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The role of social isolation in ethanol effects on the preweanling rat

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Abstract

The present experiments investigated the effects of acute ethanol exposure on voluntary intake of 0.1% saccharin or water as well as behavioral and nociceptive reactivity in twelve–day-old (P12) rats exposed to differing levels of isolation. The effects of ethanol emerged only during short-term social isolation (STSI) with different patterns observed in males and females and in pups exposed to saccharin or water. The 0.5 g/kg ethanol dose selectively increased saccharin intake in females, decreased rearing activity in males and attenuated isolation-induced analgesia (IIA) in all water-exposed pups. Ingestion of saccharin decreased IIA, and the 0.5 g/kg ethanol dose further reduced IIA. The 1.0 g/kg ethanol dose, administered either intragastrically or intraparentionally, also decreased IIA in P12 females, but not in P9 pups.

A significant correlation between voluntary saccharin intake and baseline nociceptive reactivity was revealed in saline injected animals, saccharin intake was inversely correlated with behavioral activation and latency of reaction to noxious heat after 0.5 g/kg ethanol in females. The 0.5 g/kg ethanol dose did not affect plasma corticosterone (CORT) measured 5 hours after maternal separation or 20 minutes after ethanol injection. Female pups CORT level was inversely correlated with magnitude of IIA that accompanied the first episode of STSI (pretest isolation) 1.5–2 hours before CORT measurement.

The present findings suggest that the anxiolytic properties of ethanol are responsible for enhancement of saccharin intake during STSI. Furthermore, differential reactivity of P12 males and females to STSI plays an important role in ethanol effects observed at this age.

Keywords

ethanol; social isolation; stress; neonatal; sweet taste; ingestive behavior

1. Introduction

There are substantial variations in ethanol acceptance by infant rats during early ontogeny. The developmental curve of ethanol consumption in preweanling rats can be characterized

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as a non-monotonic function of age, with peak intake rates observed between postnatal (P) days 10 and 13 [1–3]. . Several factors affect developmental changes of ethanol acceptance and intake during early ontogeny, such as prenatal experience [4, 5], peculiarities of infant taste perception [6], the reinforcing properties of ethanol [7], development of neurotransmitter systems [8, 9] and others. Interestingly, this period of maximal ethanol consumption as well as changes in sensitivity to the pharmacological effects of ethanol [10] corresponds to the "descending" phase of the stress hyporesponsive period (SHRP). When SHRP is pharmacologically manipulated during early infancy, corticosterone (CORT) response in these subjects to short-lasting stressors becomes adult –like [11, 12]. Experimental assessment of behavior in preweanling (infant) rats, including tests of ethanol intake or reinforcement typically include isolation from dam and littermates, if only for a few minutes [13, 14], and these short-term social isolation periods are certainly stressful and/or anxiogenic for the preweanling rat [15–17]. Given that sensitivity to ethanol challenge in animals exposed to acute stress is higher than that in unstressed subjects [18–21], changes in reactivity of infant rats to the stressful components of the ethanol intake test during "late" SHRP can affect pup's sensitivity to ethanol and, accordingly, can represent a substantial source of variation of ethanol intake between P10 and P14.

Importantly, , many experimental procedures employed for assessment of ethanol intake in infant rats facilitate competition between behavioral reactions to social isolation and independent ingestive behavior, with ethanol consumption being affected by this competition, and probably, vice versa. Mode and strength of the interaction between independent ingestive behavior and isolation-induced activity can change as a function of age. This represents an additional source of ontogenetic variance of ethanol intake.

Interaction between ethanol treatment and stress has been studied effectively in adult and adolescent rats with experimental manipulations of the "stressfulness" of physical and emotional stressors. Research using this experimental approach has revealed that acute and chronic ethanol effects, as well as intake of ethanol, are affected by strength and type of various stress factors, including social stressors.[19–25]. In contrast, only a few studies have explicitly compared effects of ethanol in preweanling rats across different conditions of social isolation. Recently, Arias et al [14] tested the effect of isolation stress on locomotor activity induced by a relatively high dose of ethanol (2.5 g/kg) in P15 rats. This study found that ethanol-mediated locomotor activation was expressed in pups separated from the dam but not in those grouped with littermates or kept in their home cage before the test. The present experiments extend our understanding of other interactions between effects of ethanol and effects of social isolation during early development.

The present experiments were designed to assess and compare effects of acute ethanol on voluntary saccharin or water intake and the behavior of rat pups exposed to long-term maternal isolation or short-term isolation (STSI) from siblings. Furthermore, animals isolated from the dam but kept in a group with their littermates were also tested. The hypothesis was that anxiolytic and stress-ameliorating properties of ethanol are responsible for enhancement of voluntary intake of 0.1% saccharin during STSI of P12 rats [10]. To achieve the goal of the present study we adopted a methodological approach that included combined assessment of voluntary intake and stress-related behavioral reactivity to social isolation. Voluntary intake and temporal patterns of ingestion were analyzed using a paradigm that permitted pups to freely extract fluid from an intraoral cannula [6, 10, 26]. This paradigm may be viewed as a model of early independent ingestive behavior.[27].

On the second week of postnatal life pups are capable of maintaining active behavior for some time when isolated from the dam. The response of the preweanling rat to separation from the dam is complex and various behavioral and physiological components of this

reaction have different temporal dynamics and also different adaptive significance [28]. The immediate response of infant rats to isolation from the dam and peers is emission of ultrasound vocalization (USV) accompanied by motor activation and exploration of the novel environment. Although analysis of USV was extensively used in animal models of anxiety-like behavior or early communication disorders [29, 30], there have been no studies specifically designed to assess USV in pups engaged in ingestive activity during STSI. It seems that USV emissions by isolated pups may interact with independent ingestive activity and USV rate may not be able to accurately measure distress reaction to social isolation during feeding.

Looking for alternative measures of stress reaction to social isolation we assessed stress-induced analgesia, the suppression of pain upon exposure to stressful stimuli. A decrease of response to noxious stimulation has been widely reported after exposure to various physical stressors [31, 32] or to stimuli associated with social stress [33–35]. However, reaction to stress is not the only factor associated with pain suppression. Given a dissociation between indexes of stress response, defensive behavior and changes in nociceptive reactivity [36, 37], we will refer to “analgesic responses“ observed in this study by the more specific term “isolation–induced analgesia” (IIA).

To characterize several aspects of behavioral reaction to social isolation we have designated locomotion as a measure of motor activation and exploration, rearing activity as an index of aversive reactivity to isolation, plasma corticosterone (CORT) content as a measure of systemic response to stress, and paw licking–grooming as an appetitive or aversive reaction depending on context and age of the animal. To reproduce pharmacological effects of ethanol during voluntary drinking of low (10%) concentrations of ethanol solution we administered several doses (0.5 vs. 1.0 g/kg) to P12 pups that were comparable to the amount of 10 % ethanol consumed by the naive preweanling rat during 20 minutes of free access to this fluid [6]. However, to determine pharmacological effects of ethanol independently of its orosensory effects, we exposed animals to 0.1% saccharin–a sweet tastant similar to 10% ethanol in terms of palatability [6].

Experiment 1 re-evaluated the effects of 0.5g/kg ethanol on voluntary intake of 0.1 % saccharin and water revealed earlier [10] and compared them with effects on IIA and non-ingestive behavior in pups undergoing STSI (long-term maternal deprivation plus short-term sibling deprivation). In this experiment we also sought predictors of ethanol effects on intake by assessing correlations among ethanol-mediated changes in intake, behavioral activity and nociception. Experiment 2 extended analysis of behavior of short-term-isolated animals to ethanol administered intragastrically (i.e., doses: 0; 0.5 and 1.0 g/kg). Experiment 3 analyzed the relationship between IIA and CORT response in normal and ethanol–treated animals exposed to water. Experiment 4 compared ethanol-mediated effects on nociceptive reactivity in P9 and P12 pups. Experiment 5 tested the isolation specificity of ethanol-mediated effects assessing intake, behavioral activity and changes in nociceptive reactivity in ethanol-treated pups kept in a group with littermates. Finally, Experiment 6 assessed effects of 0.5 g/kg ethanol in animals exposed to relatively long (5 hours) social isolation from both dam and siblings.

2. Materials and methods

2.1. Subjects

Experimental subjects were 9 and 12-day-old Sprague-Dawley rats bred at Binghamton University. A total of four hundred thirteen 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 pine shavings as bedding material. They were checked for births daily

between 8.00 and 10.00 a.m., and the day of birth was considered 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

2.2.1. General procedures—Testing procedures 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 (± 0.2°C) throughout each experiment using a temperature controller (FHC, Inc. Bowdoinham, ME); ambient room temperature was kept at 22–24°C. After completion of each test the plastic wall of the arena was cleaned using Kimtech wipes (Kimberly-Clark, Roswell, GA) soaked in warm water. A stream of fresh air from a Dust Destroyer pressured can (Branchburg, NJ) was also used to dry the walls and clear the paper-covered arena floor to eliminate any debris. If urination or fluid spills appeared on the arena floor, the paper was changed or the entire arena was replaced

Fluids were available to pups through an intraoral cheek cannula, which consisted of polyethylene tubing (PE10) with a flanged tip. The cannula was inserted into the anterior portion of the mouth through the cheek 2–3 mm caudal to the mystacial pad as described in previous studies [10, 38]. The cannulation procedure was accomplished within 5 – 10 sec. with no bleeding. After completion of cannulation procedure pups were kept either in holding boxes in groups (n=5) with littermates for five hours (Experiments 1, 2, 3, 4 and 5) or were isolated from littermates for the same time while being placed in a testing arena (Experiment 6). The holding boxes (size 18 × 18 cm) were filled with a mixture (1:1 proportion) of fresh and home cage shavings. The temperature of the floor of holding boxes was maintained at 30–32° C using heating pads.

A custom-made flow tracker was used to assess the volume and temporal patterns of voluntary intake through the intraoral cannula. The fluid flow tracking technique has been described in detail elsewhere [6, 10]. In brief, a piece of translucent PE-90 tubing (Clay Adams, Parsippany, NJ), 90–100 cm long, connected through a valve and adapter to an intraoral cannula was used as a reservoir for the fluid to be presented to the subject. The outer end of the PE –90 tubing was attached to the wheel of the fluid tracker and remained open. 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 directly proportional to the amount of fluid ingested. The resolution of flow measurement was approximately 0.2 μl / step. There are two units set up for simultaneous recording of flow in two subjects. A controller unit, built on a PIC16F874 microprocessor (Microchip Technology Inc), was used to program movements of step motors and to record data from 2 flow inputs and 4 switch inputs used to mark behavioral events. The data was sampled at a rate of 10 Hz and transferred to a personal computer via an RS232 port. The flow tracker was designed and programmed by W. Kashinsky.

The nociceptive thermal reactivity of the preweanling rat pups was assessed using the standard adult "hot-plate-paw-lift test". We modified a previously used procedure to fit infant rats [16, 39–42].. The test consisted of placing the animal's paw on the hot plate (temperature 49.5°C, Analgesia Meter, Model 39L, IITC Life Science, Woodland Hills, CA) and measuring latency of forepaw withdrawal. More specifically, the pup's right forelimb, two hind limbs, and trunk were gently 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 an electronic clock that was stopped upon removal of the paw. Cut-off time was set at 30 seconds. Before 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 25 seconds were removed from the experiment. Paw withdrawal latencies were measured 8 – 10 min. before and immediately after STSI or exposure to fluids (intake tests).

Rat behavior was monitored continuously during intake test and pretest STSI (see below) using a CCD video 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.

