Consolidation and expression of a shock-induced odor preference in rat pups is facilitated by opioids

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Abstract

To support nipple attachment and huddling, rat pups must learn to approach and prefer maternal odor. Similar to other altricial species, rat pups have a sensitive period for learning this odor preference, which ends around postnatal day (PN) 10 and coincides with the emergence of walking. One characteristic of this sensitive period is that an odor paired with moderate shock elicits an odor preference. After PN10, this behavioral training produces an odor aversion, although pain threshold remains unchanged. Recently, we demonstrated that the endogenous opioid system might be a key element in the acquisition of the shock-induced odor preference during the sensitive period since antagonism of this system disrupts odor preference learning. In older pups, acquisition of a shock-induced odor aversion was unaffected by opioid system manipulation. The purpose of these experiments was to further elucidate the role of opioids in infant olfactory learning through assessment of memory consolidation and expression during and after the sensitive period. In Experiment 1, we demonstrate that naltrexone (NTX), a nonspecific opioid antagonist, given immediately following odor–shock conditioning during the sensitive period, blocks odor preference formation and yields an odor aversion. However, the same treatment does not disrupt consolidation of an odor aversion in older pups. In Experiment 2, we demonstrate that during the sensitive period, NTX disrupts expression of the shock-induced odor preference, but not the learned odor aversion in older pups. Results using this model of attachment suggest that opioids have an important role in the acquisition, consolidation, and expression of early olfactory preferences. Furthermore, since prenatal drug exposure is known to alter the endogenous opioid system, these results highlight the capacity of prenatal opiate exposure to disrupt early infant learning and attachment.

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1. Introduction

During pregnancy, maternal opiate abuse results in fetal opiate exposure via the placenta, which not only alters the developing endogenous opioid system, but several aspects of neural development [12,27,44,48,84,85]. The opiates continue to exert their effects postnatally not only through an altered nervous system in the infant, but also through direct opiate exposure through the mother’s milk and in modulation of the mother’s maternal behaviors. In turn, mother–infant interactions and affiliation are altered [24,39,51]. Hence, the normal activation of endogenous opioids mediating reward, which function in concert with learning and memory circuits to facilitate mother–infant relationships, may be impaired [19]. For this reason, it is critical to elucidate the role of endogenous opioids on the neurobehavioral basis of maternal behavior and the infant’s care-seeking behaviors, with the present research focusing on the infant.

Learning and memory are critical to the normal development of mother–infant relationships, and opioids have a direct impact on this learning in both infants and adults in several species [7,8,19,55,57,67,87]. Specifically in the infant rat, opioids appear to have a facilitatory role in odor preference acquisition [3,40,57,62,67,73], and nipple–milk conditioning [59,60,64,65,74,75]. In addition, opioids appear to mediate isolation-induced ultrasonic vocalizations used to help the dam localize a displaced pup [10,25,42,56,86]. Overall, results from infant studies cohesively provide support for opioid facilitation of infant behaviors toward the mother.

However, recent data from our lab suggests that opioids may have a unique role in neonatal rat pup learning that is temporally limited to the sensitive period when pups rapidly,
and easily learn an odor attraction. The unique role for opioids was uncovered using a mammalian model of imprinting in which the neonatal rat paradoxically learns an odor preference for an odor previously paired with a moderately painful shock [9,79,80]. This paradoxical odor preference learning does not reflect a higher pain threshold in the neonate, is temporally limited to the sensitive period, and is characteristic of a number of other species, including dogs, chicks, nonhuman primates, and potentially humans [18,28,29,31,32,47,63,68]. It is possible that this paradoxical infant learning system developed through evolution to ensure that altricial animals develop an attachment to the mother regardless of the quality of care [33].

Learning may be pharmacologically manipulated during acquisition, when information is acquired [1], or during consolidation following acquisition, when memory is stored [49,52,54,69,71]. In addition, expression of learned memories may be manipulated prior to testing [16,37]. To elucidate the role of the endogenous opioid system in infant learning, we compared opioid effects on odor–shock conditioning in rat pups younger than 9 days old (during the sensitive period when odor–shock training produces an odor preference) and in older pups (after the sensitive period when odor–shock conditioning produces an odor aversion [9,79,80]). Using this odor–shock conditioning, we recently demonstrated that pretraining, systemic injections (0.5 mg/kg) of naltrexone (NTX) disrupt odor preference acquisition in young rat pups (sensitive period), but not acquisition of an odor aversion in older pups [67]. We also demonstrated that the infant-learned odor preference from this conditioning paradigm is reversible to an odor aversion if NTX is administered immediately following the training [67]. This suggests not only a critical role of opioids in infant olfactory learning during the sensitive period, but that their role may be unique to this developmental period. The goal of these experiments was to further examine potential developmental differences in the role of opioids in olfactory associations using odor–shock conditioning. In the following experiments, we examined the effect of systemic NTX on olfactory memory consolidation and expression both during and after the sensitive period.

