1986; Blass et al., 1991; Blass et al., 1993; Blass and Shide, 1994; Anseloni et al., 2002; Kozlov et al., 2012). Basically, tests consisted of placing the pup’s forepaw on a hot surface (temperature - 49.5°C) and measuring latency of paw withdrawal after contact with the hot plate. 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. Automatic withdrawal time was 30 seconds. In other words if the pup had not withdrawn the paw within 30 seconds of contact the paw was removed automatically. Paw withdrawal latencies were measured 8 minutes before and immediately after the intake test. To ensure full

recovery of the subject's nociception between consecutive measurements a 15 minute inter-test interval was implemented.

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 via 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).

2.3. Drug administration

Pups were intraperitoneally (i.p.) administered either saline or 0.5 g/kg ethanol as a 12.6% (v/v) solution in physiological saline. Animals were also given i.p. injections of three doses of a selective mu-antagonist CTOP (Sigma-Aldrich, St Louis, MO): 0, 0.1, 0.5 mg/kg. The dose range of CTOP was chosen on the basis of previous studies in preweanling rats (Nizhnikov et al., 2009). The doses of mu antagonist were prepared in physiological saline by varying the concentration of CTOP solution: 0.01 mg/ml and 0.05 mg/ml for 0.1 and 0.5 mg/kg CTOP respectively. Ethanol, saline and CTOP in all doses were given in a volume of 0.01 ml/g of animal weight.

Injections of CTOP and ethanol (or saline) were delivered sequentially (timing of pharmacological interventions is presented in Fig. 1). The mu-opioid antagonist (CTOP) was injected first (24–25 minutes before the intake test). Ethanol was then injected 18 minutes after CTOP administration, immediately following measurement of the pup's reactivity to a thermal stimulus and 6–7 minutes prior to the intake test.

2.4. Experimental Design and Data analysis

The sample sizes were 144 subjects in Experiment 1 (12 per group) and 108 pups in Experiment 2 (9 subjects per group). To avoid possible 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.

The flow tracking technique presented here has much greater accuracy than change in bodyweight for measurement of intake, with an error of 0.2 mg compared to a measurement error of 10 mg for the standard scale used to weigh infant rats. Infant rats also move while on a scale, which adds variability to weight measurements and reduces accuracy. Given these facts we used the flow tracker records for intake measurements. Furthermore, our previous work has shown that the correlation between flow and weight measurements of intake of various fluids for infant rat pups is extremely high, $0.76 < r < 0.94$ (Kozlov et al., 2008). Therefore, it seems that for this young age amount of flow is an accurate and reasonable measure of intake. Accordingly, intake of 0.1% saccharin during intake test was expressed as total volume intake or as per-minute mean rate of flow through an intraoral cannula. Behavioral activity was scored manually by experimenters blind to the 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 in P12 rat pups was mostly represented by forepaw movements aimed at the face, which frequently started as forepaw licking and then were transformed to “face wiping”.

Nociceptive reactivity was assessed using normalized difference scores 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 (greater than zero) of normalized difference of paw withdrawal latency indicated IIA since it indicated longer latencies following than prior to isolation, therefore we used the expressions “positive difference of paw withdrawal latency” and “magnitude of IIA” as interchangeable terms. It is important to note that pre and post latencies were within subject measures.

Because previous experiments indicated that effects of ethanol in P12 rats were sex-dependent (Kozlov et al., 2009, 2012) the sex factor was included in all experimental designs. Accordingly, a factorial ANOVA design with three between-group factors was used: ethanol dose (saline; 0.5 g/kg), CTOP dose (saline; 0.1; 0.5 g/kg CTOP), 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 a particular activity and were arc-sin transformed before being submitted to an ANOVA. When the ANOVA revealed significant main effects or effects of interactions, planned comparisons were performed. Planned comparisons included assessment of means of all dose groups against a saline control or 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 a repeated measure factor was found or significant effects of a 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 the time series and was compared between treatments. Although some temporal curves of flow demonstrated complex patterns during the 8-minute intake test we have applied linear fitting in temporal analysis because, as has been shown in our previous work, with longer (20 minutes) intake tests a linear trend was the best-fit model of flow changes in P12 rats (Kozlov et al., 2009). Unless otherwise specified population estimates are presented in the form of mean $\pm$ S.E.M.

3. Results

3.1. Experiment 1: Effects of ethanol and CTOP on responding to STSI

3.1.1. Intake—A 2(sex) $\times$ 2(ethanol dose) $\times$ 3(CTOP dose) ANOVA did not reveal any significant main effects, but found significant interactions: ethanol dose $\times$ CTOP dose, $F(2, 132)=3.53$, $p=0.03$, and sex $\times$ CTOP dose, $F(2, 132)=8.36$, $p<0.001$. The 0.5 mg/kg CTOP, i.p. dose, decreased saccharin intake in ethanol –intoxicated animals, $p=0.0063$, but not in saline-treated pups (Fig. 2, left column). This effect was clearly driven by CTOP’s effect on ethanol exposed females since reduction of saccharin intake with 0.5 mg/kg CTOP was observed in females, $p<0.001$, but not in males. It is important to note that saccharin intake in female pups not treated with the opioid antagonist was significantly higher than in their male counterparts, $p<0.001$. Notably, CTOP eliminated only ethanol-mediated enhancement of saccharin intake, decreasing intake to the level of saccharin consumption in non-intoxicated animals (150 – 200 μ l; Fig. 2, bottom graph in left column).