2.2.2. Research Design—The sequence and timing of behavioral procedures used in the present experiments are depicted in Figure 1. In terms of order of tests and timing of treatments two experimental protocols were adopted in this study. In the first protocol there were two exposures to fluids: the "pretest exposure" to water was followed by a main intake test during which animals were allowed to voluntarily ingest 0.1% saccharin or water through the cannula. While effects of ethanol, ingestion of fluids and variations of isolation condition were assessed during the main intake test no experimental treatments were applied during "pretest". This protocol was used in Experiments 1, 2, 3, 5, and 6 to compare behavior and intake across different conditions of social isolation and to assess sources of variance as well as possible predictors of voluntary intake. The sequence and timing of treatments and tests were the same for all experiments and isolation conditions: 1) cannulation; 2) first waiting period (duration- 3.0 hrs); 3) pretest STSI, with water freely available for 5 minutes; 4) second waiting period (duration 1.5–2.0 hrs); 5) treatment – administration of ethanol or saline; 6) delay – pups were returned to the holding box for 6–7 minutes; 7) intake test (duration - 8 minutes), during which water or 0.1% saccharin were freely available through the cannula (see Fig. 1). Social conditions during intake test and/or holding conditions were explicitly varied between experiments in order to reproduce three types of social isolation: 1) acute STSI; 2) acute long-term isolation (LTSI); 3) animals in contact with littermates. Note, however, that "social isolation" in the present study means separation from the littermates, as all pups were deprived from the dam for 3– 5.5 hours prior to behavioral tests.

The second protocol used in Experiment 4, included a single period of isolation during which animals were exposed to infused fluids (Experiment 4, Fig. 1). Experiment 4 tested effects of ethanol on the rat initial response to novelty and included comparison of IIA between P9 and P12. Experiment 4 also tested whether changes in mode of fluid access or timing of the intake test would affect effects of ethanol on nociceptive reactivity.

2.2.3. Experiments 1, 2 and 3—Experiments 1, 2 and 3 included STSI (Fig. 1, top). After removal from the dam pups were cannulated and left undisturbed in holding boxes with littermates (5 per group) for three hours. Three hours later they were exposed to first

STSI (pretest) with water being continuously available during the 5 minutes of isolation. No treatment was applied at pretest STSI. Animals were then returned to holding boxes for 1.5–2.0 hours. After this recovery period pups were exposed to the second STSI, or main intake test, during which they were placed in the same testing chamber for 8 minutes. In Experiments 1 and 3 pups were administered either saline or 0.5 g/kg ethanol (12.6% v/v in physiological saline) intraperitonealy (i.p.) 6–7 minutes prior to the second STSI (intake test). Water or 0.1% saccharin (Experiment 1) or water only (Experiment 3) was available through the cheek cannula during intake test. Latency of forepaw withdrawal from the hot plate was measured 8 min prior to, and immediately after, the pretest and intake test respectively.

Unlike Experiments 1 and 3, pups in Experiment 2 received intragastric (i.g.) administration of water or one of two doses of ethanol, 0.5 and 1.0 g/kg prior to test. For the latter doses ethanol was administered in a volume equivalent to 0.01 ml/g of body weight, as 6.3% or 12.6% v/v solution, respectively. Intragastric administration was performed 15 minutes before the intake test using a 10 cm length of polyethylene tubing (PE-10 Clay Adams, Parsippany, New Jersey) attached to a 1 ml syringe. The tubing was gently introduced through the mouth and slowly guided to the stomach.

In Experiment 3 trunk blood samples for CORT determinations were collected 4–5 minutes after the intake test that occurred about 5 hours after separation from the dam. Blood samples were centrifuged at 2 C for 10 min at 3000 rpm and plasma samples were frozen at −80 °C until further analysis. Plasma CORT levels were analyzed by radioimmunoassay using RIA kits obtained from ICN Biomedicals, Inc. (Orangeburg, NY). Treatment and timing of STSI procedures in Experiment 3 were identical to those in Experiment 1.

2.2.4. Experiment 4—Experiment 4 differed from Experiments 1–3 in two aspects: 1) a single isolation procedure was employed instead of two exposures to STSI; 2) free access to fluids during STSI that permitted voluntary control of intake was replaced by forced fluid delivery through an intraoral cannula. Cannulated animals were kept with littermates for 3 hours, baseline latency of paw withdrawal from the hot plate was measured, and then pups were given an i.p. injection of ethanol (12.6%, v/v). Pups were then returned to their littermates for 7–8 minutes. After this time elapsed animals were connected to an infusion pump and isolated (duration 8 minutes). Animals were then tested for nociception.

A custom-made rotary micro-infusion pump [43] with a mounted Gilmont syringe was used to infuse fluids (0.1% saccharin or water). The rates of infusion were set equal to the mean rates of voluntary fluid consumption that animals showed in tests employing free access to these fluids. These rates were estimated for P12 pups from data of Experiment 1 and for P9 rats from our recent study [10] using flow data obtained during the first 8 minutes of a 20-minute intake test. According to these estimations we set the rate of 0.1% saccharin infusions as 20 μl/min for P9 rats and 24 μl/min for P12 pups.

2.2.5. Experiment 5—In Experiment 5 social contact with littermates was maintained at all times except for the pretest STSI. Animals were kept in groups of 5 in two heated holding boxes filled with nest shavings during waiting periods as well as during the intake test. The size of the holding boxes (18 × 18 cm) allowed enough room for free movement, so pups were not forced to have permanent contact with peers during the intake test. We did not systematically record the time subjects spent without physical contact with littermates, but overall this time did not exceed 20% of the duration of the intake test. Two subjects were simultaneously assessed, one in each holding box. Water or 0.1 % saccharin were freely available through the intraoral cannula and voluntary intake was measured using the flow tracker. Either 0.5 g/kg ethanol or saline was injected (i.p.) 6–7 minutes prior to this intake

test.. The experimental protocol included the same pretest STSI as in Experiment 1 occurring 3–3.5 hours after maternal separation and serving as a reference procedure for assessment of baseline differences in reaction to STSI between Experiment 1 and Experiment 5.

2.2.6. Experiment 6—Experiment 6 included acute LTSI. The holding condition of animals in Experiment 6 differed from conditions in all other experiments of the present study: pups were separated from siblings immediately after cannulation, placed in the testing arena, and stayed in the same arena separated from their peers during the entire experiment (5–5.5 hrs). However, the timing of pretest and the intake test, as well as treatment procedure and doses of ethanol (saline and 0.5 g/kg, i.p.), were the same as in Experiment 1. However, unlike Experiment 1 pups were exposed to only water during pretest and 0.1% saccharin was freely available through intraoral cannula during the intake test. The latency of forepaw withdrawal from the hot plate was measured 8 min prior and immediately after each of the pretest and the main intake test.

2.3. Data analysis

The sample sizes for each of the following experiments were: Experiment 1: n=96, Experiment 2: n=60, Experiment 3: n=34, Experiment 4: n=119 (56 P9 pups and 63 P12 rats) -, Experiment 5: n=64 and, Experiment 6: n=40. 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. Numbers of males and females in each group were equated.

To allow comparisons between the pretest and the main intake test, which differed in duration (5 and 8 minutes respectively), voluntary fluid intake was indexed by mean flow rate through the intraoral cannula. Behavioral activity was scored manually by experimenters blind to treatment condition and recorded using a tracking device simultaneously with the flow data in real. Randomly selected video records and data files were compared with corresponding samples of behavioral scores to verify accuracy of manual scoring. The dependant variables were locomotion and exploration; rearing activity (wall climbing and head lifting combined); paw licking and grooming; baseline latency of paw withdrawal from hot plate; change of withdrawal latency (LT) expressed as maximum possible effect: MPE = (posttest LT - pretest LT)/(set-off time – pretest LT). Voluntary intake, locomotion, rearing and paw licking-grooming were recorded in all experiments except Experiment 4; latency of forepaw withdrawal from hot plate was measured in all experiments.

Pilot experiments as well as data from our previous study [10] indicated that the effects of ethanol on P12 rat pups are dependent on the sex of the animal. Therefore, sex was included as a factor in all experimental designs. Depending on whether animals were exposed to one or two fluids (water or 0.1% saccharin) during their intake test, 2- or 3-way ANOVAs were employed to analyze all dependant variables using sex, fluid (water, saccharin) and ethanol (0, 0.5, 1.0 g/kg) as independent factors and two fluid exposures (pretest, intake test) as repeated measures. Saline, 0.5 or 1.0 g/kg ethanol was injected into water-infused subjects in Experiment 4, while saccharin-exposed animals received only saline injections, so a 4 (treatment) × 2 (sex) ANOVA was adopted in this experiment, in which the treatment factor included four groups: saline animals exposed either to water or saccharin and water-exposed pups injected with 0.5 or 1.0 g/kg ethanol.

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 run through the ANOVA. Post hoc comparisons were conducted using the Fisher's PLSD test. Student's t-tests for independent, unequal samples were used to compare mean values of intake and behavioral data between different experiments. The type 1 error level (alpha) was set at 0.05. Effect size was expressed as Cohen's d, defined as the 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 ±S.E.M.

3. Results

3.1. Experiment 1: Effects of ethanol in P12 pups during STSI

3.1.1. Pretest STSI—No differences in water consumption, rearing activity or paw-licking-grooming were found due to treatment or sex during the pretest STSI. However, Figure 2 shows that male pups spent significantly more time in locomotion than females during first STSI (pretest), p=0.008.

Males did not differ from females in baseline thermal nociception and no differences were found in this measure between pups assigned to different treatment groups. Changes between baseline (prior to any isolation or fluid exposure) and post– first-isolation latency of paw withdrawal from the hot plate indicated that 5-minutes isolation from littermates is enough to induce an analgesic reaction in P12 pups. To clarify, since the change in normalized latency (MPE) was positive and was significantly greater than zero ($p<0.01$) it can be said that isolation from littermates for 5 minutes induced analgesia in P12 pups (see Fig. 2; bar graph for nociceptive reactivity).

3.1.2. Intake test

3.1.2.1. Flow rate: A mixed ANOVA with pretest STSI and intake test STSI treated as a repeated measure revealed main effects of fluid, $F(1,88)=38.41$, $p<0.001$, and test, $F(1,88)=23.57$, $p<0.001$, as well as effects of the interactions test × fluid, $F(1,88)=54.74$, $p<0.001$, sex × fluid × ethanol dose, $F(1,88)=7.93$, $p=0.006$, test × ethanol, $F(1,88)=6.03$, $p=0.016$, and test × sex × ethanol, $F(1,88)=4.06$, $p=0.047$.

During the intake test the rate of saccharin flow (i.e., rate of intake) was significantly higher than water flow rate ($p<0.001$; top row vs. bottom row). Comparison of subjects ingesting saccharin indicated that females treated with 0.5 g/kg ethanol took in significantly more saccharin than males under the same treatment, $p=0.013$, (Fig. 2, top), as well as more than saline–treated females, $p=0.008$. However, flow rates did not differ between saline- and ethanol-treated animals exposed to water during the intake test (Figure 2, bottom).

3.1.2.2. Locomotor activity: An ANOVA on locomotion indicated significant main effects of fluid, $F(1,88)=10.47$, $p=0.002$, test, $F(1,88)=48.32$, $p<0.001$, and their interaction, test × fluid, $F(1,88)=9.01$, $p=0.003$. Time spent in locomotion decreased during STSI associated with intake test in comparison to pretest STSI, and activation of pups ingesting saccharin was lower than that of pups exposed to water, $p=0.001$, (compare top and bottom panels in Figure 2). A significant interaction between test and sex was also revealed, $F(1,88)=4.69$, $p=0.033$, with male pups spending significantly more time in locomotion than females during pretest STSI, $p=0.008$, but not during the intake test.

