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Ketamine Blocks a Conditioned Taste Aversion (CTA) in Neonatal Rats[1]

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NMDA Antagonism Ketamine Conditioned Taste Aversion Neonate Learning Development Neural Plasticity Glutamate NMDA receptors Lithium Chloride Saccharin

WHEN studying memory formation in young animals, one is faced with the difficulty of assessing the behavior of organisms that have immature sensory and motor functioning. For these reasons, the gustatory and olfactory systems have frequently been evaluated because they are somewhat functional-late in mammalian gestation (57). In particular, conditioned taste aversions (CTAs) have been utilized in the study of early learning. CTAs may be formed when a novel taste (Conditioned Stimulus = CS) is paired with a poisonous substance (Unconditioned Stimulus = US) (17). As a result, animals develop an aversion to the novel taste. This taste aversion memory is notable for its potency and the apparent preparedness of animals to acquire it. The CTA association may be established after only one CS-US pairing (6,18) with a long interval between the taste and the malaise (52) and under a variety of circumstances in which conscious awareness of the relevant stimuli is degraded (6). In fact, the association of a gustatory trace with poisoning can proceed even under deep anesthesia (24,48,51).

Conditioned taste/olfactory aversions may be acquired during

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the perinatal period. For example, it has been reported (54) that pairing of a chemical stimulus with an intraperitoneal (i.p.) injection of lithium chloride (LiCl) on Embryonic (E) Day 17 created a conditioned suppression of rat fetal activity when subjects were re-exposed to the same stimulus 2 days later. Presentation of a taste/odor along with LiCl on either E18 (41) or E20 (55,56) created a CTA that was observed even when the taste test was conducted as long as 2.5 weeks postnatally. Further, others (53) have demonstrated conditioned olfactory aversions in 2-day old rat pups.

The neural mechanisms that subserve fetal/neonatal learning of CTAs are largely unexplored. However, much more is known about the neurochemical substrate of taste aversion learning in adult animals. For example, *N*-methyl-D-Aspartate (NMDA) glutamate receptors have been implicated in the formation of CTAs in adults. Recent findings indicate that the NMDA agonist d-cycloserine enhances CTAs and that this enhancement is not merely due to the toxic qualities of the drug (34). The NMDA antagonist DL-aminophosphovaleric acid (APV) disrupts CTA formation when it is injected into the amygdala (66). Other labs (1,61) that have documented ketamine-induced antagonism of conditioned aversions have reported similar data. Because ketamine blocks NMDA receptors (58), these findings offer an obvious parallel with the more extensive data base indicating that NMDA antagonists impair the formation of hippocampal long term-potentiation (LTP) and prevent some forms of learning (38,50).

In addition to their role in adult learning, NMDA receptors may play an important part in early neuronal development. Evidence that a mechanism very similar to LTP might operate during refinement of the retinotectal projection in fish and frogs has been offered (49). Experiments involving cell cultures have shown that NMDA exerts a trophic influence on hippocampal (9) and cerebellar neurons (4,5). Glutamate itself decreases dendritic growth and causes pruning of hippocampal cells in culture (37). Conversely, NMDA receptor antagonists block synapse elimination during brain development (3) and promote axonal elongation (9). Recent studies also suggest that NMDA antagonists can increase total dendritic length and reduce the branch loss normally seen in granule cell neurons between P14 and P24 (7).

The role of NMDA receptors in the establishment of fetal/neonatal conditioned taste aversions has not been explored outside of our laboratory (41). If NMDA receptors are indeed involved in perinatal learning, they may support acquisition by the same NMDA-mediated production of LTP as is used in mature and developing brains to assist in establishing initial connections between neurons. In fact, several parallels have been reported between the neurophysiological substrates of neural development, connectivity, and adult memory formation (28).

The role of NMDA receptors in the learning process may not be the same throughout development as the organism occupies a succession of ontogenetic niches characterized by differences in neural structure and chemistry (54). This laboratory has observed the enhancement of CTA memory formation in E18 fetuses after the administration of ketamine (41). In an attempt to further describe the role of NMDA receptors in memory formation during a different period of development, the current study attempted to condition a CTA in ketamine-treated neonatal (P0) rats. Here we report that ketamine blocked the classically conditioned taste aversion.

METHOD

Subjects

The subjects were male and female rats of the Sprague–Dawley strain obtained from Zivic–Miller Laboratories (Portersville, PA, USA). The timed-pregnant animals (from which our neonatal subjects were derived) were individually housed in plastic "shoe box" cages (44.45 × 21.59 × 20.32 cm). Rat litters were housed with the dam until postnatal Day 28 (P28) when they were weaned and placed in their own individual plastic "shoe box" cages. Rodent chow (Purina #5001) and water were available ad lib. (except as noted below). Home cage temperature was maintained at 23–26°C under a 12:12 h light/dark cycle (lights on at 0600 hours).

Adult male Sprague–Dawley rats (400–900 g) were obtained from Zivic–Miller Laboratories and used in the pilot study exploring the interactions between ketamine and LiCl. These animals were individually housed in plastic "shoe box" cages (44.45 × 21.59 × 20.32 cm) and maintained in the same vivarium as the animals described above.

Pilot Study: Brain Ketamine Assays

To determine the brain levels of ketamine that corresponded with various doses of peripherally administered drug, a series of High Pressure Liquid Chromatography (HPLC) assays were performed. On their day of birth (P0) pups received one of the following doses of ketamine: 0.1 ($n = 5$), 1.0 ($n = 6$), 10 ($n = 14$), or 70 ($n = 10$) mg/kg, i.p. Thirty minutes later, the pups were decapitated and cerebral hemispheres dissected and quickly frozen in alcohol (ETOH) cooled to −60°C (via liquid Nitrogen). Wet cerebral hemispheres were weighed, added to 500 μl, 0.1 N $HClO_4$ (perchloric acid) and sonicated. Homogenized tissue/$HClO_4$ samples were centrifuged and supernate removed for HPLC assay. Brain ketamine content was determined using an HP series 1050 Chromatograph, 50-μL sampling loop, ULTRACARB 3 ODS (20) 100 × 2 mm reverse phase column and ultraviolet detection (240 nm). Mobile phase was 5–10% Acetonitrile in 0.05 M NaH_2PO_4 buffer.