2. Materials and methods

2.1. Animals

Subjects were both male and female pups, born of Long–Evans rats (Harlan Sprague–Dawley, IN) in the animal vivarium at the University of Oklahoma. Mothers were housed in polypropylene cages with wood shavings and kept in an environment with controlled temperature (23 °C) and light (12:12 h light/dark). Food and water were available ad libitum. Cages were checked daily, and the day of parturition was termed 0 days of age. On postnatal days (PN) 1–2, litters were culled to five males and five females each. The numbers of subjects used in each experiment are listed in corresponding figure captions. All procedures were approved by the University of Oklahoma Institutional Animal Care and Use Committee and follow NIH guidelines.

2.2. General training procedure

On the day of training, pups were removed from the mother and randomly assigned to a training condition: (1) paired odor–shock, (2) backward odor–shock, and (3) odor-only. Pups were marked for identification using indelible ink, weighed and placed in individual 600-ml glass beakers, and given a 10-min adaptation period to recover from experimental handling. During a 1-h training session, pups received 14 presentations of a 30-s peppermint odor (CS) and a 1-s 0.5 mA tail shock (US), with an intertrial interval of 4 min. Paired odor–shock subjects received 14 pairings of the 30-s odor with shock during the last second of the odor presentation, while backward odor–shock subjects received a 1-s shock 2 min after an odor presentation. Odor-only subjects received only the peppermint odor presentations. Peppermint odor was presented with a flow-dilution olfactometer at 2 l/min and at a concentration of 1:10 peppermint vapor.

During training, we recorded the number of limbs moving (0 = no movement of the extremities; 5 = movement of all five extremities) 10 s before presentation of the odor, as well as during presentation of the odor [26]. Due to motoric immaturity, our rating scale measures generalized behavioral activity, and provides a general assessment of learning during training. Following training, pups were returned to the mother.

2.3. Y-maze testing procedure

On the day following training, pups were removed from the mother and tested using a Y-maze. The Y-maze consisted of a habituation chamber (7 cm long and 9 cm wide) and two alleys (22 cm long and 9 cm wide) extending at 45° angles. The habituation chamber was separated from the alleys via two removable doors. One arm of the maze contained the familiar pine wood nest odor (20 ml of clean, pine shavings in a petri dish), while the other arm contained the peppermint odor (25 μl of peppermint extract placed on a KimWipe that had been placed in a ventilation hood for 5 min). Each pup was placed in the starting chamber and given 5 s for habituation before the doors to the alleys were removed. Each subject had 60 s to make a choice, which required the pup to enter the alley. Each subject was given a total of five trials, and the floor was wiped clean (using a cloth with water) between each trial. A 30-s intertrial interval was used between testing trials, and the orientation of the pup was counterbalanced between trials when placed in the habituation chamber. Pups that failed to make at least three choices in the Y-maze were excluded from the study.
Observations of each pup were made blind to the training condition.

2.4. Drug treatment

Pups received NTX (0.5 mg/kg sc) or vehicle treatment either immediately following the odor–shock training in Experiment 1 or before testing in a Y-maze in Experiment 2. A detailed description of drug treatment is described below within the Results sections for each experiment.

2.5. Data analysis

In Experiment 1, we used an unpaired $t$ test to compare consolidation effects of NTX on the two groups trained during the sensitive period. For all other experiments, we used the analysis of variance (ANOVA) and post hoc Fisher tests to analyze differences between training conditions and drug treatment groups. ANOVA repeated measures and post hoc Fisher tests were used to analyze behavioral acquisition data from training. The two ages in each experiment were not trained together, and different litters were used for each age group. For this reason, the data from different ages were analyzed separately, although presented together to simplify data presentation.