3.1.2.3. Rearing activity: Rearing activity during the intake test was lower in subjects exposed to saccharin than in pups ingesting water, as indicated by a significant main effect of fluid, $F(1,88)=5.81$, $p=0.018$, and significant interaction between test and fluid, $F(1,88)=5.44$, $p=0.022$. A significant 3-way interaction [test × ethanol × sex × fluid,

F(1,88)=7.37, p=0.008] revealed that 0.5 g/kg ethanol significantly attenuated isolation-induced rearing only in pups ingesting saccharin, p=0.001. Animals exposed to water were not affected by ethanol.

3.1.2.4. Paw licking and grooming: ANOVA on paw licking-grooming revealed a significant main effect of test, $F(1,88)=14.79$, $p<0.001$, and significant test × fluid interaction, $F(1,88)=6.29$, $p=0.014$. Time spent in paw licking-grooming in animals ingesting saccharin decreased relative to that in pretest STSI, $p<0.001$ (Fig. 2, top), but pups exposed to water during the intake test STSI showed a level of paw licking-grooming similar to that in pretest STSI. The 0.5 g/kg ethanol did not affect paw licking-grooming.

3.1.2.5. Nociceptive reactivity: The latency of paw withdrawal from the hot plate before the intake test (second STSI), was lower than the baseline latency measured before first (pretest) STSI [main effect of test, $F(1,88)=247.02$, $p<0.001$; 9.44±0.53 s vs. 7.07±0.48 s]. The normalized differences between latency of paw withdrawal from the hot plate measured before and after STSI and computed as maximal positive effect under normalization, MPE were significantly greater than zero in all treatment groups, indicating IIA (Fig. 2).

The ANOVA on isolation–induced changes in nociceptive reactivity revealed significant interactions between test and fluid, $F(1,88)=11.59$, $p=0.001$ and between test and ethanol, $F(1,88)=6.58$, $p=0.012$. This ANOVA also indicated a marginal test-x-sex interaction, $F(1,88)=3.57$, $p=0.062$. The saline-injected pups ingesting saccharin showed significantly less IIA than saline-injected pups ingesting only water, $p=0.017$ (Figure 2, compare top and bottom line graphs). In saline pups ingesting saccharin the normalized difference between pre- and post-test response latencies did not differ from that after the first STSI, $p=0.169$, but did differ in ethanol-treated pups, $p<0.001$. 0.5 g/kg ethanol decreased IIA in pups exposed to water during intake test relative to that found after the first (pretest) STSI, $p=0.025$ (Fig. 2 bottom). This effect, however, was significantly greater in females than in males, $p=0.042$.

3.1.2.6. Correlation between intake and behavior: The comparison between mean values demonstrated that 0.5 g/kg ethanol affected not only intake of 0.1% saccharin but also behavioral reactions to STSI. Therefore, variation in intake could be related to effects of ethanol on behavior and nociceptive reactivity, so we assessed the strength of co-variation between ethanol effects on saccharin intake and changes in behavioral reactions to STSI using partial correlation analysis.

Pearson coefficients of partial correlation were computed separately for 2 sets of variables. The first set included saccharin intake and three behavioral variables – locomotion, rearing activity, licking-grooming. The second set included saccharin intake and four indexes of nociceptive reactivity, including baseline reactivity measured prior to pretest STSI or prior to intake test, and normalized differences between paw withdrawal latency after pretest STSI and that during the STSI accompanying the intake test. Accordingly, correlation coefficients represent either estimates of correlation between intake and each of three behavioral variables -- locomotion, rearing, and licking-grooming -- or correlation between intake and 4 indexes of nociceptive reactivity. Correlation between saccharin intake and the behavioral variables was analyzed separately for males and females in saline- and ethanol treatment groups. To increase the power of this correlation analysis we pooled data from the present experiments with data obtained on saline-treated animals in a study that employed the same isolation procedures, behavioral scoring and intake measurements (Kozlov et al., 2010, in preparation). The number of subjects included in this analysis, values of Pearson coefficients of bivariate and partial correlation, t–statistics values, degrees of freedom and probabilities of null hypothesis (correlation is equal to zero) are given in Table 1.

Ethanol–treated and control pups demonstrated different patterns of correlation between saccharin intake and reactions to STSI (table 1 and left-column scatterplots on Fig. 3). Saline–treated animals generally had a moderate but significant negative correlation between saccharin intake and baseline nociceptive reactivity [for males, partial r = −0.579, t(15)=−2.75, p=0.015; for females, partial r = −0.527, t(15)=−2.39, p=0.037]. The dependence of saccharin intake on baseline nociceptive reactivity clearly dominated over other correlations. Note that a causal relationship could be suggested here because baseline nociceptive reactivity was measured about 2 hrs prior to the intake test. Males expressed a significant negative correlation between saccharin intake and rearing activity during STSI, partial r=−0.562, t(16)=−2.72, p=0.015. Females also tended to demonstrate the same association, but it was weaker than in males and insignificant, partial r=−0.372, t(16)= −1.60, p=0.129.

The same relationship between baseline nociception and saccharin intake was not observed after administration of 0.5 g/kg ethanol. However, this ethanol treatment produced a different set of correlations that were sex-specific. Among ethanol–treated females there was a negative correlation between saccharin intake and behavioral measures of reaction to STSI including: time spent in locomotion, partial r=−0.660, t(15)=−3.40, p=0.004; rearing activity, partial r = −0.522, t(15)=−2.37, p=0.032; and magnitude of IIA, partial r = −0.535, t(14)=−2.37, p=0.032 (see Table 1 and Figure 4). Among males, saccharin intake tended to be positively correlated with magnitude of IIA after first STSI, partial r=0.468, p=0.078, and negatively correlated with baseline nociceptive reactivity preceding the second STSI (intake test), r = −0.494, p=0.061. Thus, 0.5 g/kg ethanol changed correlational patterns differently in males and females, with saccharin intake being directly dependent on baseline nociceptive reactivity in control animals although inversely related to locomotion among females given 0.5 g/kg ethanol.

3.2. Experiment 2: Effects of ethanol in P12 pups during STSI: intragastric ethanol administration

3.2.1. Pretest STSI—Student’s t-tests for independent, unequal samples compared behavioral and nociceptive reactivity during the first (pretest) STSI in Experiment 1 and Experiment 2. No differences were found in level of locomotion, paw licking-grooming or water intake during pretest STSI. However, rearing activity during pretest STSI was lower in Experiment 2 than Experiment 1, t(105)=2.27, p=0.025, whereas magnitude of IIA was higher in animals from Experiment 2, t(100)=3.39, p<0.001 (compare bars in the left portions of the plots for nociceptive reaction on Fig. 2 and Fig. 5), baseline nociceptive reactivity also increased in Experiment 2, but at a marginal level, t(98)=1.84, p=0.067.

3.2.2. Intake test—Repeated–measure ANOVA did not reveal significant differences between behavioral reactions to the first and second STSI during which pups were exposed to water. No significant effects of sex, ethanol or any significant effects of interaction were revealed among locomotion, rearing activity and water intake. A significant effect of test was found for paw licking-grooming, F(1,54)= 70.95, p<0.0001, with pups spending a longer time in this activity during intake test STSI than during pretest STSI (data not shown). An ANOVA revealed a significant main effect of test for IIA (expressed as MPE), F(1,54)= 21.24, p<0.0001, and a significant test × ethanol interaction, F(1,54)= 7.58, p=0.0013. The main effect of test was produced by changes of nociceptive reactivity in pups treated with ethanol. The magnitude of IIA was significantly lower in pups administered 1.0 g/kg ethanol, i.e., than in saline treated animals (Fig. 5). The highest reduction of IIA was found in females treated with 1.0 g/kg ethanol compared to saline controls, effect size d=2.03. Females treated with 1.0 g/kg ethanol even demonstrated negative difference of withdrawal latencies, indicating that post-test nociceptive reactivity was higher than before

STSI. The dose 0.5 g/kg ethanol, i.g., also significantly decreased IIA, (p=0.042) in males and females, but this reduction of IIA had a smaller effect size than that associated with 1.0 g/kg ethanol (Cohen effect size for males: d=0.77, females: d=0.52).

3.3. Experiment 3: Effects of ethanol in P12 pups during STSI: relation between IIA and CORT

3.3.1. Pretest STSI—Subjects in Experiment 3 exhibited comparable behavioral and nociceptive reactivity following first (pretest) STSI as pups in Experiment 1: the difference between experiments was not significant for baseline nociception, locomotion; rearing activity, paw licking-grooming and post test latency of paw withdrawal from the hot plate.

3.3.2. Intake test—As was found for water-exposed subjects in Experiment 1, a dose of 0.5 g/kg ethanol did not affect any of the behavioral indexes of pup reaction to STSI during the intake test. Although magnitude of IIA tended to be decreased in ethanol-treated females relative to saline-treated controls (effect size d=0.73, Fig. 6, bottom left column, line graph), this difference did not reach statistical significance [for the sex × ethanol interaction, $F(1,36)=2.28$, $p=0.141$, and for the test × sex × ethanol interaction, $F(1,36)=0.477$, $p=0.494$]. There was also no overall significant effect of ethanol on CORT level, $F(1,32)=2.42$, $p=0.129$, and no sex × ethanol interaction, $F(1,32)=2.55$, $p=0.117$. However, there was a tendency for higher plasma CORT concentration after ethanol treatment in females (Fig. 6, upper left plot).

Analysis of partial correlation was conducted to assess the relationship between CORT concentration and scores of behavioral and nociceptive reactivity to STSI. The procedure was the same as in Experiment 1: two separate analyses were performed, one testing the correlation between CORT and behavioral variables, and the second testing the correlation between CORT measurements and variables characterizing nociceptive reactivity. Because there was no effect of ethanol treatment we have pooled ethanol and saline groups in order to increase reliability of this correlation analysis. Values of Pearson coefficients of bivariate and partial correlation are present in Table 2.

There was no correlation between CORT and magnitude of IIA associated with second STSI (post- test latency of paw withdrawal was measured 5 minutes before blood sampling). . There was, however, a sex–dependent pattern of correlation between CORT and magnitude of IIA associated with first STSI (1.5 hours before blood sampling): Although in males there was no correlation between CORT level and magnitude of analgesia induced by first STSI (partial $r=0.20$, $p=0.48$), in females this correlation was inverse and relatively strong (partial $r = -0.59$, $p= 0.032$). Overall, saline– and ethanol-treated animals demonstrated similar regression trends (Fig. 6, bottom plot).

3.4. Experiment 4: Effects of ethanol on IIA: comparison between P9 and P12 pups

Nine-day old and twelve-day old pups demonstrated comparable mean values of baseline latency of paw withdrawal from hot plate: 8.74± 0.42 s on P9, 8.89± 0.48 s on P12, $t(28)= 0.21$, $p=0.839$ (see bars on Fig. 7). However, the normalized difference between pre- and post-isolation latencies (index of IIA) was significantly higher in saline– treated animals on P12 than in their counterparts on P9, 0.363± 0.067 vs. 0.148 ± 0.051; $t(28)= 2.57$, $p=0.016$ (see line graph on Fig. 7).

On P12 an ANOVA revealed a significant main effect of ethanol treatment, $F(3, 56)=2.94$, $p=0.041$, but no effect of sex or interaction between sex and treatment. The pattern of changes in nociceptive response to STSI after ethanol administration resembled that observed in Experiments 1, 2 and 3. In comparison to saline controls, the magnitude of IIA

significantly decreased in animals injected with 0.5 g/kg ethanol, p=0.006. However, after 1.0 g/kg ethanol, reduction of IIA was significant only in females, p=0.018 (Fig. 7). The reduced IIA was also observed in pups receiving saccharin through their cannula, p=0.034. Although the effects of saccharin and 1.0 g/kg ethanol on analgesic response to STSI were higher in females than in males, the sex differences were not statistically significant.