To allow comparison of the ketamine brain content of the neonates in this study with the brain content of fetuses in our previous work (41), we also performed HPLC ketamine analyses of fetal brains. In this case, fetuses ($n = 12$) received ketamine via the maternal circulation. Dams of E18 fetuses were injected with 100 mg/kg of ketamine and 10 mg/kg of xylazine (intramuscularly (i.m.)). Starting 30 min later, the fetuses were removed through cesarean section and decapitated, and cerebral hemispheres were dissected and analyzed as described above.

A one-way ANOVA (with litter as a nested factor) compared the brain ketamine levels of P0 pups after 0.1, 1.0, 10.0, or 70 mg/kg of ketamine injections. The data from E18 fetuses that had received ketamine (100 mg/kg) through the maternal circulation were also included in the analysis. This ANOVA revealed a significant difference between the drug treatment groups [$F(4,5) = 8.07$, $p < 0.05$]. Further, a Newman–Keuls post hoc analysis ($\alpha = 0.05$) showed that a dose of 70 mg/kg of ketamine, i.p., produced a level of the drug in the neonatal cerebral hemispheres that was comparable to that produced in the E18 fetus after a 100 mg/kg of ketamine dose to the dam (see Fig. 1). Likewise, the brain ketamine levels of the neonates receiving 0.1, 1.0, and 10 mg/kg of ketamine were not statistically distinguishable from each other but were significantly different from both the neonates that received 70 mg/kg and the fetuses.

Pilot Study: Ketamine and LiCl Interactions

Adult male rats were placed on 24-h water deprivation and allowed to drink water for only 0.5 h (1400–1430) each day (for 2 days). On the subsequent 2 days (i.e., 2 days before a drinking test) rats had access to 0.3% Saccharin (Sac) water in lieu of the tap water. On the drinking-test day, half of the rats ($n = 5$) were

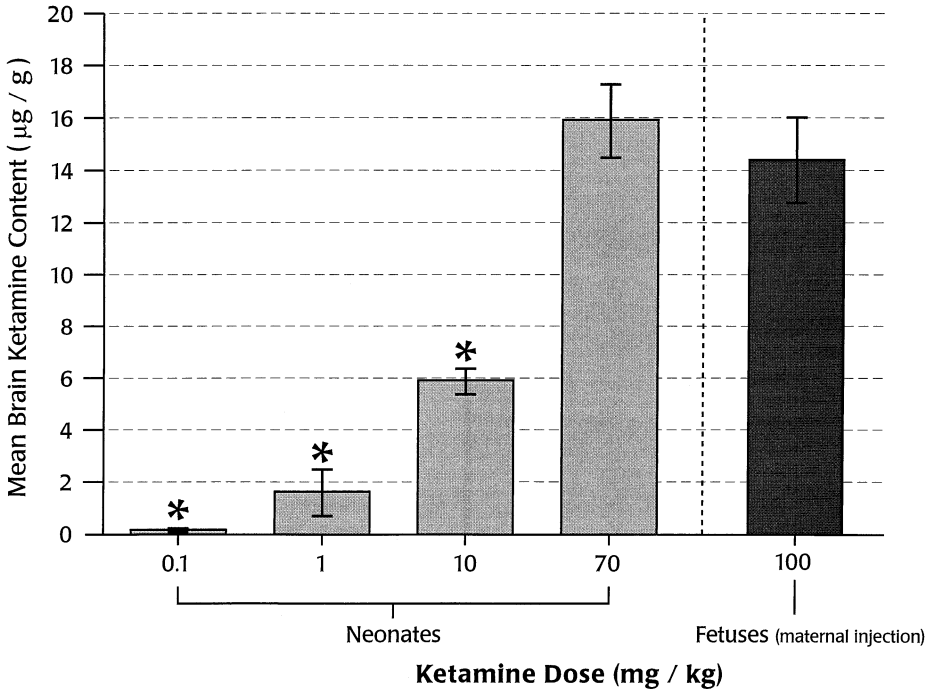

FIG. 1. Mean (± SEM) levels of cerebral ketamine 30 min after peripheral injections of various doses of the drug. Neonates received ketamine i.p. whereas fetuses received the drug through the maternal circulation. A neonatal dose of 70 mg/kg, i.p., ketamine produced brain levels similar to those found in fetuses after a 100 mg/kg, i.p., maternal injection. *Significantly different ($\alpha = 0.05$) from fetal rats and those neonates dosed with 70 mg/kg of ketamine. Statistical results were derived from a one-way ANOVA and Newman–Keuls post hoc tests.

injected with 81 mg/kg of LiCl, i.p., and then 10 mg/kg of ketamine, i.p. The other half of the rats ($n = 5$) were injected with 81 mg/kg of LiCl and then Sal (in a volume equal to the experimental group). One-half hour later 0.3% Sac water consumption was measured in a one-bottle test.

Lithium chloride reduced the volume of Sac consumed by 44% (compared to the previous day). A Student's *t*-test (between 2 independent means) revealed that the Sac consumption of rats treated with LiCl and 10 mg/kg of ketamine (Mean ± SEM = 7.66 ± 2.47 mLs) was not significantly different ($p > 0.05$) from that of LiCl + Sal-treated animals (9.80 ± 1.78 mLs). If anything, there was a trend for ketamine-injected rats to drink less Sac—suggesting that the NMDA antagonist was potentiating (albeit not significantly) the effects of LiCl. This parametric experiment used the same drug doses and Sac concentrations as employed in the current neonatal study. The data indicated that ketamine does not antagonize LiCls effects on Sac consumption (43).