A mixed within-between ANOVA, with successive one-min time intervals treated as a repeated measure, was employed to analyze the effects of ethanol and opioid antagonists on temporal patterns of saccharin flow. Because a single complex 2 (sex) $\times$ 2 (ethanol dose) $\times$ 3 (CTOP dose) $\times$ 8 (time) ANOVA is difficult to interpret for multiple interaction effects we

preferred to run four separate 3 (CTOP dose) $\times$ 8 (time) ANOVAs combined with regression analysis of temporal flow course for each of four sex – ethanol experimental groups. The ANOVAs revealed a significant main effect of time: for sober male and female subjects, $F(7, 231)=6.97$, $p<0.001$ and $F(7, 231)=10.35$, $p<0.001$ respectively; and for ethanol-intoxicated males and females, $F(7, 231)=5.85$, $p<0.001$ and $F(7, 231)=3.72$, $p<0.001$, respectively. A main effect of CTOP was found in ethanol intoxicated females, $F(2, 33)=5.31$, $p=0.01$, and in sober males, $F(2, 33)=6.37$, $p<0.01$. In females 0.5 mg/kg CTOP decreased saccharin intake, while in males the same CTOP dose increased saccharin intake. No significant interactions of time $\times$ CTOP dose were found. Saccharin flow rate declined with time in all treatment groups (Fig. 3).

Temporal flow patterns in all male groups and in all but one female group were best fit by a linear trend. In female pups intoxicated with ethanol but not treated with CTOP, a segmental linear fit was found as a significantly better approximation for the flow curve than a straight line, $F(1,93)=4.27$; $p=0.41$. This regression model was composed of two segments with a breakpoint at minute 4 (Fig. 3, bottom plot on the right). The slope in the first, earlier, interval was higher than the slope during the second phase of the flow curve ($b=-4.62\pm1.59$ vs. $b=0.27\pm1.72$), with the slope of the second phase not differing significantly from zero ($p>0.05$). A late plateau, or even a slight increase of flow at minutes 6 – 7, was responsible for the zero slope of late segment of saccharin flow after 0.5 g/kg ethanol. Minute-to-minute comparisons of the flow rates in ethanol-exposed pups revealed a significant reduction of flow at the initial phase of the intake test in CTOP-treated females (minute 1, $F(1, 33)=5.06$, $p=0.03$, 0.5 mg/kg CTOP vs. saline), and at the time of “secondary” increase of saccharin flow, (minutes 6 and 7, $F(1, 33)=14.38$, $p<0.01$; $F(1, 33)=10.02$, $p<0.01$, 0.5 mg/kg CTOP vs. saline, also in these females, Fig. 3, bottom right), with the differences being insignificant at other intervals of the intake test. A comparison between regression parameters in experimental groups with linear fits as best flow curve approximations revealed that CTOP did not significantly affect the slope of flow curves but significantly changed the value of the intercept of regression lines in sober male pups, $F(2, 20)=33.31$, $p<0.01$ and $F(2, 20)=11.43$, $p<0.01$ correspondingly. This indicated that CTOP (0.5 mg/kg) did not affect the temporal pattern of saccharin flow but shifted the flow curve upward uniformly.

3.1.2. Locomotor activity—A significant main effect of ethanol dose was revealed for percent time spent in locomotion, $F(1, 132)=8.54$, $p=0.004$. The 0.5 g/kg ethanol dose decreased locomotor activity during STSI, $p=0.004$, compared to saline (Fig. 2). Conversely, CTOP (0.5 mg/kg) increased locomotion, but only in ethanol – intoxicated animals, i.e. under the treatment that suppressed motor activation associated with STSI. This was shown by a significant ethanol dose $\times$ CTOP dose interaction, $F(2, 132)=3.30$, $p=0.04$. A 3 (CTOP) $\times$ 8 (time) ANOVA, with a successive 1-min time interval treated as a repeated measure revealed significant decreases of mean time spent in locomotor activity from the first to sixth minute of the test in sober pups $F(7, 483)=3.15$, $p=0.0029$ and from the first to third minute in ethanol-intoxicated pups, $F(7, 483)=11.43$, <0.001 . However, injection of CTOP did not affect the temporal course of locomotion, and showed no significant interactions with time in sober, $F(14, 483)=1.19$, $p=0.28$, as well as in ethanol-treated pups $F(7, 483)=1.36$, $p=0.17$.

3.1.3. Rearing activity—Injection of 0.5 g/kg ethanol significantly decreased percent time spent in rearing activity, as indicated by a main effect of ethanol dose, $F(1, 132)=6.07$, $p=0.015$. Again, CTOP was effective only in ethanol-treated animals as indicated by a significant interaction of ethanol dose $\times$ CTOP dose, $F(2, 132)=3.50$, $p=0.033$. An injection of 0.5 mg/kg CTOP increased rearing activity compared to saline injected subjects in ethanol exposed rat pups, $p=0.0068$. However, the level of activity was not different from

animals not pre-treated with ethanol ($8.02 \pm 1.82\%$). In other words, 0.5 mg/kg CTOP blocked ethanol –induced reduction of rearing activity (Fig. 2). This effect was seen in both males and females. As repeated measure (CTOP) x (time) ANOVA indicated, rearing activity decreased from the first to fourth minute in sober pups, $F(7, 483)=2.92$, $p=0.0053$, and from the second to fourth minute after injection of 0.5 g/kg ethanol, $F(7, 483)=2.97$, $p=0.0047$. CTOP did not significantly affect the temporal course of rearing activity.

3.1.4. Paw licking and grooming—An ANOVA revealed a significant main effect of CTOP, $F(2, 132)=3.20$, $p=0.044$, and significant interactions between sex and ethanol dose, $F(1, 132)=5.21$, $p=0.024$, and between ethanol dose and CTOP dose, $F(2, 132)=4.27$, $p=0.016$. Pups of both sexes injected with 0.5 mg/kg CTOP spent more time in paw licking – grooming than saline-treated animals, and this difference was significant only for ethanol-intoxicated rats, $p=0.0005$. Furthermore, saline-exposed male pups exhibited higher grooming activity than their saline exposed female counterparts, $p=0.012$. The per-minute rate of grooming activity did not change significantly in time in sober pups, $F(7, 483)=1.69$, $p=0.11$, however, intoxicated pups showed a significant interaction, CTOP x time, $F(14, 483)=1.69$, $p=0.11$. While no significant temporal changes in grooming activity was revealed in intoxicated pups after vehicle injection, 0.5 mg/kg CTOP increased time spent in grooming during the first 2 minutes of STSI, then grooming decreased to a level similar to that observed in pups injected with ethanol and vehicle.