The ingestion of saccharin and injection of ethanol also affected the magnitude of IIA in P9 animals, $F(3, 48)=7.32$, $p<0.01$. Notably, the pattern of changes in nociceptive reactivity differed at P9 and P12. While at P9 saccharin infusions significantly decreased the analgesic response to STSI comparative to the reaction seen to infusions of only water, p=0.005, ethanol did not affect IIA at P9 (Fig. 7, left panel). Nevertheless the magnitude of IIA in P9 animals treated with 0.5 or 1.0 g/kg ethanol were comparable to those in P12 pups after injections of the same doses of ethanol, $t(28)= 0.30$, p=0.763, and $t(28)= 0.33$, p=0.741, correspondingly.

3.5. Experiment 5: Effects of ethanol in P12 pups kept with littermates

3.5.1. Pretest STSI—P12 pups in Experiment 5 exhibited rearing and paw licking–grooming activity after their first (pretest) STSI that were comparable to those observed in Experiment 1 under the same conditions. However, locomotor activity of females in Experiment 5 was significantly higher than that of females in Experiment 1, $t(64)=2.34$, p=0.023. Males in Experiment 5 showed slightly higher level of locomotion (29.9±2.45%) than females (25.5±2.45%), but the difference was not significant, $t(62)=1.24$, p=0.218. The behavioral scores did not differ between animals assigned to different treatment groups.

Baseline nociceptive reactivity also did not differ between Experiment 5 and Experiment 1. However, females in Experiment 5 responded to pretest STSI with significantly higher magnitude of IIA than females from Experiment 1, $t(69)=2.67$, p=0.009, (compare bars on far -right plots in Fig. 2 and Fig. 8).

3.5.2. Intake test

3.5.2.1 Flow rate: While kept in a group with littermates saline-treated pups demonstrated a slightly lower rate of saccharin consumption (18.23± 1.39 μl/min) than their short-isolated counterparts (20.89±1.58 μl/min, Experiment 1), but the difference between isolated and grouped subjects was insignificant, $t(38)=1.26$, p=0.216. A mixed between-within ANOVA (sex × fluid × ethanol × test) revealed a significant main effect of test, $F(1,56)=7.32$, p=0.009, and of the interaction between test and fluid, $F(1,56)=15.78$, $p<0.001$. The 0.5 g/kg ethanol did not affect saccharin consumption in pups grouped with their littermates: rate of saccharin flow was higher than rate of water flow in saline- as well ethanol- treated animals, $p<0.001$ and p=0.02, respectively (Fig. 8).

3.5.2.2. Behavioral activities: Saline animals ingesting saccharin, while kept in a group with littermates, spent comparable time in locomotion with their counterparts exposed to STSI (Experiment 1). However, water-exposed short -isolated pups from Experiment 1 spent significantly more time in locomotion, $t(35)=4.64$, $p<0.001$, rearing activity, $t(37)=2.85$, p=0.008, paw licking grooming, $t(32)=3.58$, p=0.001 than their grouped counterparts in Experiment 5,. (compare Fig. 2 vs. 8). While kept together with littermates during intake test, subjects spent significantly less time in all of three behavioral activities than during pretest STSI, as reflected in significant main effects for locomotion, $F(1,56)=95.18$, $p<0.001$, rearing, $F(1,56)=57.86$, $p<0.001$, and licking and grooming, $F(1,56)=52.01$, $p<0.001$. Administration of 0.5g/kg ethanol did not affect any of these relationships (see Figure 8).

3.5.3.3. Nociceptive reactivity: Significant main effects of sex, F(1,56)=4.25, p=0.044, and test, F(1,56)=92.62, p<0.001, were revealed for the normalized difference between pre- and post-test nociceptive reactivity, but there was no significant main effects or interactions involving ethanol. Latency of paw withdrawal from the hot plate after intake test, during which pups were grouped with littermates, was significantly lower (p<0.001) than after pretest STSI. In other words, analgesia was present in pups following an isolation period, but absent if the pups grouped with littermates regardless of injection fluid (Fig. 8, right most column). Furthermore, the MPE following the intake test did not differ from zero after saline injection in animals ingesting saccharin, t(16)=1.03, p=0.316, and was slightly above zero in pups exposed to water, t(16)=2.04, p=0.059. This indicates that, in terms of analgesic response, these pups were insensitive to injection and the fluid available at intake test. The significant effect of sex was related to the higher magnitude of analgesic response to pretest STSI observed in female comparative to male pups (open and shaded bars on far –right plots, Fig. 8).]

3.6. Experiment 6: Effects of ethanol in P12 pups isolated from littermates for 5 hours

3.6.1. Pretest—Student's t-tests for independent, unequal samples found that pups isolated for 3 hours spent significantly less time during pretest in locomotion, t(85)=3.38, p=0.001, than animals in Experiment 1, which were isolated for only 5 minutes at pretest. Rearing activity was also lower in long- isolated pups than in subjects from Experiment 1, t(41)=2.25, p=0.029 (significant difference for females only -- compare bar graphs in Fig. 2 and Fig. 9). However, subjects in Experiment 6 and Experiment 1 were comparable in the mean rate of water consumption during pretest and in amount of time spent in licking-grooming. Although pups in Experiment 6 were isolated from littermates for 3 hours before pretest they did not differ in baseline nociceptive reactivity from subjects in Experiment 1 who were kept in groups with littermates during this period. However, long-isolated pups, in contrast to animals in Experiment 1, did not increase latency of paw withdrawal from hot plate after water exposure, t(39)= 1.35, p=0.184, indicating that exposure to water during pretest did not induce analgesia (see bar graphs on Fig. 9).

3.6.2. Intake test

3.6.2.1. Flow rate: During the intake test, saline-treated pups isolated from littermates for 5 hours ingested 0.1% saccharin more rapidly (28.22± 1.52 μl/min) than their short-isolated counterparts from Experiment 1 (20.87± 1.58 μl/min), t(42)=3.34, p=0.002. ANOVA revealed only significant main effects of test, F(1,36)=30.57, p<0.001, with saccharin flow at intake test being higher than that of water flow at pretest (p<0.001, Fig. 9). 0.5 g/kg ethanol did not affect saccharin intake of animals given LTSI.

3.6.2.2. Behavioral activities: Significant main effects of test were found for locomotion, F(1,36)=20.74, p<0.001, rearing activity, F(1,36)=21.26, p<0.001, paw licking and grooming, F(1,36)=6.67, p=0.014. At the time of the intake test -- after 5 hours of isolation -- pups spent less time in locomotion, rearing, licking, and grooming than during pretest after they had been isolated for 3 hours. No main effect or interaction with ethanol was found for any of the behavioral variables.

3.6.2.3. Nociceptive reactivity: After pups ingested saccharin during the main intake test, paw withdrawal latency from the hot plate did not change significantly in comparison to pretest values, F(1,36)=2.03, p=0.163 (Fig. 9, plot on the right). The 0.5 g/kg ethanol did not affect nociceptive reactivity in pups isolated from their peers for 5 hours, F(1,36)=0.651, p=0.425.

4. Discussion

The present study compared effects of ethanol across three conditions of social isolation for P12 rats. The general results indicated that low dose (0.5 g/kg) ethanol can alleviate the effects of short-term (8 min) isolation from littermates among pups subjected to relatively long-term isolation from their dam. Similar ethanol-induced alleviation did not occur for pups isolated from littermates (and dam) for a much longer period (5 hr). No effects of ethanol were revealed in pups kept in a group with littermates for the same period.

Various forms of social isolation using rodants have been used in preclinical studies for characterization of anxiolytic drugs, with rate of USV being employed in these tests as a measure of anxiety and distress reaction [30] [44]. For technical reasons we did not record USV of individual rat pups, but have instead measured an “analgesic response” to isolation. Stress-induced analgesia is not one, but probably a set of heterogeneous phenomena with different mechanisms of pain control being activated by different stressors [31, 32]. Because many findings of the present study are related to assessment of pain suppression in response to social isolation, and because some hypothesized mechanisms of ethanol effects are based on specific interpretations of “analgesic response”, we first discuss the functional significance of this phenomenon and then analyze variations of IIA and changes of other behavioral activities as a function of isolation conditions and ethanol treatment.

Rat pups in the present study were given experimentally controlled social stressors – long lasting maternal deprivation and short-term separation from littermates -- as well as transient (phasic) physical stressors inextricably associated with a variety of short-term tests and treatments. Because analgesic responses can be induced in preweanling rats by separation from the dam as well as by isolation from siblings [45], the “raw measure” of nociceptive reactivity used in the present experiments (latency of paw withdrawal from hot plate) was probably affected by the combined effects of long lasting maternal separation stress, STSI from littermates and procedures associated with treatments. To measure an analgesic response to short–term interventions separately from effects of maternal separation, we calculated the normalized difference between pretest and post latencies. With this index long-term variations in nociceptive reactivity were “dissected” from effects caused by the isolation procedure and transient physical stressors associated with experimental treatment and measurements.

Nociceptive reactivity is quite sensitive to novelty and anxiogenic situations. Transient analgesia has been reported in adult rats and mice tested in an elevated maze or open field - procedures traditionally used for assessment of anxiety-like behavior [46–48]. However, increased pain sensitivity has also been found in several situations that produce fear and anxiety [49, 50]. Pain suppression in the preweanling rat is most effectively elicited by exposure to an unfamiliar adult male, a threat that generates fear and passive defensive behavior [33]. Alternatively, magnitude of IIA in preweanling rats is correlated with specific characteristics of USV [16], which is considered an ethologically valid measure of the intensity of an aversive affective reaction and/or anxiety. The literature suggests that pain attenuation observed in experimental subjects after STSI most probably reflects anxiety and fear. Accordingly, reduction of IIA with ethanol indicates that at this age, low doses of ethanol known to induce appetitive reinforcement are generally anxiolytic and fear suppressive. This interpretation is consistent with a large body of data that has documented anxiolytic effects of various doses of ethanol in the adult, adolescent and infant rat [7, 51] [52–55]. In particular, effects of attenuation of IIA resemble the calming effect of low doses of ethanol on socially isolated animals assessed in terms of USV [56]. Moreover, the effects of ethanol-mediated reduction of IIA were relatively robust against changes in the route of ethanol administration (i.p. or i.g.) and mode of fluid delivery.

To what extent is IIA affected by the systemic hormonal response to STSI? Given that subjects in the present experiments were examined during the stress hyporesponsive period of ontogeny, characterized by low glucocorticoid activation to short lasting stressors (injections, handling, short-term exposure to novel environment), it seems reasonable that the impact of CORT on nociceptive reactivity should be low. This notion was supported by lack of a correlation between CORT concentration and magnitude of analgesia caused by STSI immediately preceding CORT measurement (Experiment 3). The fact that CORT and IIA effects of ethanol treatment diverge from each other (see Figure.6) also indicate that IIA was not caused by CORT release associated with STSI.

Consideration of IIA as a measure of anxiety rather than a more general expression of the stress response can help explain a seemingly paradoxical, inverse correlation between CORT measured 5 hours after separation and magnitude of pain reduction to pretest STSI that occurred 1.5 hours before blood sampling for CORT measurement. As mentioned above, behavioral and physiological components of the infant rat's reaction to social isolation differ in their temporal dynamics and adaptive significance [28]. While the initial phase of the pup's response to isolation -- motor activation, USV production and decrease in pain sensitivity -- can be viewed as an alarm–arousal reaction that facilitates reunion with the dam, activation of a neurohormonal cascade (expressed as CORT elevation and increased responsiveness to stressors) represents a delayed adaptation response that helps to maintain homeostasis against continuing environmental challenge. It could be hypothesized that these two phases of reaction to maternal separation and/ or social isolation represent complementary processes and CORT activation is delayed in animals displaying an intensive and/or long anxiety-arousal reaction.