Taste-Aversion Conditioning

Rat pups underwent a taste aversion conditioning procedure on P0. Initially pups were weighed and then received either 0.1, 10, 70 mg/kg of ketamine HCl, i.p., or an equal volume of Sal (control injection). One-half hour later, rats received either an oral injection of the conditioned stimulus (CS = 10 μl of 0.3% Sac) or a control vehicle (10 μl distilled water = H_2O). Immediately after CS administration, pups received a second injection comprised of the unconditioned stimulus (US = 81 mg/kg of LiCl, i.p.) or a control vehicle injection of an equal volume of Sal. All rats from a particular litter received the same injections. Ideally, one would prefer to administer different CS-US combinations to different pups within the same litter. However, potential confounds associated with this procedure convinced us to do otherwise. It was important for the survival of the neonates that they were reunited with their dam as soon as possible after the conditioning procedure. Prolonged separation of the pups and dam causes a lowered acceptance of the pups and compromises the thermal and nutritional wellbeing of the neonates. If a pup that has just received an oral injection of Sac is placed back with the dam, we have found that saccharin from this animal's mouth can contaminate a nipple. Other neonates (in other experimental conditions) subsequently attaching to that nipple may come in contact with a sweet taste not intended by the experimenter. Consequently, the same CS-US combination was administered to all the pups in a litter and to assess litter effects via statistical means (see below).

Four combinations of fetal injections defined the main treatment groups:

1) Sac + LiCL: 10 μL of 0.3% Sac (oral) + 81 mg/kg of LiCl, i.p.. This was the main taste aversion conditioning group. The following number of pups received this treatment: 0.1 mg/kg of ketamine, $n = 26$ (two litters); 10.0 mg/kg of ketamine, $n = 14$ (two litters); 70.0 mg/kg of ketamine, $n = 20$ (two litters); Sal, $n = 27$ (four litters).

2) Sac + Sal: 10 μL of 0.3% Sac (oral) + Sal (vehicle for LiCl, i.p.). This group controlled for the nonconditioned effects of the CS alone. The following number of pups received this treatment: 0.1 mg/kg of ketamine, $n = 31$ (four litters); 10.0 mg/kg of ketamine, $n = 41$ (four litters); 70.0 mg/kg of ketamine, $n = 17$ (two litters); Sal, $n = 23$ (four litters).

3) H_2O + LiCl: 10 μL of distilled water (vehicle for Sac; oral) + 81 mg/kg of LiCl, i.p. This group controlled for the nonconditioned effects of the US alone. The following number of pups received this treatment: 0.1 mg/kg of ketamine, $n = 29$ (three litters); 10.0 mg/kg of ketamine, $n = 24$ (two litters); 70.0 mg/kg of ketamine, $n = 18$ (two litters); Sal, $n = 19$ (two litters).

4) H_2O+Sal: 10 μL of distilled water (vehicle for Sac; oral) + Sal (vehicle for LiCl, i.p.). This group controlled for the effects of the injection procedure. The following number of pups received this treatment: 0.1 mg/kg of ketamine, $n = 27$ (four litters); 10.0 mg/kg of ketamine, $n = 27$ (four litters); 70.0 mg/kg of ketamine, $n = 18$ (two litters); Sal, $n = 23$ (three litters).

Note Throughout this paper the Sac + Sal, H_2O + LiCl, and H_2O + Sal groups are frequently referred to as the "CTA control groups."

State-dependent Learning Controls

Possible state-dependent effects (44) of 10 mg/kg of ketamine were investigated by using a 2 × 2 factorial design. Four groups of animals that received Sac + LiCl pairings on P0 were included in this manipulation. Two of the groups are described above: (1) pups that received a Sal control injection before conditioning and Sal again 30 min before the nipple taste test (NTT) (S + S; $n = 27$ from four litters) and, (2) pups that received 10 mg/kg of ketamine before conditioning and Sal 30 min before the NTT (K+S; $n = 14$ from two litters). In addition, two other groups of animals were run: (3) pups that received 10 mg/kg of ketamine before conditioning and 10 mg/kg of ketamine again 30 min before the NTT (K + K; $n = 18$ from three litters); (4) pups that received a Sal control injection before conditioning and 10 mg/kg of ketamine 30 min before the NTT (S + K; $n = 26$ from seven litters).

Taste-preference Tests

Neonatal nipple preference taste test. Procedures used previously (55,56) were adapted to assess the taste preferences of neonatal rats injected *in utero*. On postnatal Day 15 ± 0.06 (Mean ± SEM), infant rats were isolated from their dam for 6.5 h. Neither food nor water was available to the pups during this period. Immediately before the test the dam was anesthetized with 50 mg/kg of sodium pentobarbital (i.p.). Atropine sulfate (0.4 mg/kg, i.p.) was used as a pre-anesthetic to control salivation. The dam was then placed in a plastic "shoe box" cage with her ventral surface exposed. The under-side of the cage was warmed with a heating pad and maintained at approximately 35°C.

The nipple preference taste test procedure was divided into two parts which were both conducted, in succession, on the same day: (1) initial nipple preference, in which nipple taste was not manipulated and spontaneous nipple preferences were detected; and (2) nipple taste test (NTT), in which nipples were painted with either Sac or water and taste preferences were detected.

To determine initial nipple preference, individual pups were placed at the end of the cage, facing the cage wall furthest from the dam. The pup was allowed to turn and approach the dam to suckle. [Note, however, that anesthesia is known to interfere with normal milk ejection (55,56) and the presence of milk was not detected during this procedure.] A trial was considered complete after a pup had been attached to a nipple for 15 s. Pups were then removed from the nipple and immediately placed at the far end of the cage for the start of another trial. If a nipple attachment did not occur within 2 min, that trial was ended and a new one was begun. Each pup was given a maximum of thirteen trials in which to achieve five nipple attachments. If the pup met this nipple-attachment criterion, it progressed to the NTT. Otherwise, the animal was eliminated from the study. Pups usually showed a spontaneous preference for two or three nipples. In addition to noting the particular nipple attachments, the latency to attach to the nipple was also recorded.

