

Short Communication

Bilateral 6-OHDA lesions of the locus coeruleus impair associative olfactory learning in newborn rats

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Abstract

On postnatal day 4 (PN4) Wistar rat pups were anesthetized and received bilateral infusions of 6-OHDA into the locus coeruleus or received vehicle infusions. On PN6 pups were trained in a classical conditioning paradigm with intra-oral milk infusions as the UCS and citral odor as the CS. Pups were trained in either 'paired', 'odor-only', 'milk-only' or 'backward' (milk then odor) conditions. On PN7 acquisition of a learned odor preference to the CS was tested in a two-odor choice test. HPLC analysis showed that locus coeruleus lesions significantly reduced olfactory bulb NE content but had no effect on olfactory bulb DA or 5-HT levels compared to controls. Pups receiving locus coeruleus lesions did not differ in behavioral response patterns during training compared to their littermate, vehicle controls. However, locus coeruleus lesions impaired acquisition of conditioned odor preferences. These results suggest that NE output from the locus coeruleus is critical for early olfactory learning.

Key words: Locus coeruleus; Olfactory bulb; Norepinephrine; Dopamine; Serotonin; Associative learning; Memory; Rat pup

Associative olfactory learning in newborn rats involves and requires norepinephrine (NE). Systemic administration of the NE β -receptor antagonists, propranolol or timolol, prior to conditioning impairs acquisition of learned odor preferences [9,13]. Conversely, association of an odor with the NE β -receptor agonist, isoproterenol, is sufficient to produce a subsequent preference for that odor [9,13]. NE antagonists injected before testing, however, do not impair expression of a previously learned response [11]. Thus, in infant rats, NE appears to be critical for acquisition but not expression of a learned odor preference.

Correlated with the learned behavioral responses is a structural [19] and physiological [2,8,17] modification of the olfactory bulb. The physiological changes appear to be dependent on NE during acquisition [9,13]. In fact, localized infusions of propranolol into the olfactory bulb during conditioning blocks acquisition of learned odor preferences [14]. Thus, one critical site of NE action during early learning is the olfactory bulb.

The primary source of olfactory bulb NE is the locus coeruleus, and this projection develops early in the perinatal period [6,15]. Furthermore, the neonatal locus coeruleus has been shown to be particularly sensitive to stimuli which function as UCS's in newborns [7]. The present study, therefore, examined the effects of locus coeruleus lesions on olfactory learning in rat pups.

Wistar rat pups, born in our colony to dams from Hilltop Lab Animals (Scottsdale, PA), were used as subjects. Day of birth was considered postnatal day 0 (PN0). Litters and dams were housed in polypropylene cages lined with wood chips and supplied with ad libitum food and water. Lights were kept on a 12/12 h cycle with lights on at 07.00 h. No more than one pup per litter was used in any training/lesion condition.

On PN4, pups were anesthetized by hypothermia and placed in a stereotaxic apparatus. The skull was aligned such that lambda and bregma were in the same horizontal plane. Holes were drilled through the skull at 1.4 mm posterior to lambda, and ± 0.6 mm from the midline. A 30 gauge, blunt tipped cannula, attached to a 10 μ l syringe, was lowered 5.5 mm from the surface

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of the skull, which placed the tip near the locus coeruleus. The infusion consisted of 1 μ l of either saline vehicle or 6-OHDA HCl (4 mg/ml; Sigma). The volume was infused slowly in two 0.5 μ l increments. The procedure was then repeated on the contralateral side. The scalp was closed and the pups were allowed to recover on a heating pad (32°C) for 1 h before being returned to the dam.

On PN6, pups were implanted with intra-oral cheek cannulas (PE10 tubing) under ether anesthesia and deprived of maternal care for 6 h on a heating pad (32°C). Following the deprivation period, pups were placed in individual glass beakers for a 10 min habituation period followed by a 30 min training session. Pups were assigned to one of 4 classical conditioning groups. *Paired*. Vehicle, $n = 9$; lesion, $n = 9$. Pups received an odor CS paired with 10, intra-oral milk infusions. The odor CS was citral (Sigma; approximately 2.5 μ l) placed on a Kimwipe suspended 9 cm above the floor of the beaker. The milk UCS (Half-and-Half at room temperature) was delivered by a Hamilton syringe pump for 10 s at 0.05 ml/min with an inter-trial interval of 3 min.

Odor. Vehicle, $n = 9$; lesion, $n = 9$. Pups received only the odor for 30 min.

Milk. Vehicle, $n = 9$; lesion, $n = 9$. Pups received only the milk infusions. This group was trained in a room separate from the odor conditions.

Backward. Vehicle, $n = 8$; lesion, $n = 7$. Pups received 10 milk presentations (3 min ITI) followed by the 30 min odor presentation.

The milk and odor presentations were done in different rooms with at least 30 min between milk and odor presentations. Twenty minutes after training, the cheek cannulas were removed and the pups were returned to the dam.

To examine behavioral responsiveness to the UCS, pups in the Paired condition were monitored during training. Behavioral activity levels were observed immediately pre- and post-UCS onset. Pup behavior was rated on a 0–5 scale as described by Hall [4], with 0 corresponding to no movement and 5 corresponding to vigorous movement of all extremities.

On PN7, learned relative odor preferences were assessed in all pups with a two-odor choice test. The test apparatus consisted of a Plexiglas arena (24 cm long \times 14 cm wide) with a metal grating floor. A neutral zone (2 cm) bisected the arena perpendicular to the long side. On either side of the neutral zone, beneath the grating, was a citral-scented Kimwipe or clean wood shavings. The Kimwipe was scented with 5 μ l of citral and allowed to stand 30 min before testing began. A testing trial began with the pup placed on the neutral zone. The time spent over each odor was recorded. Each pup received three 60 s trials and the direction the pup was placed in the arena was counter-

balanced across trials. Between trials, the pup was removed from the arena and the floor wiped clean with water.