3.1.5. Nociceptive reactivity—Saline non-intoxicated controls demonstrated positive values of normalized differences between post- and pre- isolation latencies of paw withdrawal from the hot plate (scored as MPE), $t(23)=2.44$, $p=0.023$, indicating IIA. On the other hand, pups injected with 0.5 g/kg ethanol showed MPE values that did not significantly differ from zero, $t(23)=1.24$, $p=0.228$ (Fig. 2 right most panel). In other words, 0.5 g/kg ethanol eliminated IIA. No significant main effects of CTOP dose or ethanol dose x CTOP dose interactions were found for magnitude of IIA or for pretest latency of paw withdrawal from the hot plate.

3.2. Experiment 2: Effects of ethanol and CTOP on responding to LTSI

3.2.1. Intake—A $2(\text{sex}) \times 2(\text{ethanol dose}) \times 3(\text{CTOP dose})$ ANOVA failed to reveal significant main effects or interactions for saccharin flow rate (Fig. 4, left column). Analysis of effects of ethanol and CTOP on temporal patterns of saccharin flow [with mixed – ANOVAs, $3(\text{CTOP}) \times 8(\text{time})$], found significant main effects of time and no CTOP x time interaction for all treatment groups. Similar to the results seen with STSI, saccharin flow decreased with time after exposure to LTSI (Fig. 5). However, the temporal pattern of flow during the intake test after LTSI differed in some experimental groups from the temporal course of saccharin intake observed during STSI (compare Fig. 3 and 5). First, in some non-intoxicated groups the best fitting temporal course of saccharin flow was one-phase exponential decay ($Y=(Y_0 - A_0)*\exp(-K*t)+A_0$) characterized by a decrease of decay over time, proportional to the reduction of per minute flow rate. Second, ethanol-treated females isolated for 5 hours did not demonstrate the two-phase flow curve, with zero decay of flow rate during the second phase, characteristic of their counterparts exposed to STSI. The slope of this decrease in flow ($b=-4.4 \pm 0.56$) was significantly higher than the rate of flow reduction in females administrated 0.5 mg/kg CTOP ($b=-2.3 \pm 0.45$; $F(2, 18)=4.79$, $p<0.05$). No significant differences were found between parameters of linear fits for temporal courses of saccharin flow in other treatment groups.

3.2.2. Behavioral activities—No significant main effects or interactions were found for time spent in locomotion or for time spent in rearing activity (Fig. 4). A sex x CTOP interaction was revealed after analysis of time spent in paw licking – grooming, $F(2,$

96)=4.61, $p=0.012$. Males injected with 0.5 mg/kg CTOP spent more time in grooming than their female counterparts, $p<0.001$. Locomotion and grooming did not change across time in sober pups but showed a time -depended decline after injection of 0.5 g/kg ethanol, for locomotion, $F(7, 357)=4.07$, $p<0.001$, and grooming, $F(7, 357)=3.65$, $p<0.001$. In contrast, rearing activity significantly decreased with time in sober pups, $F(7, 357)=2.69$, $p=0.01$, but not in ethanol-intoxicated pups, $F(7, 357)=1.77$, $p=0.092$. CTOP did not change the time course of locomotion, rearing and grooming either in sober or ethanol-treated pups.

3.2.3. Nociceptive reactivity—Neither ethanol nor CTOP affected baseline nociceptive reactivity or MPE (normalized post- test latency of paw withdrawal from a hot plate). However, mean values of MPE after the intake test did not significantly differ from zero in pups not treated with CTOP, in both ethanol and saline exposed groups, $t(17)=0.70$, $p=0.493$; $t(17)=1.01$, $p=0.324$, respectively, indicating that no “analgesic” response developed during the intake test in pups isolated from littermates for 5 hours (Fig. 4, right most panel).

4. Discussion

The present experiments indicate that pharmacological blockade of mu-opioid receptors interacts with the rat pup’s reaction to social isolation. Some effects of the mu-opioid antagonist CTOP were observed only in animals exposed to STSI but not in pups socially isolated from littermates for 5 hours. Given that the saline pretreated pups in the STSI (Experiment 1) but not in the LTSI (Experiment 2) showed significant differences in several of our dependent measures and that CTOP blocked these effects, one may conclude that there is a higher level of endogenous mu opioid activity in pups isolated for 8 minutes than in animals kept alone for 5 hours. Differences in expression of opioid activity in response to brief vs. prolonged social isolation has previously been found in studies assessing patterns of immunoreactivity to ligands bound to mu–opioid receptors in 10-day-old pups exposed to 5 or 60 minutes of isolation (Carden et al., 1996). The radioimmunoassay analysis revealed that patterns of change in peptide activity evoked by social isolation was region–specific, with high levels of immunoreactivity to opioid peptides being observed in ventral striatum, hypothalamus and some midbrain nuclei – such as periaqueductal gray (Shoemaker and Kehoe, 1995; Carden et al., 1996). It is important to note that mu–opioid receptors in the periaqueductal gray also mediate some components of fear reactions in preweanling rats (Wiedenmayer and Barr, 2000). During STSI pups were placed alone in a novel box, a procedure known to induce fear and anxiety in rodents (Kavaliers and Innes, 1988). Thus, the present findings along with data from previous studies suggest that fear and anxiety associated with STSI can stimulate endogenous opioid activity in preweanling rats.

Pharmacological studies of effects of opioid antagonists on behavior of socially isolated preweanling rats have produced conflicting reports (Kehoe and Blass, 1986; Carden and Hofer, 1990; Winslow and Insel, 1991; Shair et al., 2005). This is not surprising since the response of preweanling rats to separation from the dam represents a complex set of behavioral and physiological reactions with differing temporal dynamics and dependence on multiple neurotransmitter systems (e.g. GABAergic, monoaminergic, and opioidergic) (Hofer, 1996). Recent experiments have shown that ethanol- and novelty--induced motor activation in the preweanling rat are attenuated by the nonselective opioid antagonist naloxone (Arias et al., 2009a; Arias et al., 2009b). Importantly, these opioid-mediated effects of ethanol were expressed only during STSI. In the present study we also revealed a dramatic difference between rats exposed to STSI and LTSI in terms of involvement of opioid activity in ethanol-mediated effects. Although CTOP decreased initial ingestion of 0.1% saccharin in ethanol pre-treated pups following LTSI (Fig. 5 right panel), a significant reduction of total intake of saccharin after pharmacological blockade of opioid receptors was

only found in ethanol – treated female pups exposed to STSI (Figure 2 left bottom). Several factors may be responsible for the different effects of CTOP on saccharin intake in STSI versus LTSI.