During the NTT, all of the preferred nipples (determined in the Initial Nipple Preference test) were then painted with 0.3% Sac (i.e., the same Sac solution used during taste aversion conditioning). Additional nipples were also randomly painted to bring the total number of Sac-painted nipples to six. The remaining (nonpreferred) nipples were painted with distilled water (Sac vehicle). Each pup was then given additional trials using the procedures described. Although most pups readily attached to nipples, some animals did not (e.g., subjects that received ketamine before the NTT. See Results.). Rats were run for a maximum of thirteen trials. However, the test stopped whenever the subject achieved either five nipple attachments (irrespective of order) or five nonattachments in a row. The number of Sac- or water-painted nipple attachments, as well as the latency to attach to the nipple, were recorded. Between pups, the nipples were swabbed with 10% ethanol and gently dried with cotton gauze. As previously reported (56), this procedure was not severe enough to interfere with subsequent nipple attachment.

Young adult taste preference test. In preparation for a two-bottle taste preference test, rats were weaned and individually housed on P28. On P40 ± 0.2 (mean ± SEM) rats were given an additional test of their Sac preference/aversion using the following procedure. Water was removed on Monday afternoon at approximately 1500 hours. At the same time on Tuesday, Wednesday, and Thursday rats were given 0.5-h access to two bottles that both contained distilled water. The two bottles were placed on opposite sides of the cage top. Pilot work has shown that 24-h water-deprived rats drink almost all of their liquid within the first 5–10 min of the 0.5-h test. They tend to drink voraciously from the first bottle they encounter. Once their initial thirst is satiated, much of the animal's time is spent grooming or eating solid food. To facilitate the subject's sampling of the liquids in both bottles, it was necessary to switch the bottle positions 3 min after the beginning of water access. We used the same procedure on Friday (test day); however, on this day, one of the bottles contained the 0.3% Sac solution whereas the other contained water. The amount of each liquid consumed was recorded at the end of the 30-min drinking period.

Additional Behavioral Measures

It has been reported (19) that there exists a characteristic motor syndrome in adult animals that received chronic dosing of MK-801 (NMDA receptor antagonist) as neonates. Although the doses of NMDA antagonist that produced this syndrome were significantly higher than those used in the current study, we selected and observed several indices of functioning and maturity to evaluate possible sensory/motor deficits that might influence our nipple-attachment measure. Animals that were not sensing their environment or were incapable of smoothly locomoting to the dam and attaching to nipples would have been handicapped on the NTT. Therefore, during, the initial nipple preference test, eye opening/closure were recorded as well as the presence of tremors during movement. The eyes of neonatal rats are closed for the first 2 weeks after birth and were opening about the time of our NTT. Eyes were recorded as "open" only if both eyes were observed as open in the period before and during the initial nipple preference test. Tremors were defined as whole-body or head-shaking during standing or locomotion. Finally, as an indicator of sensory/motor capability and/or motivational level, the number of times rats failed to attach to a nipple were recorded during the initial nipple

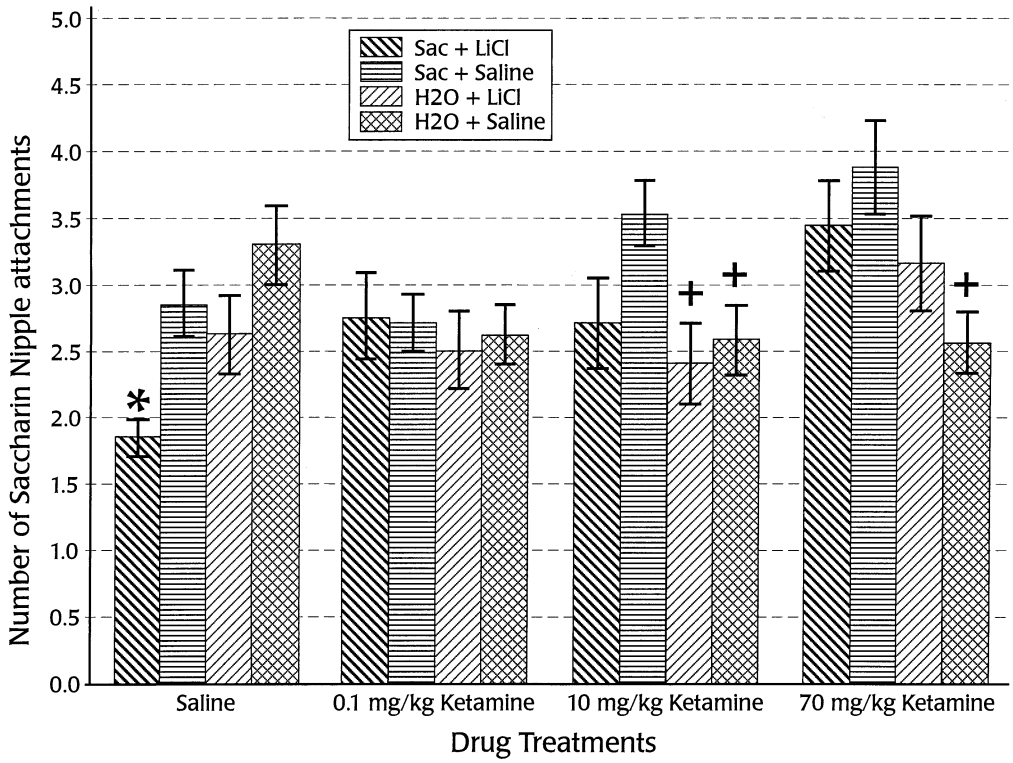

FIG. 2. Mean (± SEM) number of times (out of five trials) that neonatal pups attached to dam's Sac-painted nipples during a taste aversion test. Neonates that received Sal (Control) injections before a Sac + LiCl paring showed a significant avoidance of Sac-painted nipples. However, neonates that received a ketamine pretreatment (either 0.1, 10, or 70 mg/kg) failed to show the aversion. Pups that received 10 or 70 mg/kg of ketamine and oral injections of water during the conditioning stage of the experiment attached to fewer Sac-painted nipples than those that tasted Sac. *Significantly different ($\alpha = 0.05$) from all other groups of Sal-treated animals; $^{+}$Significantly different from Sac + Sal treated animals within the same drug treatment group. Statistical results were derived from one-way ANOVAs and Newman–Keuls post hoc tests.

preference test, i.e., before nipples had been painted with Sac or water.