3. Results

3.1. Experiment 1

This experiment assessed whether blocking the opioid system immediately post-training would alter consolidation of the newly acquired olfactory memory. Our previous work had suggested that opioids may be altering acquisition through its action on consolidation [67], but we had not examined the effects of opioids on memory consolidation in older pups.

3.1.1. Subjects

A total of 62 pups derived from 18 litters were used at either PN7–8 (13.1–19.9 g) or PN11–12 (22.2–31.5 g). Only healthy pups with similar weights were chosen from each litter and no more than one male and one female from a given litter were used for a given treatment/drug/age condition. Since the PN7–8 data were replications of previously published results [67], pups were trained with only paired presentations of odor and shock as described above to reduce the number of animals required for this experiment as recommended by NIH Institutional Animal Care and Use Committee guidelines. All conditioning groups were used for older pups. Immediately following odor–shock training, pups were injected (subcutaneously at the nape of the neck) with either 0.5 mg/kg NTX (naltrexone HCl, Sigma, St. Louis, MO) or equal volume isotonic saline. Pups were given 15 min to recover from injections in a 27 °C incubator before being returned to the mother. The following day, they were tested using a Y-maze as described above.

3.1.2. Results

During the sensitive period, a post-training injection of NTX blocked the consolidation of an odor preference, which replicated our previous results [67]. Specifically, an unpaired $t$ test showed a significant effect of drug treatment following paired presentations of odor–shock, with NTX producing significantly fewer choices toward the conditioned odor [$t(12) = -5.26, P < .01$] (Fig. 1a). This difference between paired NTX- and saline-treated pups was not due to differences in training behavior, since pups responded similarly to odor and shock presentations, and indeed, both groups showed acquisition curves that did not differ significantly (data not shown).

After the sensitive period, NTX had no effect on consolidation (Fig. 1b). ANOVA analysis showed a significant main effect of training condition [$F(2,42) = 10.65, P < .01$]. There was no main effect of drug treatment or a Training Condition × Drug Effect. Post hoc Fisher tests showed that both saline- and NTX-treated subjects in the paired odor–shock groups chose the peppermint odor significantly less than all other subjects in the experiment, indicating a shock induced odor preference [$P < .05$]. Analysis of behavior during odor–shock conditioning indicated that pups from both the paired NTX and saline conditions showed significant acquisition curves [$F(12,252) = 7.316, P < .01$], and that acquisition curves were not significantly different from each other (data not shown).

3.2. Experiment 2

This experiment assessed whether blocking the opioid system during testing would alter the expression of the learned behavior.

3.2.1. Subjects

A total of 78 pups derived from 15 litters were used. PN6–7 pups (11.5–19.2 g) and PN11–12 pups (24.4–32.4 g) were trained without any drug manipulation during odor–shock conditioning. Pups were returned to the mother until testing. On the following day, subjects received systemic injections of 0.5 mg/kg of NTX or saline 15 min before Y-maze testing.

3.2.2. Results

NTX delivered just prior to testing eliminated the expression of the shock-induced odor preference learned during the sensitive period (Fig. 2a). ANOVA analysis revealed a significant main effect of training condition [$F(2,36) = 6.25, P < .01$], a main effect of drug treatment [$F(1,36) = 16.24, P < .01$], and a significant interaction between training condition and drug treatment [$F(2,36) = 30.59, P < .01$]. Post hoc tests showed that NTX had no
significant effect in the odor-only or backward groups, while NTX treated subjects in the paired odor–shock condition chose the conditioned odor significantly less than their saline controls in the paired condition (P < .01). NTX treated subjects in the paired condition were not significantly different from the other control subjects, with the exception of the NTX treated subjects in the odor-only condition (P < .05), although the odor control group did not

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**Fig. 1.** Effect of opioid antagonism on post-training memory consolidation following odor–shock conditioning. (a) Number of approaches toward the conditioned odor for PN7–8 rat pups receiving NTX (n = 8) or SAL (n = 6) immediately following paired presentations of odor–shock. (b) Number of approaches toward the conditioned odor for PN11 or PN12 rat pups receiving either paired (SAL, n = 9; NTX, n = 10) or backward (SAL, n = 7; NTX, n = 6) odor–shock presentations or odor-only (SAL, n = 8; NTX, n = 8) presentations. Bars represent mean values and vertical lines indicate S.E.M. There is no error bar for the odor-only saline condition in the older pups. SAL = saline; NTX = naltrexone.