For example, in addition to numerous findings implicating opioid activity in natural reinforcement based on the orosensory properties of palatable solutions (Doyle et al., 1993; Berridge, 2000; Ferraro et al., 2002), the ability of opioid antagonists to reduce intake can depend on the rate of basal fluid consumption. Specifically, rats that consumed sweet solutions at high rates showed greater suppression of intake (in terms of percent of base line) following opioid antagonists than low consumers (Cichelli and Lewis, 2002). This type of interaction suggests opioid-mediated reinforcement: ingestion of sweets stimulates opioid activity and the greater the initial intake the greater the decrease in intake following opioid blockade. Since we have shown that ethanol treated females drink significantly more than all other groups, it would follow that opioid blockade should have a more pronounced effect on STSI ethanol pretreated females.

Opioid-mediated mechanisms of intake modulation may also have differential expression across time. Several studies have demonstrated delayed opioid modulation of intake that usually occurred after subjects consumed sweet fluids for an extended period of time (Siviy et al., 1982; Frisina and Sclafani, 2002; Shimura et al., 2006; Woolley et al., 2007). These findings suggest an engagement of opioid-mediated mechanisms of natural reinforcement: ingestion of sweets stimulates opioid activity, and release of opioids, in turn, enhances ingestive and affective reactions to sweet solution. We propose that both changes in taste-hedonic and natural reinforcement mechanisms were involved in the opioid-mediated effects of 0.5 g/kg ethanol on saccharin intake of P12 pups, and that differences in expression of these two mechanisms can explain some of the differing effects seen between STSI and LTSI.

The increase in saccharin consumption following 0.5 g/kg ethanol pre-treatment was observed at the very beginning of the STSI trial, suggesting increased sensitivity to sweet taste. Ethanol-treated pups also demonstrated the late, “secondary”, increase in saccharin consumption (Fig. 3) indicating that ethanol facilitated time – dependent enhancement of ingestive activity during STSI. Blockade of opioid activity decreased saccharin flow at the initial phase of intake, but opioid antagonists also virtually eliminated the late-phase increase of flow (on minutes 6 – 7, Fig. 3). CTOP decreased saccharin flow after exposure to LTSI as well, but the effect was observed only during the first minute of the intake test (Fig. 5). It is important to note that in contrast to short-isolated animals, saccharin flow rate in pups isolated for 5 hours sharply declined from the beginning to the end of the intake test. The relatively low arousal of pups kept alone for 5 hours could be responsible for this time-dependent decline in rate of saccharin ingestion, lack of opioid-mediated enhancement of intake at late phases of the test, and ultimately, the failure of 0.5 g/kg ethanol to increase total intake of saccharin during LTSI (to compare levels of locomotion between STSI and LTSI see Fig. 2 and Fig. 4). We have demonstrated previously that during 20-min isolation of P9 or P12 rat pups, voluntary intake through an intraoral cannula depends on behavioral arousal as well as duration of wakefulness episodes (Kozlov et al., 2009). In the present free intake paradigm pups need to be aroused and behaviorally active to extract fluid from a cannula and to sustain voluntary ingestive activity for some time which may account for the lower intake seen following LTSI.

Consistent with our recent work (Kozlov et al., 2012) 0.5 g/kg ethanol decreased the magnitude of IIA, indicating ethanol-mediated amelioration of anxiety and stress associated with social isolation. In the present study socially isolated saline-treated animals also showed increased IIA, and ethanol exposure removed this effect. Recently, we also found

that rate of saccharin consumption in females receiving 0.5 g/kg ethanol was negatively correlated with magnitude of IIA (Kozlov et al., 2012). Given that in the context of social isolation, lower values of IIA correspond to lower anxiety (Butler and Finn, 2009), we suggest that the high sensitivity of females to the anxiolytic effects of ethanol facilitates opioid-mediated enhancement of intake. In agreement with this suggestion females placed in a novel environment are less vulnerable than males to stress-induced suppression of feeding (Zylan and Brown, 1996; Faraday, 2002). Thus, it seems that the effects of opioid activity associated with STSI combined with the anxiolytic effects of low doses of ethanol to enhance consumption of 0.1 % saccharin [and perhaps 10 – 12% ethanol as a fluid with taste properties comparable to those of 0.1 % saccharin]. CTOP was also ineffective in modulation of nociceptive reactivity in pups isolated from the dam for 5 hours. A floor effect could be responsible for the inefficacy of the opioid antagonist under this isolation condition, since the analgesic response to fluid exposure was close to zero after 5 hours of isolation. The low magnitude of IIA after LTISI could be the result of habituation to experimental settings as well as a consequence of sweet taste stimulation during the intake test.

Interestingly, administration of the selective mu-opioid antagonist CTOP not only attenuated ethanol induced enhancement of saccharin intake but also reversed the ethanol mediated reduction of locomotor activity (in females; Figure 2 bottom row 2nd panel) and aversive reactions (paw licking, grooming) to STSI. Recently, we revealed an inverse correlation between saccharin intake and locomotion in females exposed to STSI that indicated a competition between ingestion and locomotor activity (Kozlov et al., 2012). Given this negative correlation and contrasting changes of intake and locomotion caused by CTOP we suggest that change in locomotion (and rearing) caused by blockade of opioid activity was related to the effects of CTOP on saccharin intake. In other words, when animals performed two competing activities (ingestion and locomotion), as in the present tests, stimulation of consumption of sweet fluid by opioid release to STSI reciprocally decreased the level of motor activation associated with STSI. Alternatively, decreases of motor and aversive reactivity to STSI due to the anxiolytic and sedative properties of 0.5 g/kg ethanol could facilitate the effects of opioid stimulation on saccharin intake. Support for this hypothesis also comes from our previous study (Kozlov et al., 2012), but more experiments are required to elucidate details of the complex interaction between ethanol-mediated and opioid-stimulated changes in behavior and ingestive activity during social isolation.