Statistical Analyses

Unless otherwise specified below, the CTA data were analyzed via one-way ANOVAs [using a general linear model (SAS™, SAS Institute, Inc., Cary, NC) compensating for unequal *n* values] comparing the different CTA treatments within each drug treatment (ketamine or Sal control). The same CTA treatments were administered to all the pups in a litter (see rationale above in "CTA Conditioning"). Therefore, to evaluate the contribution of litter effects, we adopted procedures previously recommended (13) wherein litter was included as a nested factor within CTA treatment. The *F* statistic associated with a particular treatment was computed using the mean square (MS) for the litter effect as the denominator. Further, the df associated with the litter effect were used in these calculations (13). If initial ANOVAs did not reveal a significant effect of litter, subsequent analyses were run without this nested factor. Individual group comparisons were accomplished by using Newman–Keuls post hoc tests (31). When appropriate, the post hoc tests used the MS-litter and the harmonic mean of the Ns to compensate for the unequal group sizes (64). An $\alpha = 0.05$ was adopted throughout these analyses. The data from both male and female rats were combined in these studies because preliminary ANOVAs confirmed that there were no sex differences on the measures reported here.

RESULTS

Our data suggest that the NTT is capable of revealing conditioned taste aversions learned on P0. Neonates treated with Sal and then conditioned with a pairing of Sac + LiCl, later avoided Sac-painted nipples [$F(3,88) = 5.38$, $p < 0.0019$] as compared to other animals in the Sal-treated CTA control groups (i.e., Sac + Sal, H_2O + LiCl and H_2O + Sal) (see Fig. 2).

This CTA was not observed in ketamine-treated rats however. ANOVAs comparing saccharin-painted nipple attachments showed that neonates injected with 0.1 mg/kg of ketamine before taste aversion conditioning exhibited no differences between CTA groups. However, the pups treated with 10 or 70 mg/kg of ketamine exhibited differential patterns of saccharin-nipple attachment depending on CTA treatment [10 mg/kg: $F(3,102) = 4.29$, $p = 0.0068$; 70 mg/kg: $F(3,69) = 5.02$; $p = 0.0033$] (see neophobia discussion below), Newman–Keuls post hoc comparisons indicated that pups treated with ketamine and then Sac + LiCl attached to approximately the same number of Sac-painted nipples as did the pups in the CTA control groups receiving the same dose of ketamine.

A one-way ANOVA compared the number of Sac-coated nipples selected by Sac + LiCl-treated rats (rats treated with Sac + Sal, H_2O + LiCl or H_2O + Sal were excluded from this analysis) injected with either Sal or various doses of ketamine. The ANOVA indicated that there was a dose-dependent drug effect [$F(3,83) = 6.28$, $p = 0.0007$]. Post hoc analyses showed that Sal-treated

neonates selected significantly fewer sweet nipples than did animals treated with 70 mg/kg of ketamine at conditioning. However, rats receiving lower doses of ketamine (0.1 or 10 mg/kg) were not significantly different from the Sal-injected control animals.

Although not consistent over all drug treatment groups, some of the animals receiving water as a CS control during conditioning showed a significant neophobia for Sac painted nipples (see Fig. 2) when compared to rats that received Sac + Sal treatments (Newman–Keuls, ($\alpha = 0.05$). This effect was seen in the pups treated with 10 or 70 mg/kg of ketamine but not in those treated with Sal or 0.1 mg/kg of ketamine.

A comparison of the latencies to Sac-nipple attachment did not reveal any reliable differences between the CTA or drug-treatment groups. The mean time for all subjects to attach to a Sac-painted nipple was 32.9 ± 1.9 s (mean ± SEM). Sal-treated animals that received Sac + LiCl required 37.1 ± 3.9 s to attach to a Sac-painted nipple, whereas the average latency for all the Sal-treated control pups was 33.0 ± 5.1 s.

The CTA revealed during the NTT of neonates (see above) was not detectable later during the bottle preference test of 40-day-old weaned rats. During this second CTA test, subjects could select between drinking bottles containing Sac or water. Sal-treated neonates that received a Sac + LiCl pairing on P0, did not exhibit reliably lower percent-Sac-consumed scores (i.e., total Sac consumed/(total Sac + water consumed); mean ± SEM = 0.27 ± 0.04) than did all of the Sal-treated CTA controls (mean ± SEM = 0.29 ± 0.04). Likewise, the total volume (mL) of Sac consumed was similar for these animals (Sal-treated Sac + LiCl rats = 4.49 ± 0.53; all other Sal-treated controls = 4.39 ± 0.43). The Sac consumption of the Sac+LiCl-treated subjects in the four drug treatment groups was also not significantly different.

Not all subjects progressed from the Initial Nipple Preference Test to the NTT. If they did not attach to nipples on five trials during the first phase of the experiment (see Methods) they were eliminated from the study. To determine if ketamine treatment on P0 influenced the animal's ability to attach to nipples during Initial Nipple Preference testing, we recorded the number of pups/litter that progressed (or failed to progress) to the NTT phase of the study. Data were pooled for all ketamine-treated rats and compared to those of the Sal-treated rats. A higher percentage of ketamine-treated rats (mean ± SEM = 89.2 ± 2.8) met the nipple-attachment criteria of the Initial Nipple Preference test than did the Sal-treated rats (65.9 ± 7.1). A $\chi 2$ analysis revealed that ketamine-treated rats were more likely than Sal-treated rats to meet the nipple-attachment criteria and progress to the NTT [χ^2 (1) = 32.57; $p < 0.01$].