The present experiments replicated the finding of our recent study [10] in which 0.5g/kg ethanol selectively increased voluntary intake of 0.1% saccharin in P12 female pups. The important new finding presented here are that ethanol–mediated enhancement of saccharin intake was confined to situations of short-term (8 minutes) social isolation and was not observed neither in pups separated from their peers for 4–5 hours nor in animals kept together with littermates prior to and during the intake test.

Upon exposure to STSI on P12 pups injected with only saline demonstrated decreased nociceptive reactivity, more rearing and paw licking-grooming than pups kept with littermates (compare Figures 1 and 8) or those isolated for 5 hours (Experiment 6, Figure 9). This suggests that STSI is more anxiogenic and aversive to P12 pups than the other two situations in which intake was measured. Importantly, animals isolated for a short time and injected with saline consumed less saccharin than counterparts that had adapted to the test environment for 5 hours, suggests that anxiety during STSI has inhibitory effects on intake of a palatable fluid. Low dose (0.5 g/kg i.p) ethanol attenuated IIA, decreased isolation - induced rearing but enhanced intake of saccharin. Moreover, during STSI saccharin flow rate was inversely correlated with behavioral activation (locomotion), aversive activity (rearing) and magnitude of IIA in ethanol-treated females. In other words, for female pups given 0.5 g/kg ethanol, the less the behavioral activation, aversive activity and pain suppression to isolation, the more saccharin they ingested during STSI. Taken together, these data indicate an association between anxiolytic, stress-ameliorating properties of low doses of ethanol and facilitatory effects of the same doses on voluntary intake of palatable fluids in P12 rats.

The other interesting, but paradoxical, finding of the present study is that not only ethanol, but also voluntary ingestion of 0.1% saccharin during STSI attenuated IIA. At first glance this finding contradicts the well established "analgesic" effect of sweet substances observed in preweanling rats as well as in human infants [39, 42, 57]. However, the analgesic effect of

sweet solutions has not been studied in pups isolated for periods as short as the present 8-min duration. Rather, this effect was shown in other studies after an infusion of sweet fluids to suckling pups or animals kept in contact with littermates [39, 58]. These situations clearly differ in behavioral activities displayed by pups and they probably differ in mechanisms of pain control. We suggest that ingestion of 0.1% saccharin decreases analgesia related to anxiety and distress during STSI by reducing stress, while infusions of sweet solutions to socially non-deprived or attached pups induce analgesia through activation of the endogenous opioid system as proposed by Blass [16]. It should be noted, however, that 0.1% saccharin in the present study (Experiment 5) did not induce analgesia in pups grouped with littermates, contrasting with findings of Anseloni et al [39] who observed an analgesic response to infusions of sweet solutions under similar condition. Differences in fluids, mode of fluid delivery and, accordingly, intensity and duration of sweet stimulation can account for differences in the analgesic effects observed in the present experiments and previous studies. Further investigation is required to determine which factors are responsible for the different analgesic effects of sweet substances reported for the preweanling rat.

If one were willing to assume that, in terms of taste reactivity, 0.1% saccharin is equivalent to 10% ethanol [6], it could be hypothesized that orosensory properties of low concentration ethanol and the pharmacological effects of low doses of ethanol act in additive fashion to reduce isolation-induced stress and/or anxiety. Note that in control animals the amount of saccharin consumed was inversely correlated with baseline latency of nociceptive reaction established prior to any treatment or social isolation. The increased latency of nociceptive reaction indicates pain suppression due to maternal separation and the inverse correlation between this measure and saccharin ingestion suggests that pups exhibiting a high analgesic response to separation from the dam consumed small amounts of saccharin during STSI from littermates. Given an association of pain inhibition with the anxiety reaction to stress [31, 46], apparently the more anxious the pups were after separation from their dam the less saccharin they took in during the intake test. On the other hand, the inverse correlation between saccharin intake and locomotion in ethanol-treated female pups could indicate a competition between ingestion and motor activation to STSI. Taken together, these findings suggest that anxiety must be relatively low for appetitive (hedonic) signals from oral sweet stimulation to control intake in short-isolated animals. By reducing anxiety and motor activation in isolated pups, ethanol apparently increases their sensitivity to appetitive taste stimulation that ordinarily engage these pups in ingestive activity and decreases time spent in concurrent motor activity, further reducing anxiety. This hypothetical mechanism of positive interaction between appetitive taste stimulation and the anxiolytic effect of low doses of ethanol can explain heightened consumption of 10% ethanol by P12 female rats in a situation resembling the procedure of STSI in the present study [6].

The present experiments have found that many effects of ethanol and correlations between behavioral reaction to isolation and intake were sex-dependent.. Sex differences appeared only when pups were exposed to STSI and many of the effects were found only in females or were more pronounced in females than in males. It seems that a female preweanling rat, when isolated from littermates for a short time, is more sensitive to the anxiolytic effects of ethanol than her male counterpart. This notion is supported by other sex–specific effects of ethanol: selective enhancement of saccharin intake in short-isolated females, stronger reduction of IIA in females, and selective quieting of females exhibiting high saccharin intake after 0.5 g/kg ethanol. Importantly, male and female 12-day old rats differed not only in reaction to ethanol but in their baseline reactivity to STSI. Males were more active than females during their initial exposure to STSI, but only females demonstrated the inverse correlation between magnitude of their analgesic response to first (pretest) STSI and CORT measured 1.5–2 hrs later. These data provide support for the idea that P12 males and females

are fundamentally different in their mode of responding to STSI and this difference plays an important role in effects of ethanol observed at this age [6].

Adult male and female rodents differ in many aspects of stress-related behavior and, in particular, in mechanisms of pain control engaged in response to stress [34, 59–62]. Sex differences in anxiety reactions and responses to novelty have also been widely reported for adult animals [34, 63, 64], [see [65]for extensive review]. Although findings vary between studies, with expression of sex differences being dependent upon a variety of factors such as assessment conditions, age, and the behavioral model used, studies generally agree that females are less anxious than males in a novel situation [51, 66, 67]. Infant female rats in the present study also demonstrated less motor activation, aversive activity, and analgesic response to STSI then male rats, which is reminiscent of the pattern of sex difference observed in adults in many (but not all) tests for anxiety.

In comparison with the abundant literature on sex differences in adults, data on gender–specific reaction to social isolation and ethanol treatment in infant rats are scarce. To our knowledge, this study and our previous experiments [10, 68] are the first to report a sex difference in the effects of ethanol on voluntary intake and isolation-induced reaction in the preweanling rat. Nevertheless, our findings of higher female sensitivity to anxiolytic effects of ethanol are in agreement with data obtained in adolescents [53]. The female–specific effect of ethanol- mediated enhancement of voluntary intake is also reminiscent of the observation that juvenile female rats (P22) consume more 30% ethanol than male counterparts [69].

Sex differences in the P12 rat can emerge from heterochronous developmental changes of GABA-related neurotransmission [70–72]. On P12, when the sex-related differences appeared in the present experiment, more GABA dependent networks supposedly have switched to an adult mode of signaling in females than in males. Given the role of GABA in many anxiolytic effects of ethanol in adults [73, 74], it could be suggested that the differential rate of transition from immature- to adult-characteristics of GABA signaling in males and females is responsible for sex differences in ethanol's effect on the socially isolated P12 rat. Another possible explanation for sex differences in effects of ethanol is that hedonic evaluation of food and palatability differs between males and females, with females being more sensitive to modulation of palatability by ethanol. Sex differences in taste preference for sweets have been described in adult rats [75] and late preweanling or juvenile rats [76]. We also found (Kozlov, unpublished observation) that taste reactivity varied between P12 males and females as a function of concentration of sweet solutions. Ethanol can increase the perception of sweetness of the saccharin solution, as recently demonstrated with taste reactivity assessment in the P12 rats [10]. However, sensitization of taste reactivity with ethanol was dependent on rate of taste stimulation. Given some indications of an opioid link connecting acute effects of ethanol with sensitization of taste reactivity to sweets [77] [68], it could be suggested that differential sensitivity of the taste system to opioids in male and female P12 rat was responsible for the sex difference in effects of ethanol on voluntary intake of palatable fluids. It should be noted, however, that in an ontogenetic perspective, the sex-dependent effects are difficult to interpret and even harder to generalize. For example, because of heterochronous maturation of several brain and neurochemical systems in males and females [78, 79] some sex effects potentially could be linked to a specific ontogenetic period and might disappear during development.

The present study also revealed that effects of ethanol on IIA are age dependent: although 0.5 or 1.0 g/kg ethanol attenuated IIA on P12, the same doses of ethanol did not affect nociceptive reactivity of P9 pups (Experiment 4 Fig. 5). A floor effect is not responsible for negative results of this ethanol treatment, because infusions of 0.1% saccharin not only

reduced but reversed IIA in P9 pups. Relatively low analgesic response to STSI on P9 as well as much lower motor activation of P9 pups compared to P12 rats [10] suggests a lower distress reaction to isolation at P9. Thus, it is possible that doses of 0.5 and 1.0 g/kg ethanol did not reduce IIA in P9 pups because these animals did not react to STSI with distress of the same magnitude as P12 rats did. However, this suggestion does not agree with USV data indicating a higher intensity of distress reaction on P9 than on P12 [80, 81], nor can it explain why exposure to saccharin could nevertheless attenuate IIA in P9 pups. It seems that an age-related difference in baseline stress reactivity does not fully account for the differential efficacy of low doses of ethanol in P9 and P12 pups exposed to STSI.

During the second postnatal week there are important developmental transformations of neural circuits supporting basic behavioral capabilities of infant rats. Noradrenergic transmission in locus coeruleus becomes an adult mode of regulation after P9 [82, 83], GABA signaling switches from an excitatory to an inhibitory mode between P7 and P17 [78] and control of stress–induced activity at various levels of the hypothalamic-pituitary-adrenal axis also dramatically changes between P9 and P14 [12]. Other adaptive changes in mode of operation of several brain systems of the infant rat alter the age-specific expression of conditioned and unlearned fear and aversive reactions [82, 84, 85]. Thus, it is not surprising that developmental changes in effects of ethanol between P9 and P12 encompass not one but several behavioral activities -- locomotion, aversive reactions, ingestive behavior, nociceptive reactivity and sleep-waking transitions. Interestingly, however, ingestion of saccharin induced the same effects on P9 and P12: response latency to nociceptive stimulation decreased as demonstrated in the present study, but the latency of sleep onset increased relative to water-exposed subjects as was found in previous work [10]. In contrast, effects of ethanol on saccharin intake differed between P9 and P12: 0.5 g/kg ethanol depressed ingestive activity of P9 pups and increased saccharin intake in P12 pups [10]. Assuming perceptual equivalence of 0.1% saccharin and 10 % ethanol for P9 and P12 rats [6], and developmental dissociation between effects of low doses of ethanol and effects of appetitive taste stimulation, we suggest that the increase of ethanol consumption during the second postnatal week [1, 2] is determined by changes in sensitivity to the pharmacological effects of ethanol, with impact of the orosensory properties of ethanol solutions of low-to-moderate concentration being insignificant during this ontogenetic period.

Taken together, the present findings support the hypothesis that ontogenetic changes in ethanol acceptance are associated with increased sensitivity of developing rats to stressogenic and anxiogenic events between postnatal days 9 – 12, and that anxiolytic and stress-ameliorating properties of ethanol rather than gustatory properties are responsible for the substantial increase in ethanol intake during this period. The present experiments also suggest that analysis of the interaction between stress and ethanol treatment represents a useful approach in determining factors that affect ethanol acceptance during early ontogeny.