Sex differences play an important role in the effects of opioid antagonists revealed in this study, despite the pre-pubertal age of the rats. For example, CTOP was effective in reducing saccharin intake in females intoxicated with 0.5 g/kg ethanol but not in males. CTOP did not significantly reduce intake in males exposed to STSI, perhaps because males did not exhibit ethanol-mediated enhancement of saccharin intake while females did. Recently we found that the pattern of isolation-induced changes in intake, behavioral activity, and nociception, as well as strength of the correlation between behavioral and hormonal response to STSI, differed between male and female P12 pups (Kozlov et al., 2012). Females also differed from male pups in their reactivity to an ethanol challenge. Taken together the data from the present and previous studies suggest that twelve-day-old male and female pups are fundamentally different in their patterns of reactivity to STSI and this difference may underlie sex-specific effects of opioid antagonists.

In summary, the findings of the present study indicate that pharmacological blockade of mu-opioid receptors interacts with ethanol-mediated effects in socially deprived P12 rat pups. The mode of opioid modulation of ethanol effects differs, however, as a function of the duration of social isolation. In general, pups separated from their siblings for 8 minutes were

more sensitive to blockade of mu-opioid activity than animals socially deprived for 5 hours. A comparison of the present findings with other data obtained in socially deprived infant rats (Arias et al., 2009b; Arias et al., 2009c) suggests a difference in the role of opioid activity with low (0.5 g/kg) and high (2.5 g/kg) doses of ethanol.

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References

- Anseloni VCZ, Weng HR, Terayama R, Letizia D, Davis BJ, Ren K, Dubner R, Ennis M. Age-dependency of analgesia elicited by intraoral sucrose in acute and persistent pain models. *Pain*. 2002; 97:93–103. [PubMed: 12031783]
- Arias C, Mlewski EC, Miller S, Molina JC, Spear NE. Novelty modulates the stimulating motor effects of ethanol in preweanling rats. *Pharmacol Biochem Behav*. 2009a; 92:448–456. [PubMed: 19463258]
- Arias C, Mlewski EC, Molina JC, Spear NE. Naloxone and baclofen attenuate ethanol's locomotor-activating effects in preweanling sprague-dawley rats. *Behav Neurosci*. 2009b; 123:172–180. [PubMed: 19170442]
- Arias C, Molina JC, Spear NE. Differential role of mu, delta and kappa opioid receptors in ethanol-mediated locomotor activation and ethanol intake in preweanling rats. *Physiol Behav*. 2009c; 99:348–354. [PubMed: 19954749]
- Arias C, Solari AC, Mlewski EC, Miller S, Haymal B, Molina JC, Spear NE. Social isolation and stress related hormones modulate the stimulating effect of ethanol in preweanling rats. *Behav Brain Res*. 2010; 211:64–70. [PubMed: 20226814]
- Barr GA, Wang S, Carden S. Aversive properties of the kappa opioid agonist u50,488 in the week-old rat pup. *Psychopharmacol (Berl)*. 1994; 113:422–428.
- Becker HC, Lopez MF, Doremus-Fitzwater TL. Effects of stress on alcohol drinking: A review of animal studies. *Psychopharmacology*. 2011; 218:131–156. [PubMed: 21850445]
- Berridge KC. Measuring hedonic impact in animals and infants: Microstructure of affective taste reactivity patterns. *Neurosci Biobehav Rev*. 2000; 24:173–198. [PubMed: 10714382]
- Birge WJ, Rose AD, Haywood JR, Doolin PF. Development of the blood cerebrospinal fluid barrier to proteins and differentiation of cerebrospinal fluid in the chick embryo. *Dev Biol*. 1974; 41:245–254. [PubMed: 4452410]
- Blass EM, Cramer CP, Fanselow MS. The development of morphine-induced antinociception in neonatal rats: A comparison of forepaw, hindpaw, and tail retraction from a thermal stimulus. *Pharmacol Biochem Behav*. 1993; 44:643–649. [PubMed: 8451267]
- Blass EM, Fillion TJ, Weller A, Brunson L. Separation of opioid from nonopioid mediation of affect in neonatal rats: Nonopioid mechanisms mediate maternal contact influences. *Behav Neurosci*. 1990; 104:625–636. [PubMed: 2169762]
- Blass EM, Jackson AM, Smotherman WP. Milk-induced, opioid-mediated antinociception in rats at the time of cesarean delivery. *Behav Neurosci*. 1991; 105:677–686. [PubMed: 1667730]
- Blass EM, Shide DJ. Some comparisons among the calming and pain-relieving effects of sucrose, glucose, fructose and lactose in infant rats. *Chemical Senses*. 1994; 19:239–249. [PubMed: 7914461]
- Blass EM. Interactions between contact and chemosensory mechanisms in pain modulation in 10-day-old rats. *Behav Neurosci*. 1997; 111:147–154. [PubMed: 9109633]
- Boyce-Rustay JM, Cameron HA, Holmes A. Chronic swim stress alters sensitivity to acute behavioral effects of ethanol in mice. *Physiol Behav*. 2007; 91:77–86. [PubMed: 17363014]
- Browne JB, Robinson SR, Smotherman WP. Fetal experience with milk or an artificial nipple alters appetitive and aversive responses to perioral cutaneous stimuli. *Behav Neurosci*. 1994; 108:606–613. [PubMed: 7917053]