Additional behavioral observations made at the time of Initial Nipple Preference determination revealed that tremors were just as likely to be seen in ketamine-treated (mean ± SEM = 27.6 ± 4.5%) as Sal-treated pups (31.5 ± 6.8%) (χ^2; $p > 0.05$). However, neonates that were treated with ketamine on P0 were less likely than Sal-treated pups to have their eyes closed during the Initial Nipple Preference test [mean ± SEM = 29.94 ± 5.4% and 51.5 ± 7.4%, respectively; χ^2 (1) = 22.79; $p < 0.01$]. These data seem to indicate that the P0 ketamine treatments employed in this study did not significantly retard (and perhaps even enhanced) some maturational measures.

The behavioral patterns of P0 neonates that received Sal before Sac + LiCl conditioning and then ketamine (10 mg/kg, i.p.) 30 min before the NTT were very different from the rats in other treatment groups. First, casual observation indicated that the ketamine produced acute behavioral effects that included locomotor hyperactivity and response perseveration (see below). In addition, many of these pups failed to attach to nipples during the NTT. On average, a pup that had 10 mg/kg of ketamine 30 min before the test failed to attach on 2.7 ± 0.6 trials (mean ± SEM) and exhibited a nipple-attachment failure rate of 37% (i.e., percent of trials when the pup did not attach to a nipple within 120 s). Nondrugged rats, however, rarely failed to attach to nipples (8%; mean ± SEM = 0.4 ± 0.1 trials/subject). A *t*-test comparing 2 independent means showed that the percent of nipple-attachment failures was significantly higher in pups that had received 10 mg/kg of ketamine 30 min before the NTT than in nondrugged subjects [$t(51) = 4.00$; $p = 0.0001$]. None of the nondrugged rats failed to meet the criterion of attaching to nipples on five trials during the NTT, whereas 23% of the ketamine-treated pups failed to meet this criterion.

Pups that received ketamine for the first time before the NTT also failed to attach to nipples more often (mean ± SEM = 2.7 ± 0.6 trials/pup) than did the rats that had ketamine both at conditioning (P0) and test (P15) (0.3 ± 0.2 trials/pup). Moreover, a Student's *t*-test indicated that the percent of nipple-attachment failures was significantly higher [$t(42) = 2.74$; $p = 0.004$] in pups that had received just one dose of ketamine (before the NTT) (mean = 31%) as compared to the neonates that received ketamine both on P0 and before the NTT (mean = 8%). These data suggest that 10 mg/kg of ketamine significantly interfered with the conduct of the NTT by reducing the likelihood of any nipple attachment. Further, the data suggest that rats that received their first dose of ketamine before the NTT were more drastically impaired than rats that had previous experience with the drug (on P0).

The fact that rats receiving ketamine before the NTT sometimes failed to attach to five nipples during the test made comparisons with animals in other groups problematic. For example, a rat attaching to one Sac-painted nipple out of five attachments should not be considered equivalent to an animal that attached to one Sac-painted nipple only (i.e., failing to attach on any nipples on any other trials). In an attempt to deal with this ketamine-induced difference in nipple attachment we computed a percent statistic (number of Sac-painted nipples attached to/total nipple attachments) to investigate state-dependent effects in this study. Four groups of animals that received Sac + LiCl pairings on P0 were included in this analysis: (1) pups that received 10 mg/kg of ketamine before conditioning and 10 mg/kg of ketamine again before the NTT (K + K); (2) pups that received 10 mg/kg of ketamine before conditioning and Sal before the NTT (K + S); (3) pups that received a Sal control injection before conditioning and 10 mg/kg of ketamine before the NTT (S + K);(4) pups that received a Sal control injection before conditioning and Sal again before the NTT (S ± S).

As expected, the S + S neonates attached to a significantly lower percentage [$F(3,12) = 4.59$, $p = 0.02$; Newman–Keuls post hocs, $p < 0.05$] of Sac-pained nipples (mean ± SEM = 37 ± 3%) as compared to the other three treatment groups (K + K = 70 ± 5%; K + S = 54 ± 9%; S + K = 66 ± 6%). However, the percentages of Sac-nipple attachments of the K + K, K + S, and S + K animals were not significantly different from one another. The fact that K + K and K + S pups both failed to avoid Sac-painted nipples might suggest that state-dependent effects are not prominent in these animals. However, it should be noted that the difference between the S + K and S + S pups was most likely produced by ketamine's acute effects during the NTT (see Discussion). A two-way ANOVA [Drug administered at conditioning (Sal or ketamine) × Drug administered at test (Sal or ketamine); with litter as a nested factor] of the percent attachment to Sac-painted nipples revealed an effect of the drug administered at time of test [$F(1,12) = 12.37$, $p = 0.004$]. Pups attached to a higher percentage of sweet nipples if tested after 10 mg/kg ketamine (combined S + K and K + K group (mean + SEM = 67 ± 4%) rather than Sal (combined S + S and K + S group (mean ±

SEM = 42 ± 4%) (Newman–Keuls post hocs, $p < 0.05$). These data are consistent with the interpretation that ketamine, given before the NTT, impairs the expression of the CTA formed on P0. The CTA is apparently acquired in S + S neonates because they show a significant avoidance of Sac-painted nipples. However, the CTA is not expressed if ketamine is injected before the NTT.

This failure of S + K subjects to exhibit a CTA may be a reflection of the demands of the dependent variable measured (i.e., nipple attachments). We noted that S + K pups exhibited oral/facial patterns (e.g., head shakes, gapes, and face wipes) characteristic of taste aversion (see Discussion and 20, 21) despite their failure to avoid Sac-painted nipples. These aversive behaviors were not observed when S + K rats came in contact with water-painted nipples. Thus, in this case, the CTA may depend on the particular behavioral measure and the "state" of the animal at time of testing. The failure to detect a state-dependent effect of ketamine by comparing the nipple attachments of the K + S and K + K groups may represent both ketamine's ability to impair memory formation at the time of conditioning on P0 as well as its acute ability to impair performance on the NTT.