Acknowledgments

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Research Highlights

- Ethanol raised saccharin intake in females and decreased analgesia (IIA) in all water group pups.
- Ingestion of saccharin decreased IIA, and the 0.5 g/kg ethanol dose further reduced IIA.
- The 1.0 g/kg ethanol dose, also decreased IIA in P12 females, but not in P9 pups.
- The anxiolytic properties of ethanol enhance saccharin intake during short isolation stress.
- Furthermore, sex is an important factor mediating responding to ethanol and stress at P12

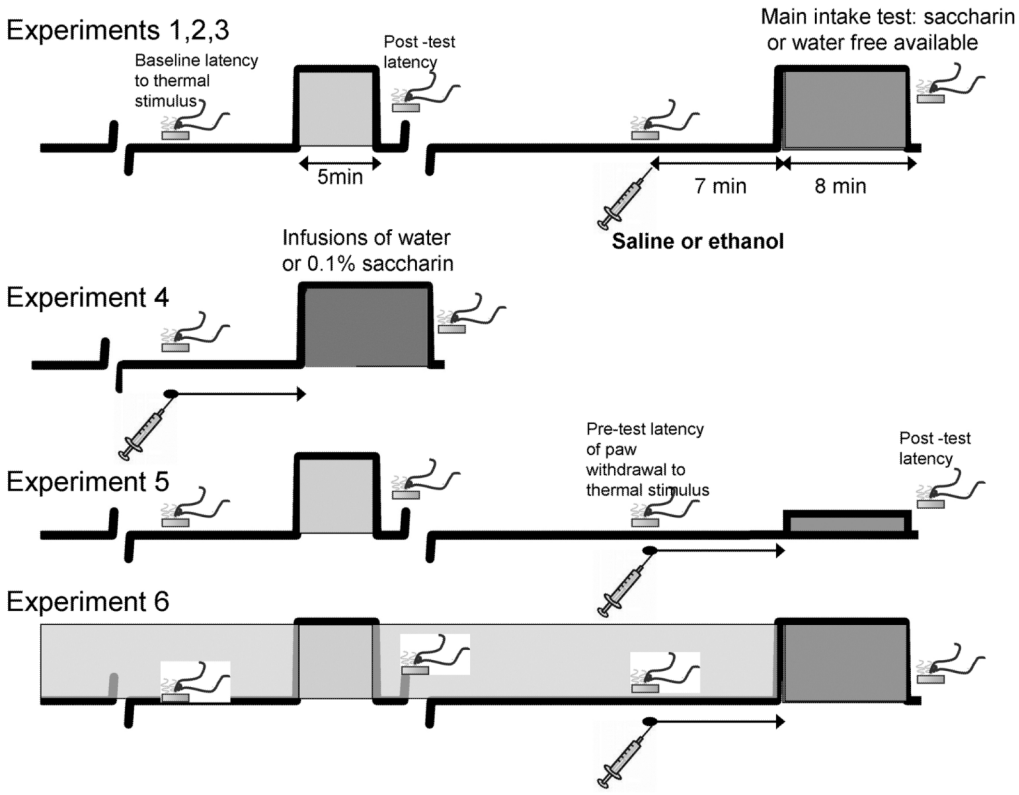

Fig. 1.

Behavioral conditions and timing of tests in different experiments. Protocol of Experiments 1–3, 5 and 6 included two exposures to fluids with first exposure (“pretest”, 3–3.5 hrs after separation from dam) being used to assess pups baseline reactivity and second exposure (“intake test”, 5 hrs after separation) –to assess effects of ethanol. Single exposure to fluids was used in Experiment 4. Conditions of social isolation differed between experiments: pups were isolated from littermates (isolation marked by upward deflections of time line) only during the period when they were exposed to fluids in Experiments 1–4 (short-term isolation, STSI), kept together with littermates during fluid exposure (small deflection) in Experiment 5 or isolated from littermates for the whole time of the experiment (5 hours, Experiment 6). Water only (light shading) and either water or 0.1% saccharin (dark shading) were freely available during pretest and intake test accordingly in Experiments 1–3, 5 or 6 and were infused in Experiment 4. Measurements of latency of paw withdrawal from hot plate and injections of saline or ethanol are indicated by pictograms.

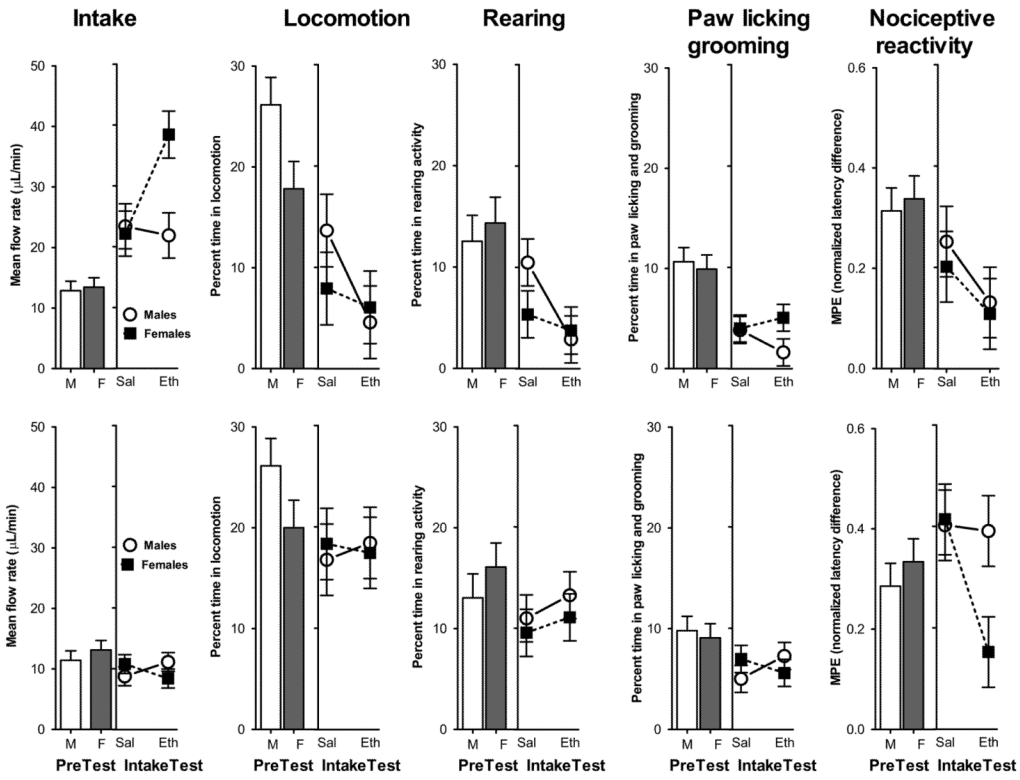

Fig. 2.

Effects of 0.5 g/kg ethanol on intake, behavior, and nociceptive reactivity of P12 pups isolated from littermates for 8 minutes. Top row, pups exposed to 0.1% saccharin; bottom row, pups exposed to water during intake test. Bar graphs illustrate “baseline” water flow rate and behavioral reactions to first (pretest) STSI, no treatment was given at pretest STSI and data were collapsed across treatment groups, however, data for males (M) and females (F) are presented separately. All animals were exposed to water during first STSI. Line graphs show effects of saline (Sal) and 0.5 g/kg ethanol (Eth) on pups during second STSI (intake test). Data are presented as mean ± S.E.M.

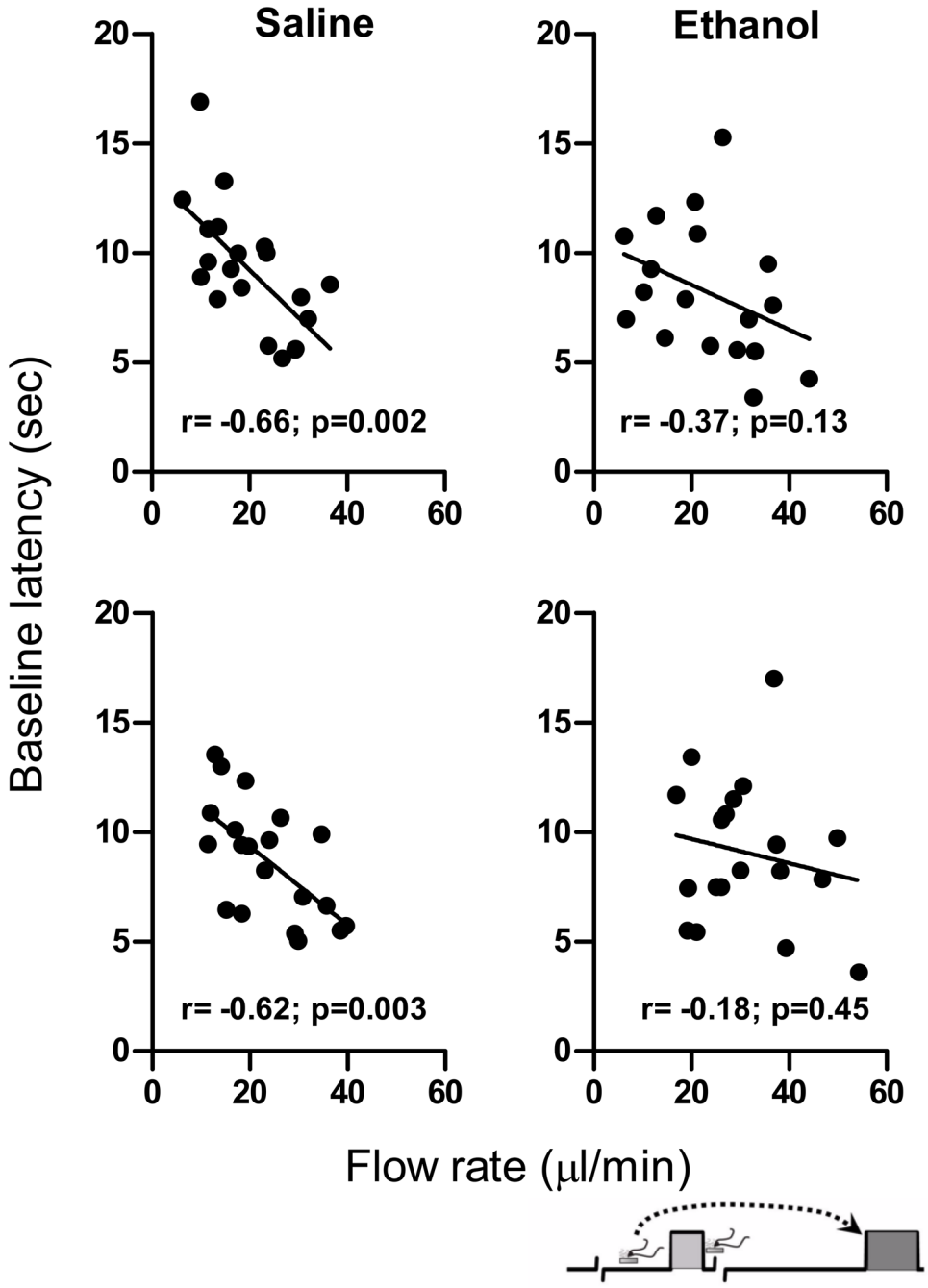

Fig. 3.

Correlation between saccharin intake (indexed as flow rate) and baseline latency of paw withdrawal from hot plate measured before first STSI –pretest. Data from male pups are presented on scatterplots in the top row, data from females –in the bottom row. Regression lines as well as Pearson coefficients of bivariate correlation and probabilities of null hypothesis (correlation is equal to zero) are given on scatterplots. Schematic on the bottom illustrates temporal relationship between correlated variables.