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**Fig. 2.** Effect of opioid antagonism on expression of a shock-induced olfactory preference or aversion. (a) Number of approaches toward the conditioned odor for PN6–7 rat pups receiving either paired (SAL, n = 6; NTX, n = 8) or backward (SAL, n = 7; NTX, n = 7) odor–shock presentations or odor-only (SAL, n = 7; NTX, n = 7) presentations. Drugs were only present during testing on PN7–8. (b) Number of approaches toward the conditioned odor for PN11–12 rat pups receiving either paired (SAL, n = 6; NTX, n = 7) or backward (SAL, n = 4; NTX, n = 7) odor–shock presentations or odor-only (SAL, n = 5; NTX, n = 7) presentations. Drugs were only present during testing on PN12–13. Bars represent mean values and vertical lines indicate S.E.M. SAL = saline; NTX = naltrexone.
differ from other control groups. This difference between paired NTX- and saline-treated pups was not due to differences in training behavior, since pups responded similarly to odor and shock presentations. Indeed, both groups showed acquisition curves \[ F(12,216) = 23.12, \ P < .01 \], which were not significantly different from each other (data not shown).

NTX delivered prior to testing had no effect on the learned odor aversion after the sensitive period (Fig. 2b). ANOVA analysis showed a significant effect of training condition \[ F(2,30) = 14.82, \ P < .01 \]. There was no main effect of drug treatment or any interaction effects between the independent variables. Post hoc tests revealed that both saline- and NTX-treated subjects in the paired odor–shock training condition made significantly less choices to the odor in comparison to all other subjects in the experiment \( P < .05 \). There was no effect of drug treatment in the backward and odor-only conditions, and saline-treated subjects in the paired condition were not significantly different than NTX-treated subjects in the paired condition. Analysis of behavior during odor–shock conditioning indicated that pups from both the paired NTX and saline conditions showed acquisition curves \[ F(12,180) = 4.56, \ P < .01 \], which were not significantly different from each other (data not shown).

4. Discussion

Our data suggest that the opioid system has a uniquely important role during the sensitive period when pups exhibit a heightened learning ability. Specifically, in neonatal rats, the opioid system appears necessary to acquire [67], consolidate, and express odor preferences (present studies). After the sensitive period, disruption of the endogenous opioid system still permits acquisition [67], consolidation, and expression of novel olfactory aversions. These data suggest that opioids modulate neurocircuitry mediating pup learning of maternal odor. This odor learning is critical for pup orientation and nipple attachment, which provide pups with the food and warmth necessary for survival.

4.1. Opioid’s role in infant’s attachment to the mother

Our results suggest that opioids play a crucial role in infant-care seeking behaviors at a time when learned odor aversions would thwart proper mother–infant bonding. As pups grow older, crawling and walking emerge [6], and they prepare for an environment without care from the mother, disruption of the opioid system does not appear to disrupt acquisition, memory formation, or expression of novel odor aversions. These data suggest that the brain is designed for the needs of specific developmental periods, and changes accordingly when pups begin to leave the nest.

Previously, we demonstrated the important role of the opioid system in the shock-induced odor preference in neonatal rats [67]. Systemic NTX delivered before odor–shock training disrupted odor preference formation. In addition, delivery of NTX post-odor–shock conditioning yielded an odor aversion, and our results in Experiment 1 replicated these previous results. In this study, we now demonstrate that the expression of the shock-induced odor preference is disrupted by alteration of the opioid system as well. Similarly, Shide and Blass [73] demonstrated that NTX delivered before testing of an odor preference induced by intra-oral infusions of sucrose or corn oil prevented preference expression, and experimental pups reacted to the odor in the same manner as control pups that had not received training. Overall, results from use of this odor–shock model of attachment and an opioid antagonist have provided further evidence for the paramount role of the endogenous opioid system in odor preference learning and memory formation during the sensitive period.

We previously gave PN12 pups systemic injections of NTX (0.5 mg/kg) before odor–shock conditioning and found that pups still demonstrated an odor aversion although their acquisition behavior from training was significantly different from their saline controls in the paired condition [67]. In this study, we demonstrate that NTX delivered to the same age pups after conditioning did not disrupt memory formation of an odor aversion, nor did NTX disrupt expression of the odor aversion when delivered to the pups before testing. Indeed, by day 10, adult-like behavior and learning emerge. Pups begin walking [6] and noxious stimuli lose their ability to produce conditioned odor preferences [9,79,80]. Additionally, by the second and third postnatal weeks, the opioid system is more adult-like [13,43,58,76]. It is important to note that we did not detect any significant enhancement in the aversive learning as would be expected in comparison to adult studies. Specifically, studies in adults suggest that learning is disrupted by opiates [34,36,38,72] and facilitated by injections of opioid antagonists [20–22,34–36], suggesting that we would have expected to see a stronger odor aversion in our older pups. However, it is important to note that the Y-maze used in testing and the behavioral rating scores we employed during training may not adequately reflect increased or decreased intensities of olfactory aversions.