DISCUSSION

The data presented here suggest that ketamine (a well-known noncompetitive glutamate NMDA receptor antagonist; 58) can block the formation/expression of a CTA in neonates. These findings are consistent with previous experiments that used adult subjects and suggested that NMDA receptor antagonism impairs learning/performance of a variety of tasks [e.g., water maze (62), delayed alternation (23), or classic fear conditioning (65)]. Olfactory learning is impaired after post-training NMDA receptor blockade in neonatal (P5) rats (60). Further, it has been reported (1,61) that ketamine can block CTAs in adult rats.

The mechanisms by which ketamine may block CTAs is controversial. Does NMDA receptor antagonism inhibit the association between CS and US? Alternatively, could it be that ketamine reduces the ability of the neonate to sense the sweetness of the Sac (CS) or to experience the malaise produced by the lithium chloride (US)? The current data do not allow us to fully address these issues. However, they have been treated by several other investigators (1,36,61) as well as by work within our own laboratory.

Several lines of evidence in the literature suggest that ketamine does not eliminate an animal's ability to sense a sweet taste. For example, it has been reported (61) that ketamine does not impair an animal's ability to recognize a Sac solution during a bottle test. Likewise when phencyclidine (PCP; an NMDA receptor antagonist, like ketamine) was given before the pairing of Sac with LiCl, rats avoided consuming Sac after a subsequent injection of PCP. Conversely, when PCP was given before a non-poisoned exposure to Sac, subjects readily consumed the Sac after a subsequent PCP injection (36). Other investigators (1) have shown that ketamine does not alter the slope of a curve representing habituation to sucrose neophobia.

Likewise, some of the findings reported here are not consistent with the interpretation that ketamine induces functionally significant alterations in sensory capacities. For example, we report that animals treated with ketamine (10 or 70 mg/kg, i.p.) and then taste water on P0, exhibit a Sac neophobia compared to ketamine-treated animals that tasted Sac on P0. These data suggest that the taste of Sac was experienced and retained whereas pups were under the influence of ketamine. Our casual observations in pups that received ketamine before the nipple taste test (S + K group) also indicate that these animals exhibited very different oral/facial expressions after encounters with Sac-painted nipples than when they encountered nipples painted with water. Again, these data would seem to indicate that ketamine does not eliminate the ability to experience a sweet taste.

Could ketamine have more-subtle effects by slightly reducing the saliency of saccharin so that it is perceived as less intense or concentrated? Could a lack of a CTA in rats treated with ketamine at the time of conditioning be simply explained as a case of generalization decrement along the dimension of flavor intensity? These explanations also seem unlikely, however, because (in K + Ss animals) the sweet taste at time of test would be relatively greater than at time of conditioning. One study (30) indicates that generalization gradients can be influenced by the intensity of the test stimuli. If a response is conditioned to a particular stimulus and then tested with stimuli that are both weaker and stronger than the training stimulus, the amount of generalization will be greater for the stronger stimulus than it will be for the weaker. "In experiments on the generalization of classically conditioned responses, this stimulus-intensity effect may be so powerful as to obscure the generalization gradient." (p. 359) (30)

The data presented above suggest that the taste of Sac may be unchanged by ketamine. However, others (1) have shown that CTA formation was somewhat slowed by ketamine (although asymptotic levels of sucrose consumption were reached at the same time by ketamine- and Sal-pre-treated rats). Could this phenomenon be produced by ketamine's inhibition of the LiCl US? We attempted to address this question by performing a pilot study investigating ketamine-LiCl interactions (see Methods section and Ref. 43). These data suggested that ketamine did not attenuate the ability of LiCl to reduce Sac consumption. Our data are consistent with the literature indicating that ketamine does not reduce the US properties of LiCl. In fact, under some circumstances, ketamine (and other NMDA receptor blocking agents) can produce a taste aversion (albeit mild) when injected after exposure to a novel taste (15,27,61). The results of our experiments do not allow, by themselves, a clear conclusion as to what the precise mechanism is through which ketamine impairs acquisition of a flavor aversion. However, given the data presented above, it is difficult to conclude that ketamine's blockade of CTA formation is entirely due to ketamine-induced changes in taste or the drug's antagonism of the LiCl US on P0.

As we have noted, some animals that tasted water on the conditioning day attached to fewer Sac-painted nipples during the NTT than did neonates that tasted Sac and were injected with Sal on P0. This finding suggests that rats encountering the taste of Sac for the first time during the NTT have a prominent neophobia, i.e., avoiding a novel taste (1). This neophobia is most prominent in the rats that received ketamine in the highest doses (10, 70 mg/kg). It was not observed in Sal-treated rats nor in animals that received 0.1 mg/kg of ketamine. The design of the current study does not offer a clear explanation for this dose-dependent phenomenon. The literature suggests that ketamine can produce hyperactivity (22,25,26) and perseverative responding (33,39). Further, our laboratory has recently reported Sac-induced perseverative mouthing responses (42). It may be the case that sufficiently high doses of the drug can create oral motor patterns that allowed a prolonged tasting of Sac on P0. Thus, the fact that animals that tasted Sac on P0 also attached to more Sac-coated nipples during the NTT, may be a reflection of ketamine-induced hyperfamiliarity with the sweet gustatory experience. This might explain the disparity between the Sac-treated and water treated animals in the context of ketamine's ability to alter motor capabilities. Further experimental work is needed to determine if this is indeed the case.

We ran additional animals in an attempt to clarify state-dependent effects of the 10 mg/kg of ketamine treatment. Pups that received ketamine before conditioning with Sac + LiCl (on P0) failed to avoid Sac-painted nipples independent of whether or not

they had ketamine or Sal before the NTT. These data suggest that state-dependent effects are not prominent in these animals. However, we also discovered that ketamine given 30 min before the NTT may interfere with the expression of a CTA. Pups that had ketamine before the taste test did not show a CTA, but the drug also altered the behavioral topography of the animal (see description below) by significantly reducing the frequency of attachment to any nipple.