- Brumley MR, Robinson SR. Facial wiping in the rat fetus: Variation of chemosensory stimulus parameters. *Dev Psychobiol.* 2004; 44:219–229. [PubMed: 15103732]
- Brunell SC, Spear LP. Effect of stress on the voluntary intake of a sweetened ethanol solution in pair-housed adolescent and adult rats. *Alcohol Clin Exp Res.* 2005; 29:1641–1653. [PubMed: 16205364]
- Butler RK, Finn DP. Stress-induced analgesia. *Prog Neurobiol.* 2009; 88:184–202. [PubMed: 19393288]
- Carden SE, Davachi L, Hofer MA. U50,488 increases ultrasonic vocalizations in 3-, 10-, and 18-day-old rat pups in isolation and the home cage. *Dev Psychobiol.* 1994; 27:65–83. [PubMed: 8112489]
- Carden SE, Hofer MA. Independence of benzodiazepine and opiate action in the suppression of isolation distress in rat pups. *Behav Neurosci.* 1990; 104:160–166. [PubMed: 2107826]
- Carden SE, Tempel A, Hernandez N, Hofer MA. Isolation alters striatal met-enkephalin immunoreactivity in rat pups. *Physiol Behav.* 1996; 60:51–53. [PubMed: 8804642]
- Chester JA, Barrenha GD, Hughes ML, Keuneke KJ. Age- and sex-dependent effects of footshock stress on subsequent alcohol drinking and acoustic startle behavior in mice selectively bred for high-alcohol preference. *Alcohol Clin Exp Res.* 2008; 32:1782–1794. [PubMed: 18652593]
- Cichelli MJ, Lewis MJ. Naloxone nonselective suppression of drinking of ethanol, sucrose, saccharin, and water by rats. *Pharmacol Biochem Behav.* 2002; 72:699–706. [PubMed: 12175467]
- Cornelio AM, Nunes-De-Souza RL. Open elevated plus maze-induced antinociception in rats: A non-opioid type of pain inhibition? *Physiol Behav.* 2009; 96:440–447. [PubMed: 19059275]
- Doyle TG, Berridge KC, Gosnell BA. Morphine enhances hedonic taste palatability in rats. *Pharmacol Biochem Behav.* 1993; 46:745–749. [PubMed: 8278455]
- Drolet G, Dumont AC, Gosselin I, Kinkead R, Laforest S, Trottier JF. Role of endogenous opioid system in the regulation of the stress response. *Prog Neuro-Psychopharmacol Biol Psychiatry.* 2001; 25:729–741.
- Faraday MM. Rat sex and strain differences in responses to stress. *Physiol Behav.* 2002; 75:507–522. [PubMed: 12062315]
- Ferraro FM, Hill KG, Kaczmarek HJ, Coonfield DL, Kiefer SW. Naltrexone modifies the palatability of basic tastes and alcohol in outbred male rats. *Alcohol (Fayetteville, NY).* 2002; 27:107–114.
- Frisina PG, Sclafani A. Naltrexone suppresses the late but not early licking response to a palatable sweet solution: Opioid hedonic hypothesis reconsidered. *Pharmacol Biochem Behav.* 2002; 74:163–172. [PubMed: 12376164]
- Goodwin GA, Barr GA. Developmental changes in the behavioral and autonomic effects of kappa opioid receptor stimulation of the midbrain periaqueductal gray. *Dev Psychobiol.* 2005; 46:47–56. [PubMed: 15690388]
- Hall, WG. The ontogeny of ingestive behavior. Changing control of components in the feeding sequence. In: Stricker, EM., editor. *Handbook of behavioral neurobiology*; v.10. *Neurobiology of food and fluid intake.* New York: Premium Press; 1990.
- Hall WG, Bryan TE. The ontogeny of feeding in rats: II. Independent ingestive behavior. *Journal of Comparative & Physiological Psychology.* 1980; 94:746–756.
- Hebb, ALO.; Laforest, S.; Drolet, G. Endogenous opioids, stress, and psychopathology. In: Steckler, T.; Kalin, NH.; Reul, JMHM., editors. *Handbook of stress and the brain - part 1: The neurobiology of stress.* 2005. p. 561–583.
- Heilig M, Koob GF. A key role for corticotropin-releasing factor in alcohol dependence. *Trends Neurosci.* 2007; 30:399–406.
- Herz A. Endogenous opioid systems and alcohol addiction. *Psychopharmacology.* 1997:129.
- Hofer MA. On the nature and consequences of early loss. *Psychosom Med.* 1996; 58:570–581. [PubMed: 8948005]
- Kalueff AV, Tuohimaa P. Grooming analysis algorithm for neurobehavioural stress research. *Brain Res Brain Res Protoc.* 2004; 13:151–158. [PubMed: 15296852]
- Kavaliers M, Innes DG. Novelty-induced opioid analgesia in deer mice (*Peromyscus maniculatus*): Sex and population differences. *Behav Neural Biol.* 1988; 49:54–60. [PubMed: 3345191]