4.2. Comparison of adult and neonatal learning circuitry

Data from adult studies support arguments that opioids modulate memory storage through norepinephrine (NE) released from the locus coeruleus (LC), which activates β-adrenergic receptors in the amygdala [11,22,23,35,50]. The modulatory role of NE in adults is in sharp contrast with the necessary and sufficient role of NE in infant learning [45,79]. Additionally, work from our lab suggests that the amygdala may not be activated during odor–shock conditioning in 9-day-old or younger pups [79,80]. This sug-
gests pups are using a learning circuitry distinct from the adult, and the altered behavior produced in the sensitive-period pups in our study may not be from opioid antagonism within the amygdala. One possible site of this opioid action in these younger pups is the LC [82]. Indeed, neonate olfactory learning is dependent upon NE from the LC [33,79], while in older pups, the LC appears to have a more modulatory role in learning [30,66,70]. Opioids have been shown to inhibit NE in as early as gestational day 17 cortical slices [14]. Given the importance of NE in neonatal acquisition and consolidation during the sensitive period but not after the sensitive period [33], blocking opioids may have altered NE levels, thus offering an explanation for our results.

An alternative explanation is that opioid antagonism altered any reward normally associated with the odor–shock conditioning during the sensitive period, thus a conditioned odor preference would not be expected if areas normally associated with mediating reward were altered in normal activity. Opioid antagonism in conditioned place paradigms, both in the adult [15,46,53] and infant [3,61], as well as in juvenile social play paradigms [83] disrupt formation of preferences. Indeed, the development of the endogenous opioid system shows tremendous changes corresponding to the end of the sensitive period, particularly in areas known to mediate olfaction, reward, and emotional learning in adults [13,43,58,76], suggesting that our results may be due to disruption of the neural circuitry mediating reward.

However, since we delivered the drug systemically, we cannot rule out pain-induced activation of the endogenous opioid system as a plausible mechanism mediating our results. Nociceptive and antinociceptive responses differ in neonates and adults [2] due to immature spinal sensory processing [18], which recently Beland and Fitzgerald [5] have suggested may be due to downregulation of opioid receptors in dorsal root ganglia during postnatal development. Behavioral studies on the ontogeny of nociception suggest that the infant rat responds to noxious stimuli in the same manner as an adult. Flexor withdrawal reflex in response to pinching or heating is present at birth, but C-fiber-mediated processes, such as the response to mustard oil, do not emerge until PN10 [2]. However, the threshold [78] and behavioral response [17] to shock does not change with the end of the sensitive period. Research on the efficacy of morphine as an effective analgesic in neonates has produced various results. Morphine was found to be an effective analgesic in PN5 pups [77], but in other studies it was found not to be effective until PN12–14 [4]. Kehoe and Blass [41] showed an analgesic response at PN10, and Thornton et al. [81] demonstrated that morphine was less effective at PN3, but reached peak potency at PN9, suggesting an age-dependent increase in anti-nociception. Overall, the differences in behavioral studies with noxious stimulation and the studies with morphine-induced analgesia overall reflect the fact that the nociceptive system is not fully mature until close to weanling [2,18].

4.3. Significance

In conclusion, our results suggest that the opioid system mediates both neural and behavioral mechanisms to facilitate survival in the nest at a time when olfactory preference formation is of paramount concern. It appears that during this time, opioids function to modulate mother–infant interactions, particularly olfactory preferences, and to enhance the retention and expression of this behavior in pups. Future experiments are necessary to examine the location of this opioid action and to distinguish between central and peripheral effects, particularly with the odor–shock conditioning paradigm. Since prenatal opiate exposure has been demonstrated to produce several maladaptive consequences in infant neural development and behavior, particularly with development of the endogenous opioid system, our results highlight the capacity of prenatal opiate exposure to alter neural mechanisms mediating postnatal learning, specifically mediating mother–infant relationships.

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