On the surface this would seem to indicate that the animals treated with Sal on P0 (at the time of Sac + LiCl pairing) and ketamine before the NTT did not acquire a significant Sac taste aversion. However, an analysis of the response topography makes us reluctant to draw this conclusion. Rats conditioned with Sac + LiCl, and then first exposed to ketamine before the nipple test, exhibited a set of aversive behaviors when they came in contact with Sac-painted nipples. Typically, after making initial oral contact with a Sac-painted nipple, pups would shake their heads, gape, and wipe their faces. All of these behaviors are well-established indicators of taste aversions (20,21). Why then, would ketamine-treated pups eventually attach to an "aversive-tasting" sweet nipple? We can only speculate that the acute effects of ketamine must play a role in developing this response pattern. Ketamine has been shown to produce perseverative responding in rats and pigeons (33,39). This was confirmed by casual observations made during the course of the current experiments. For example, nondrugged pups will frequently approach the dam and burrow under her. This is a productive nipple-seeking strategy if the dam is laying with her ventral surface down. However, during the nipple test, anesthetized dams were placed on their backs to expose their nipples for observation. Pups that had not received ketamine might burrow under the dam once or twice but would eventually vary their behavior to locate the exposed nipples. However, ketamine-treated rats burrowed for many trials in a row. Perseveration induced by an acute dose of ketamine may help explain the circumstances under which an animal might exhibit some behavioral indicators of taste aversion (e.g., face wipes and head-shakes) while persisting to make oral contact with a Sac-painted nipple.

Although Sac + LiCl, Sal-pretreated pups showed a significant CTA during the NTT, an analysis of the bottle test data (recorded 25 days later) did not reveal a CTA. The lack of CTA at bottle-test time may reflect a forgetting of the CTA or a changing capability of the animals to retain this information as they develop. There are also alternative explanations. Because, during the nipple test, pups tasted Sac without subsequent malaise (produced by a US), the failure to detect a CTA weeks later might also reflect an extinction of the aversion learned on P0. This could be tested directly by forgoing the NTT and only testing the rats on the bottle test. However, pilot data from a previously published study (41) indicates that extinction may not play a prominent role in our inability to detect a CTA on the bottle test. Rats classically conditioned in utero on E18 exhibited a CTA on a nipple taste test but, even if this test was not conducted, failed to show a CTA on a bottle test. It should also be recognized that the two behavioral tests are, in some ways, quite dissimilar. For example, the 15-day-old rat pup may attach to the dam's nipple for reasons other than nutritional value, even after a 6.5 h dam-deprivation period. On the other hand, the 24-h water-deprived 40-day-old rat is drinking water for rehydration. Thus, the two tests employed in this study measure two different behaviors motivated by different factors.

Pups that received ketamine for the first time before the NTT failed to attach to nipples more often than did the rats that had ketamine both at conditioning (P0) and test (P15). These data are consistent with the interpretation that neonates with previous experience with the drug (on P0) developed a degree of behavioral tolerance to the drug. Although the tolerance hypothesis seems unlikely because only two doses of ketamine were separated by 2 weeks, others have reported the rapid development of ketamine tolerance (2,12,29).

Both the current neonatal data and the data from adult rats suggesting that ketamine blocks CTAs may be contrasted with previous findings indicating that ketamine potentiates a CTA in fetuses (41). This disparity in learning outcomes between fetuses and neonates is evident even when peripheral doses of ketamine were matched to yield similar levels of the NMDA receptor blocking drug in the brain. Our laboratory is currently engaged in studies that may help reconcile these findings. Future studies will focus on changes in NMDA receptor populations and functions during the perinatal period. The literature already suggests that NMDA receptor populations and neuroanatomical distribution are linked to particular stages in development. Both the expression of various subtypes of NMDA receptors and NMDA receptor ligand affinity are labile and tied to particular developmental time periods (16,47,63). For example, the mRNA encoding the NMDA receptor NR2C subunit in the rat brain can be detected in large quantities in the rat hippocampus during P7-P14. However, there is no hybridization signal in the adult hippocampus (45). Likewise, the distribution of NMDA receptor subunit mRNA shifts to different brain locations during different periods of perinatal development (14,59).

In parallel to changing receptor populations, the functional role for NMDA receptors may change as the organism develops and establishes new capabilities (10). NMDA-receptor blocking drugs are only transiently effective in modulating hippocampal morphology (11) and electrophysiological responses (8) during development. In addition, experience-dependent plasticity in the visual cortex of kittens (32) and early postnatal olfactory learning in rat pups (35) seem to depend on NMDA-receptor stimulation at appropriate times. In the rat superior colliculus, a sharp rise in the amount of mRNA for the NMDA receptor in the second postnatal week, parallels the refinement of the topographical map of this brain nucleus (46). These data present a complex picture of changing populations, distributions, and functions of NMDA receptors that may accommodate differing needs of an organism as it develops. The laboratory data corroborate clinical findings suggesting that when ketamine is used as a human anesthetic, it has very different effects on infants and adults (40). Apparently, information about the stage of brain development in which NMDA receptor blockers are administered helps predict both experimental and clinical outcomes.

In conclusion, the current data indicate that neonatal CTAs may be blocked by the NMDA receptor antagonist ketamine. Ketamine is capable of altering both the acquisition and expression of the aversion. It is worth noting that ketamine seems to block associative learning of a CTA but, under some circumstances, fails to block the nonassociative learning of an encounter with a novel sweet taste (neophobia). These results may be contrasted with data collected from fetal rats (41) indicating that ketamine can potentiate CTAs in these animals. Thus, glutamate receptor blockade may shape associative memory formation in a manner that is dependent on the stage of brain development.

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