- Kehoe P, Blass EM. Opioid-mediation of separation distress in 10-day-old rats: Reversal of stress with maternal stimuli. *Dev Psychobiol.* 1986; 19:385–398. [PubMed: 3732628]
- Kehoe P, Blass EM. Behaviorally functional opioid systems in infant rats: I. Evidence for olfactory and gustatory classical conditioning. *Behav Neurosci.* 1986; 100:359–367. [PubMed: 3730143]
- Kehoe P, Boylan CB. Behavioral effects of kappa-opioid-receptor stimulation on neonatal rats. *Behav Neurosci.* 1994; 108:418–423. [PubMed: 8037885]
- Kozlov AP, Petrov ES, Varlinskaya EI, Spear NE. Taste differentiation in the context of suckling and independent, adultlike ingestive behavior. *Dev Psychobiol.* 2006; 48:133–145. [PubMed: 16489597]
- Kozlov AP, Nizhnikov ME, Varlinskaya EI, Spear NE. Pharmacological effects of ethanol on ingestive behavior of the preweanling rat. *Behav Brain Res.* 2009; 205:162–174. [PubMed: 19549546]
- Kozlov AP, Nizhnikov ME, Varlinskaya EI, Spear NE. The role of social isolation in ethanol effects on the preweanling rat. *Behav Brain Res.* 2012; 227:43–57. [PubMed: 22051944]
- Kozlov AP, Petrov ES, Kashinsky W, Nizhnikov ME, Spear NE. Oral compression activity on a surrogate nipple in the newborn rat: Nutritive and nonnutritive sucking. *Dev Psychobiol.* 2003; 43:290–303. [PubMed: 15027412]
- Kozlov AP, Varlinskaya EI, Spear NE. Ethanol, saccharin, and quinine: Early ontogeny of taste responsiveness and intake. *Alcohol Clin Exp Res.* 2008; 32:294–305. [PubMed: 18162068]
- Land BB, Bruchas MR, Lemos JC, Xu M, Melief EJ, Chavkin C. The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. *J Neurosci.* 2008; 28:407–414. [PubMed: 18184783]
- Lightman SL, Young WS. Changes in hypothalamic preproenkephalin a mrna following stress and opiate withdrawal. *Nature.* 1987; 328:643–645. [PubMed: 3614366]
- Lynch WJ, Kushner MG, Rawleigh JM, Fiszdon J, Carroll ME. The effects of restraint stress on voluntary ethanol consumption in rats. *Exp Clin Psychopharmacol.* 1999; 7:318–323. [PubMed: 10609966]
- Mogil JS, Sternberg WF, Balian H, Liebeskind JC, Sadowski B. Opioid and nonopioid swim stress-induced analgesia: A parametric analysis in mice. *Physiol Behav.* 1996; 59:123–132. [PubMed: 8848471]
- Nizhnikov ME, Pautassi RM, Truxell E, Spear NE. Opioid antagonists block the acquisition of ethanol-mediated conditioned tactile preference in infant rats. *Alcohol (Fayetteville, NY).* 2009; 43:347–358.
- Nizhnikov ME, Varlinskaya EI, Petrov ES, Spear NE. Reinforcing properties of ethanol in neonatal rats: Involvement of the opioid system. *Behav Neurosci.* 2006; 120:267–280. [PubMed: 16719691]
- Parker LF, Radow BL. Isolation stress and volitional ethanol consumption in the rat. *Physiol Behav.* 1974; 12:1–3. [PubMed: 4855694]
- Pautassi RM, Camarini R, Quadros IM, Miczek KA, Israel Y. Genetic and environmental influences on ethanol consumption: Perspectives from preclinical research. *Alcohol Clin Exp Res.* 2010; 34:976–987. [PubMed: 20374217]
- Pautassi RM, Nizhnikov M, Molina JC, Boehm SL, Spear N. Differential effects of ethanol and midazolam upon the devaluation of an aversive memory in infant rats. *Alcohol (Fayetteville, NY).* 2007; 41:421–431.
- Pautassi RM, Nizhnikov ME, Spear NE. Assessing appetitive, aversive, and negative ethanol-mediated reinforcement through an immature rat model. *Neurosci Biobehav Rev.* 2009; 33:953–974. [PubMed: 19428502]
- Pautassi RM, Nizhnikov ME, Acevedo MB, Spear NE. Early role of the kappa opioid receptor in ethanol-induced reinforcement. *Physiol Behav.* 2012a; 105:1231–1241. [PubMed: 22261437]
- Pautassi RM, Nizhnikov ME, Fabio MC, Spear NE. Early maternal separation affects ethanol-induced conditioning in a nor-bni insensitive manner, but does not alter ethanol-induced locomotor activity. *Pharmacol Biochem Behav.* 2012b; 100:630–638. [PubMed: 22108648]

- Petrov ES, Nizhnikov ME, Varlinskaya EI, Spear NE. Dynorphin a (1–13) and responsiveness of the newborn rat to a surrogate nipple: Immediate behavioral consequences and reinforcement effects in conditioning. *Behav Brain Res.* 2006; 170:1–14. [PubMed: 16621051]
- Petrov ES, Varlinskaya EI, Smotherman WP. The first suckling episode in the rat: The role of endogenous activity at mu and kappa opioid receptors. *Dev Psychobiol.* 2000; 37:129–143. [PubMed: 11044861]
- Phillips TJ, Roberts AJ, Lessov CN. Behavioral sensitization to ethanol: Genetics and the effects of stress. *Pharmacol Biochem Behav.* 1997; 57:487–493. [PubMed: 9218273]
- Ribeiro SC, Kennedy SE, Smith YR, Stohler CS, Zubieta J-K. Interface of physical and emotional stress regulation through the endogenous opioid system and mu-opioid receptors. *Prog Neuro-Psychopharmacol Biol Psychiatry.* 2005; 29:1264–1280.
- Sacharczuk M, Juszczak G, Sliwa AT, Tymosiak-Zielinska A, Lisowski P, Jaszczak K, Pluta R, Lipkowski A, Sadowski B, Swiergiel AH. Differences in ethanol drinking between mice selected for high and low swim stress-induced analgesia. *Alcohol.* 2008; 42:487–492. [PubMed: 18760717]
- Saunders NR, Ek CJ, Habgood MD, Dziegielewska KM. Barriers in the brain: A renaissance? *Trends Neurosci.* 2008; 31:279–286. [PubMed: 18471905]
- Shair HN, Brunelli SA, Hofer MA. Lack of evidence for mu-opioid regulation of a socially mediated separation response. *Physiol Behav.* 2005; 83:767–777. [PubMed: 15639162]
- Shimura T, Imaoka H, Yamamoto T. Neurochemical modulation of ingestive behavior in the ventral pallidum. *Eur J Neurosci.* 2006; 23:1596–1604. [PubMed: 16553623]
- Shoemaker WJ, Kehoe P. Effect of isolation conditions on brain regional enkephalin and beta-endorphin levels and vocalizations in 10-day-old rat pups. *Behav Neurosci.* 1995; 109:117–122. [PubMed: 7734067]
- Siviy SM, Calcagnetti DJ, Reid LD. A temporal analysis of naloxone's suppressant effect on drinking. *Pharmacol Biochem Behav.* 1982; 16:173–175. [PubMed: 7058209]
- Smotherman WP, Robinson SR. Kappa opioid mediation of fetal responses to milk. *Behav Neurosci.* 1992; 106:396–407. [PubMed: 1317189]
- Smotherman WP, Robinson SR. Classical conditioning of opioid activity in the fetal rat. *Behav Neurosci.* 1994; 108:951–961. [PubMed: 7826517]
- Sperling RE, Gomes SM, Sypek EI, Carey AN, McLaughlin JP. Endogenous kappa-opioid mediation of stress-induced potentiation of ethanol-conditioned place preference and self-administration. *Psychopharmacology.* 2010; 210:199–209. [PubMed: 20401606]
- Suchecki D, Nelson DY, Van Oers H, Levine S. Activation and inhibition of the hypothalamic-pituitary-adrenal axis of the neonatal rat: Effects of maternal deprivation. *Psychoneuroendocrinology.* 1995; 20:169–182. [PubMed: 7899536]
- Takahashi LK, Turner JG, Kalin NH. Development of stress-induced responses in preweanling rats. *Dev Psychobiol.* 1991; 24:341–360. [PubMed: 1661243]
- Terman GW, Morgan MJ, Liebeskind JC. Opioid and non-opioid stress analgesia from cold water swim: Importance of stress severity. *Brain Res.* 1986; 372:167–171. [PubMed: 3708354]
- Trigo JM, Martin-García E, Berrendero F, Robledo P, Maldonado R. The endogenous opioid system: A common substrate in drug addiction. *Drug Alcohol Depend.* 2010; 108:183–194. [PubMed: 19945803]
- Van Dorpe S, Adriaens A, Polis I, Peremans K, Van Bocxlaer J, De Spiegeleer B. Analytical characterization and comparison of the blood-brain barrier permeability of eight opioid peptides. *Peptides.* 2010; 31:1390–1399. [PubMed: 20347901]
- Varlinskaya EI, Doremus-Fitzwater TL, Spear LP. Repeated restraint stress alters sensitivity to the social consequences of ethanol in adolescent and adult rats. *Pharmacol Biochem Behav.* 2010; 96:228–235. [PubMed: 20478326]
- Vázquez, DM.; Levine, S. Hypothalamic-pituitary-adrenal axis in postnatal life. In: Steckler, T.; Kalin, NH.; Reul, JM., editors. *Handbook of stress and the brain - part 2: Stress: Integrative and clinical aspects.* Elsevier; 2005. p. 3-21.
- Wee S, Koob GF. The role of the dynorphin-kappa opioid system in the reinforcing effects of drugs of abuse. *Psychopharmacology.* 2010; 210:121–135. [PubMed: 20352414]