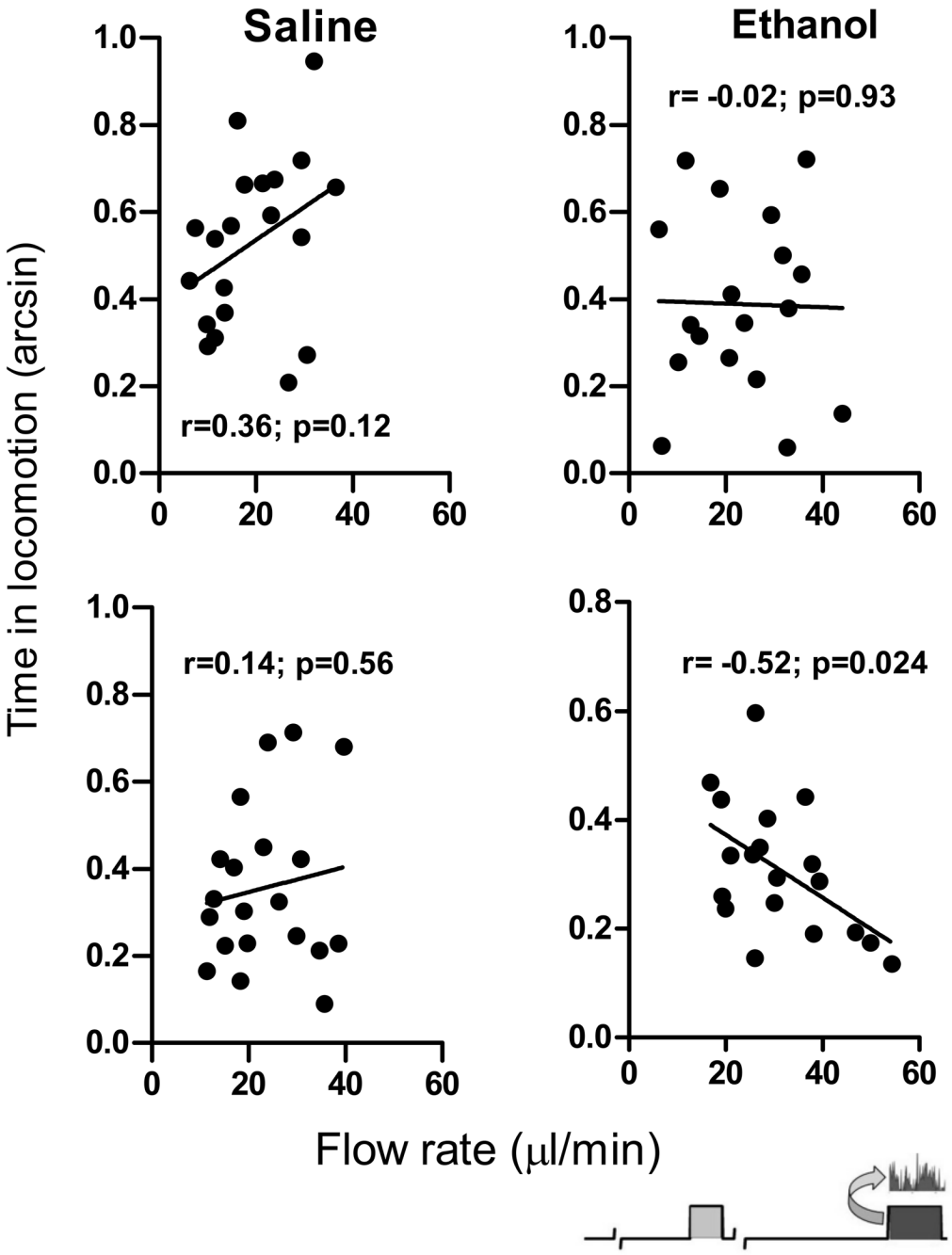

Fig. 4.

Correlation between saccharin intake and time spent in locomotion during intake test. Data from male pups are presented on scatterplots in the top row, data from females –in the bottom row. Regression lines as well as Pearson coefficients of bivariate correlation and probabilities of null hypothesis (correlation is equal to zero) are presented on scatterplots. Schematic on the bottom illustrates temporal relationship between correlated variables.

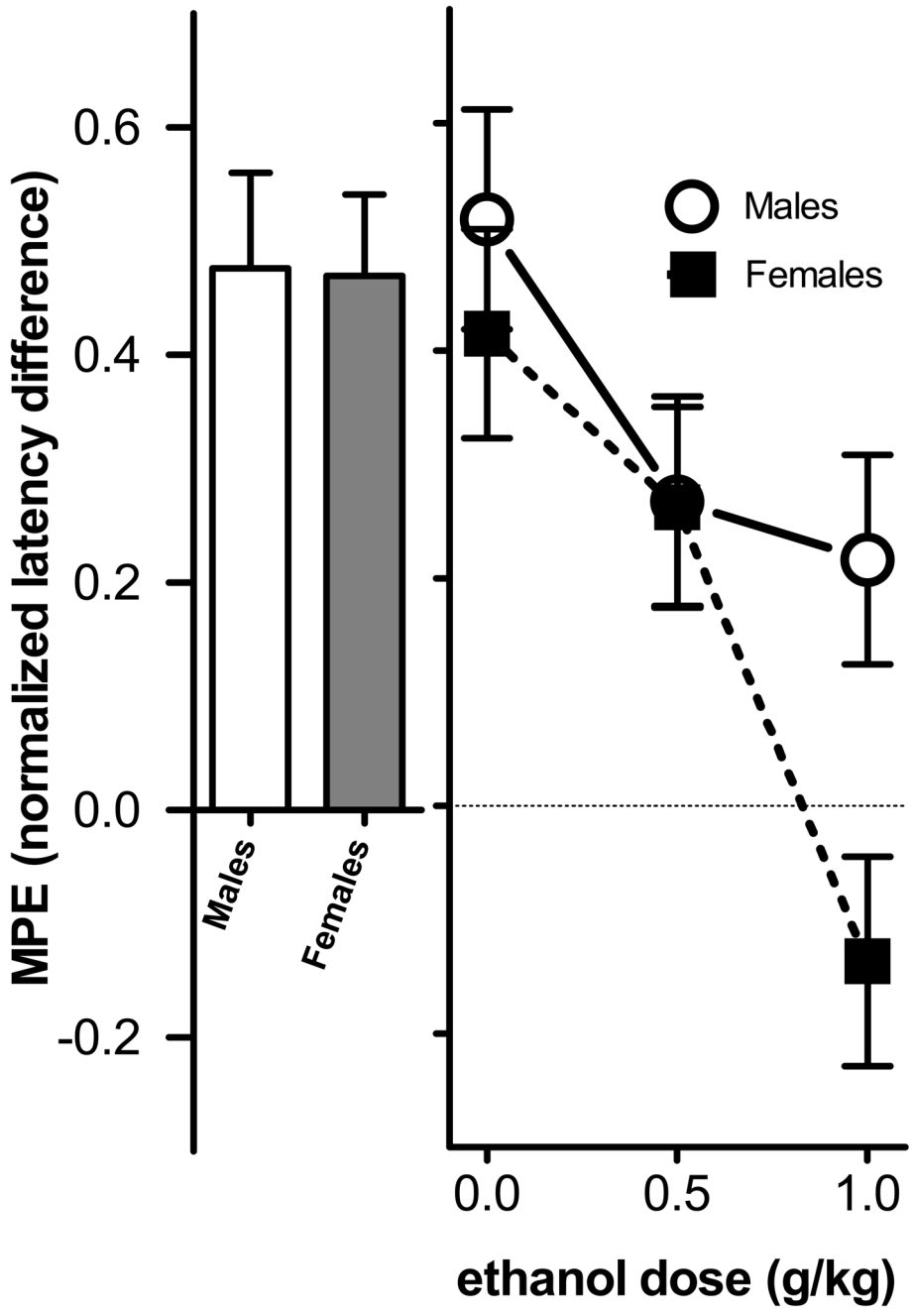

Fig. 5.

Effects of intragastric injection of 0.5 and 1.0 g/kg ethanol on nociceptive reactivity in P12 pups isolated from littermates for 8 minutes. Bar graphs on the left show normalized difference of latency of paw withdrawal from hot plate after first (pretest) STSI, no treatments was used at pretest STSI and data were collapsed across treatment groups. Data for males and females are presented separately. Line graphs demonstrate effects of three doses of ethanol on changes of nociceptive reactivity after second STSI (intake test). Pups were exposed to water during intake test. Positive values of normalized difference of response latency indicate on isolation-induced analgesia. Data are shown as mean values ± S.E.M.

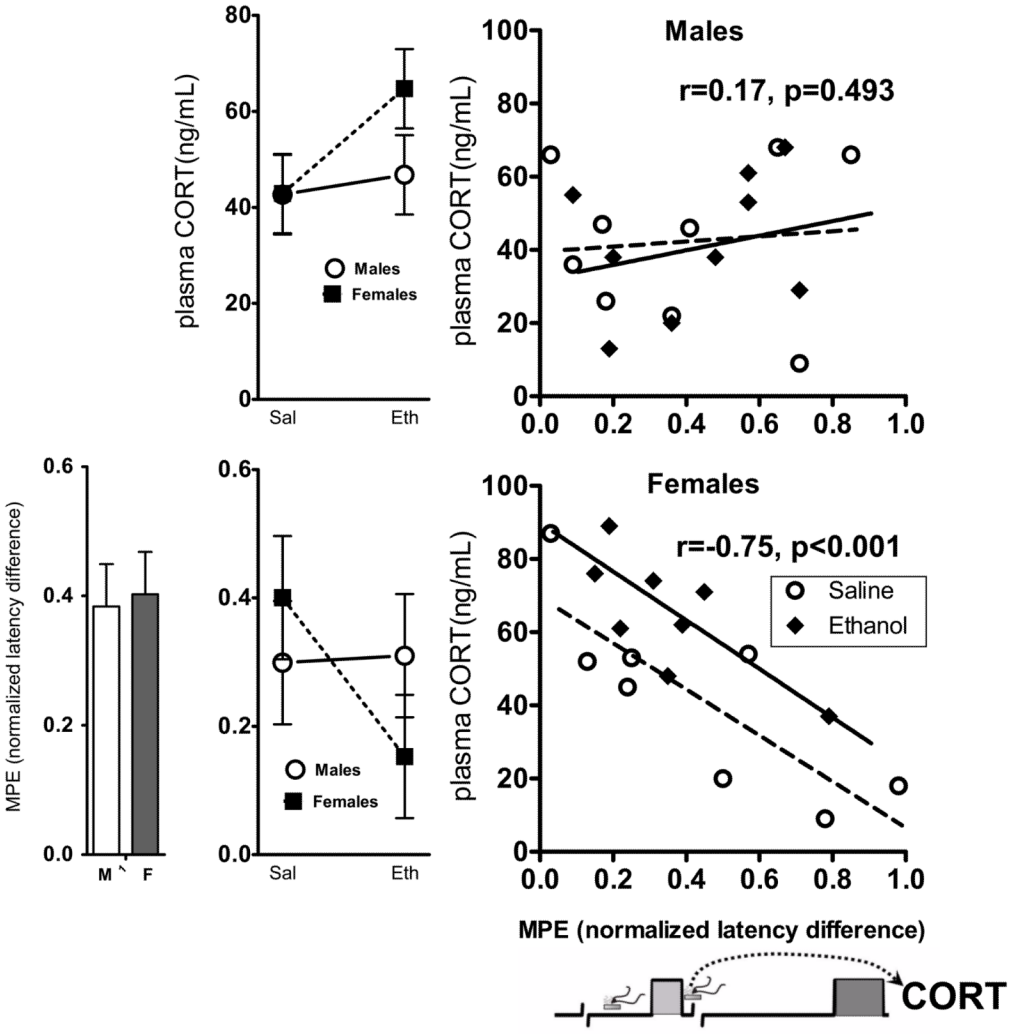

Fig. 6.

Plasma CORT concentration (left column, top) and normalized difference of latency of paw withdrawal from hot plate (left column, bottom) after i.p injections of saline and 0.5 g/kg ethanol. Bar graphs -changes of nociceptive reactivity after pretest STSI. Data are given as means ± S.E.M. Right column – scatterplots illustrating correlation between CORT and normalized latency difference after pretest STSI in males (top plot) and in females (bottom plot). Data from animals treated with saline and ethanol were combined because no significant effect of ethanol on CORT concentration was revealed. However, separate regression lines are presented for saline (broken line) and ethanol groups (solid line). Pearson coefficients of bivariate correlation were computed for pooled data.

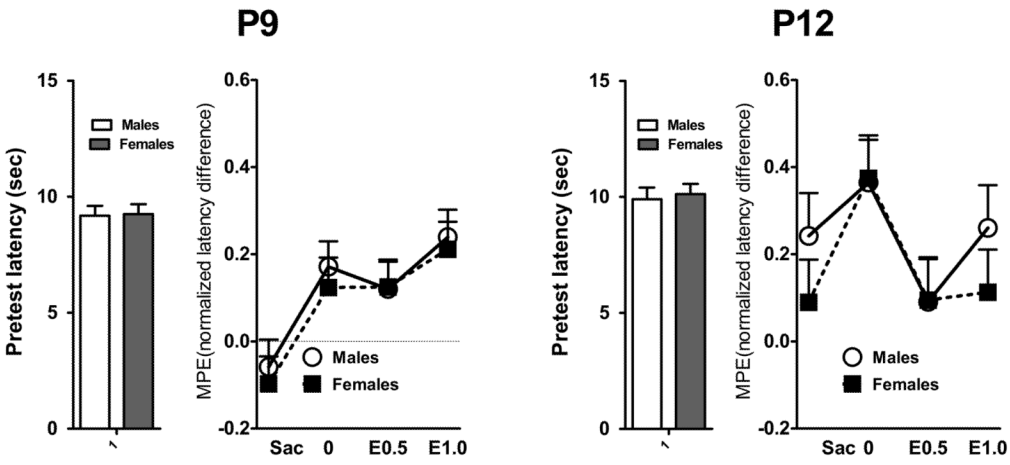

Fig. 7.

Effects of 0.5 and 1.0 g/kg ethanol (i.p.) on IIA in P9 and P12 pups. Bar graphs illustrate baseline latency of paw withdrawal before STSI, data were collapsed across treatment groups. Line graphs - changes of nociceptive reactivity after STSI. Positive values of normalized difference of response latency indicate IIA. Data are shown as mean values ± S.E.M.

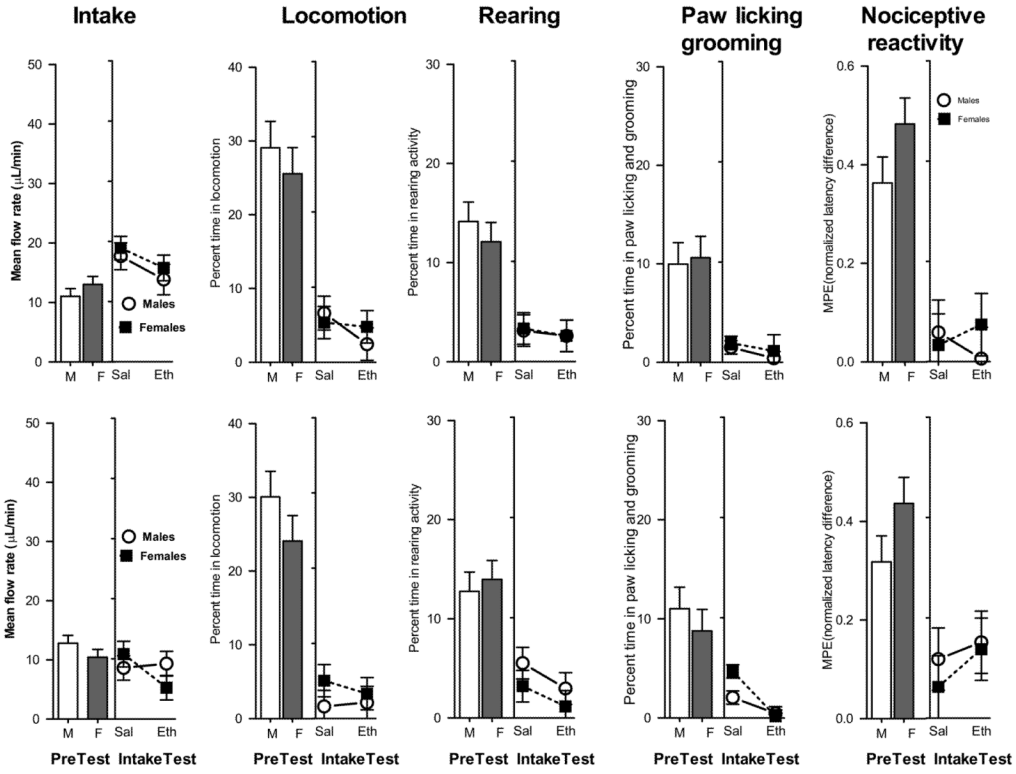

Fig. 8.

Effects of 0.5 g/kg ethanol on intake, behavior and nociceptive reactivity of P12 pups kept with littermates during main intake test. Bar graphs illustrate “baseline” intake rate and behavioral reactions to STSI (pretest), no treatment was given at pretest and data were collapsed across treatment groups. All animals were exposed to water during pretest STSI. Line graphs show effects of saline and 0.5 g/kg ethanol on pups exposed to 0.1% saccharin (top) or water (bottom row) during main intake test. Data are shown as mean values ± S.E.M.

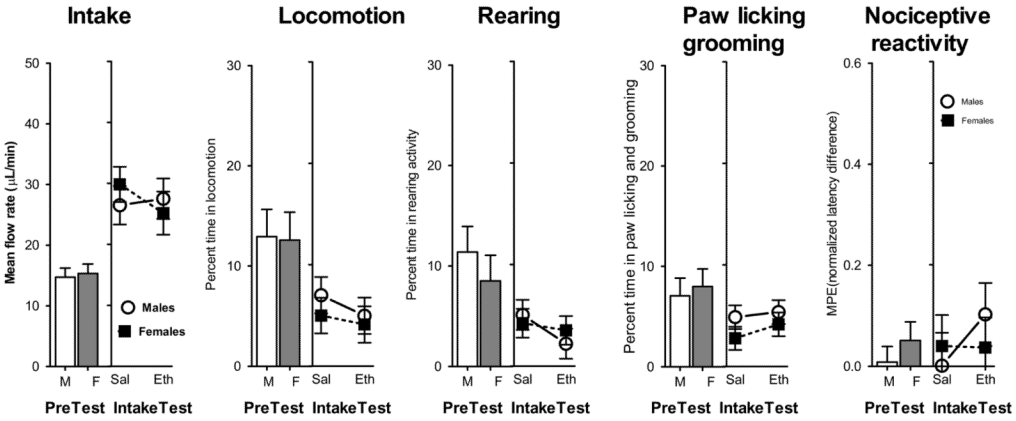

Fig. 9.

Effects of 0.5 g/kg ethanol on intake, behavior and nociceptive reactivity of P12 pups isolated from littermates for 5 hours. Bar graphs illustrate intake rate and behavioral activities after 3 hours of isolation from dam and littermates (pretest), no treatment was given at pretest and data were collapsed across treatment groups. All animals were exposed to water during pretest. Line graphs show effects of saline and 0.5 g/kg ethanol on pups exposed to 0.1% saccharin during main intake test that occurred 5 hours after separation of the animals from dam and littermates. Data are shown as mean values ± S.E.M.

Table 1

Correlation between saccharin intake, behavioral activities and indexes of nociceptive reactivity of P12 rat exposed to STSI

		saline					ethanol 0.5g/kg				
sex	**behavioral variable**	**bivariate r**	**partial r**	**t value**	**df**	**p**	**bivariate r**	**partial r**	**t value**	**df**	**p**
males	baseline latency 1	−0.66	−0.58	−2.75	15	0.015*	−0.37	−0.18	−0.65	13	0.53
	baseline latency 2	−0.10	−0.02	−0.08	15	0.93	−0.34	−0.49	−2.05	13	0.061*
	post-isolation latency 1	−0.22	−0.11	−0.41	15	0.69	0.38	0.47	1.91	13	0.08
	post-isolation latency 2	−0.17	−0.08	−0.29	15	0.77	−0.08	−0.11	−0.42	13	0.68
	locomotion	0.36	0.02	0.09	16	0.93	−0.02	−0.08	−0.30	14	0.77
	rearing	−0.49	−0.56	−2.72	16	0.015*	0.00	0.00	0.01	14	0.99
	licking	0.11	0.42	1.83	16	0.09	0.13	0.33	1.30	14	0.22
females	baseline latency 1	−0.62	−0.53	−2.40	15	0.037*	−0.18	0.00	0.01	14	1.00
	baseline latency 2	0.05	0.05	0.19	15	0.85	0.08	−0.19	−0.73	14	0.48
	post-isolation latency 1	−0.05	−0.10	−0.37	15	0.72	0.37	0.29	1.14	14	0.27
	post-isolation latency 2	−0.26	−0.10	−0.37	15	0.71	−0.51	−0.53	−2.37	14	0.032*
	locomotion	0.14	−0.16	−0.66	16	0.52	−0.52	−0.66	−3.40	15	0.0039*
	rearing	−0.32	−0.37	−1.60	16	0.13	−0.45	−0.52	−2.37	15	0.032*
	licking	0.20	0.29	1.19	16	0.25	−0.28	−0.09	−0.34	15	0.74

r -Pearson coefficients of bivariate and partial correlation;

p - two-tailed probabilities of null hypothesis (correlation is equal to zero);

baseline latency 1 and 2 refers to reactions to noxious heat stimuli presented *before* pretest STSI and intake test respectively;

post- isolation latency 1 and 2 refers to reactions to noxious heat stimuli presented *after* pretest STSI and intake test respectively. Post- isolation latency was normalized to baseline latency as described in Methods.

*-significant correlation.

Table 2

Correlation between plasma CORT concentration, behavioral activities and indexes of nociceptive reactivity of P12 rat exposed water during STSI

		saline and ethanol groups combined				
sex	**behavioral variable**	**bivariate r**	**partial r**	**t value**	**df**	**p**
males	baseline latency 1	0.13	0.18	0.67	13	0.52
	baseline latency 2	0.11	−0.01	−0.02	13	0.99
	post-isolation latency 1	0.17	0.20	0.73	13	0.48
	post-isolation latency 2	−0.08	−0.16	−0.63	13	0.54
	locomotion	0.25	0.19	0.73	14	0.48
	rearing	0.08	−0.07	−0.27	14	0.79
	licking	0.21	0.12	0.46	14	0.66
females	baseline latency 1	0.15	−0.04	−0.15	11	0.89
	baseline latency 2	−0.16	−0.12	−0.41	11	0.69
	post-isolation latency 1	−0.75	−0.59	−2.45	11	0.032*
	post-isolation latency 2	−0.24	−0.12	−0.40	11	0.69
	locomotion	−0.46	−0.35	−1.29	12	0.22
	rearing	−0.37	−0.19	−0.68	12	0.51
	licking	0.15	−0.15	−0.54	12	0.60

p - two-tailed probabilities of null hypothesis (correlation is equal to zero);

baseline latency 1 and 2 refers to reactions to noxious heat stimuli presented *before* pretest STSI and intake test respectively;

post- isolation latency 1 and 2 refers to reactions to noxious heat stimuli presented *after* pretest STSI and intake test respectively. Post- isolation latency was normalized to baseline latency as described in Methods.

*-significant correlation.