- Wiedenmayer CP, Barr GA. Mu opioid receptors in the ventrolateral periaqueductal gray mediate stress-induced analgesia but not immobility in rat pups. *Behav Neurosci.* 2000; 114:125–136. [PubMed: 10718268]
- Winslow JT, Insel TR. Endogenous opioids: Do they modulate the rat pup's response to social isolation? *Behav Neurosci.* 1991; 105:253–263. [PubMed: 1645977]
- Woolley JD, Lee BS, Taha SA, Fields HL. Nucleus accumbens opioid signaling conditions short-term flavor preferences. *Neuroscience.* 2007; 146:19–30. [PubMed: 17320293]
- Yamada K, Nabeshima T. Stress-induced behavioral responses and multiple opioid systems in the brain. *Behav Brain Res.* 1995; 67:133–145. [PubMed: 7779288]
- Zylan KD, Brown SD. Effect of stress and food variety on food intake in male and female rats. *Physiol Behav.* 1996; 59:165–169. [PubMed: 8848477]

Highlights

Effects of ethanol and CTOP on intake and behavior in infant rats differed between short-term and long-term social isolation.

CTOP blocked ethanol-mediated enhancement of saccharin intake only in short-isolated females.

Ethanol decreased but CTOP increased locomotor and aversive reactions to social isolation.

Fig. 1. A timeline for administration of ethanol, CTOP, measurements of response to nociceptive stimulation and intake test

Pups were exposed to 0.1% saccharin for 8 minutes 4.5–5 hours after separation from the dam. Effects of ethanol (0, 0.5g/kg, i.p.) or CTOP (0; 0.1; 0.5 mg/kg, i.p.) on behavior and saccharin consumption were assessed during intake test. The decrease of thermal nociceptive reactivity after social isolation (isolation-induced analgesia, IIA) was measured as difference between latencies of paw withdrawal from hot plate before and after intake test. Conditions of social isolation differed between experiments: pups were isolated from littermates only during the intake test in Experiment 1 (short-term isolation, STSI) or isolated from littermates for the whole time of maternal separation in Experiment 2 (4.5–5 hours, long-term isolation, LTSI). Periods of isolation are marked by grey shading.

Fig. 2.

Effects of i.p. administration of selective mu- opioid antagonist CTOP (0; 0.1; 0.5 mg/kg) on intake, behavior (from left to right- locomotion, rearing and paw licking- grooming), and nociceptive reactivity of P12 pups exposed to STSL. Pups injected with saline (top row) or 0.5 g/kg ethanol (bottom row) were exposed to 0.1% saccharin during the intake test. Data are presented as mean $\pm$ S.E.M.

Fig. 3.

Effects of CTOP on temporal course of saccharin flow during STSI and after i.p. injection of saline (top) and 0.5 g/kg ethanol (bottom). Data are presented as per minute mean flow rate $\pm$ SEM. Lines indicate best-fit regression of flow temporal course.

Fig. 4.

Effects of i.p. CTOP (0; 0.1; 0.5 mg/kg) on intake, behavior (from left to right- locomotion, rearing and paw licking- grooming) and nociceptive reactivity of P12 pups isolated from dam and littermates for 5 hours prior to intake test. Pups injected with saline (top row) or 0.5 g/kg ethanol (bottom row) were exposed to 0.1% saccharin during intake test.

Fig. 5.

Effects of CTOP on temporal course of saccharin flow (intake) in pups socially isolated for 5 hours and injected with saline (top) or 0.5 g/kg ethanol (bottom) before intake test. Data are presented as per minute mean flow rate $\pm$ SEM. Lines indicate best-fit regression of flow temporal course.