Following behavioral testing (within 4–18 h), pups were decapitated and their brains quickly removed. The olfactory bulbs and brainstems were dissected, weighed, frozen and stored at -70°C until analysis. Brain tissue samples were sonicated in cold pH 5.0 acetate buffer which contained dihydroxybenzylamine (DHBA) as an internal standard. Samples were centrifuged at $16,000 \times g$ for 10 min and 50 μ l of supernatant was directly injected into the high-performance liquid chromatography system coupled with dual coulometric electrochemical detectors described by Hall et al. [3]. Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), NE, serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were detected with serial electrochemical detectors; chromatographic peaks were identified by retention times and standard-addition protocols. Whole tissue levels (ng/g wet wt. of tissue) were calculated using calibration curves for each compound and recovery using the internal standard DHBA [3]. Left and right olfactory bulbs were analyzed separately. The analysis, however, revealed comparable NE depletion in both bulbs in all but a very few cases, thus for statistical comparisons both bulbs from an individual animal were combined.

As shown in Table 1, bilateral 6-OHDA infusions into the locus coeruleus significantly reduced (83–91% reduction) olfactory bulb NE content (2×3 ANOVA, main effect of lesion, $F_{1,49} = 108.50$, $P < 0.001$). Post-hoc tests revealed NE content in lesioned pups was significantly lower than vehicle control pups ($P < 0.01$). Olfactory bulb DA and 5-HT levels and indices of turnover, however, were not significantly affected. Brainstem NE levels in lesioned pups were also markedly decreased compared to vehicle pups, though not as much as seen in the bulb (41%–54% reduction; 2×3 ANOVA, main effect of lesion, $F_{1,47} = 14.48$, $P < 0.001$).

Body weight gain of lesioned pups was slightly, though significantly, reduced compared to vehicle controls (Fig. 1, top left). By PN7, 3 days after the lesion, lesioned pups weighed approximately 1 g less than controls (5.7% decrease). A repeated measures ANOVA revealed a significant age vs. lesion interaction ($F_{3,174} = 11.88$, $P < 0.001$).

During training, however, both lesioned and vehicle control pups responded similarly to UCS intra-oral milk infusions (Fig. 1, top right). Activity levels in pups in both groups were increased by the milk compared to pre-milk levels (2×2 ANOVA, main effect of milk, $F_{1,19} = 176.75$, $P < 0.001$). There was no significant interaction between activity levels and lesion condition.

Vehicle infused pups trained in the Paired condition acquired a relative odor preference for the CS odor

Table 1
Results of HPLC analysis of olfactory bulb and brainstem catecholamine content and turnover indices

Area/analysis	Condition	Training condition			
		Paired	Odor	Milk	Backward
Bulb NE (ng/g)	Sham	84.9 ± 8.8	96.7 ± 10.7	127.6 ± 30.9	104.4 ± 11.8
Bulb NE (ng/g)	Lesion	12.2 ± 3.9 **	8.5 ± 1.9 **	21.3 ± 10.5 **	15.0 ± 11.8 **
Bulb DA (ng/g)	Sham	60.0 ± 13.0	66.6 ± 20.2	57.2 ± 10.6	62.9 ± 15.5
Bulb DA (ng/g)	Lesion	50.6 ± 3.0	46.2 ± 6.6	46.0 ± 5.7	49.5 ± 5.0
Bulb 5-HT (ng/g)	Sham	77.3 ± 10.1	100.8 ± 16.2	111.7 ± 10.4	90.3 ± 11.0
Bulb 5-HT (ng/g)	Lesion	94.8 ± 14.1	98.7 ± 13.8	76.6 ± 13.3	87.9 ± 15.2
Bulb DA/HVA	Sham	1.16 ± 0.63	0.78 ± 0.34	0.60 ± 0.08	0.73 ± 0.26
Bulb DA/HVA	Lesion	0.57 ± 0.74	0.51 ± 0.08	0.48 ± 0.06	
Bulb 5HT/5HIAA	Sham	0.65 ± 0.11	0.66 ± 0.17	0.94 ± 0.18	0.69 ± 0.06
Bulb 5HT/5HIAA	Lesion	0.64 ± 0.12	0.74 ± 0.13	0.49 ± 0.07	0.65 ± 0.11
Brainstem NE (ng/g)	Sham	190.9 ± 37.0	182.6 ± 31.1	183.7 ± 32.9	159.4 ± 35.0
Brainstem NE (ng/g)	Lesion	113.0 ± 34.8	89.2 ± 21.5	83.9 ± 21.4	91.8 ± 28.7

Data are means ± S.E.M. Asterisks represent significantly different from sham operated pups ($P < 0.01$). Number of samples ranges between 5 and 9.

compared to vehicle infused pups in conditioning control groups (Fig. 1, bottom). Locus coeruleus lesions blocked this learned odor preference without significantly affecting behavior in the conditioning control groups (4×2 ANOVA, training group vs lesion interaction, $F_{3,61} = 3.84$, $P < 0.02$). Post-hoc tests revealed that Paired-vehicle pups spent significantly more time over the CS odor than any other group ($P < 0.05$).

The present results demonstrate that the NE containing nucleus locus coeruleus is critically involved in

early olfactory associative learning. This impaired learning was not associated with a change in behavioral responsiveness to the UCS. Furthermore, it was not associated with a change in behavioral responsiveness to the CS, as demonstrated by the similarity in odor-guided behavior of lesioned and vehicle infused conditioning control pups (Odor, Milk and Backward) in the two-odor choice test. Additionally, prior work has shown that NE is not required for expression of previously learned odor responses in pups [11]. Thus, the

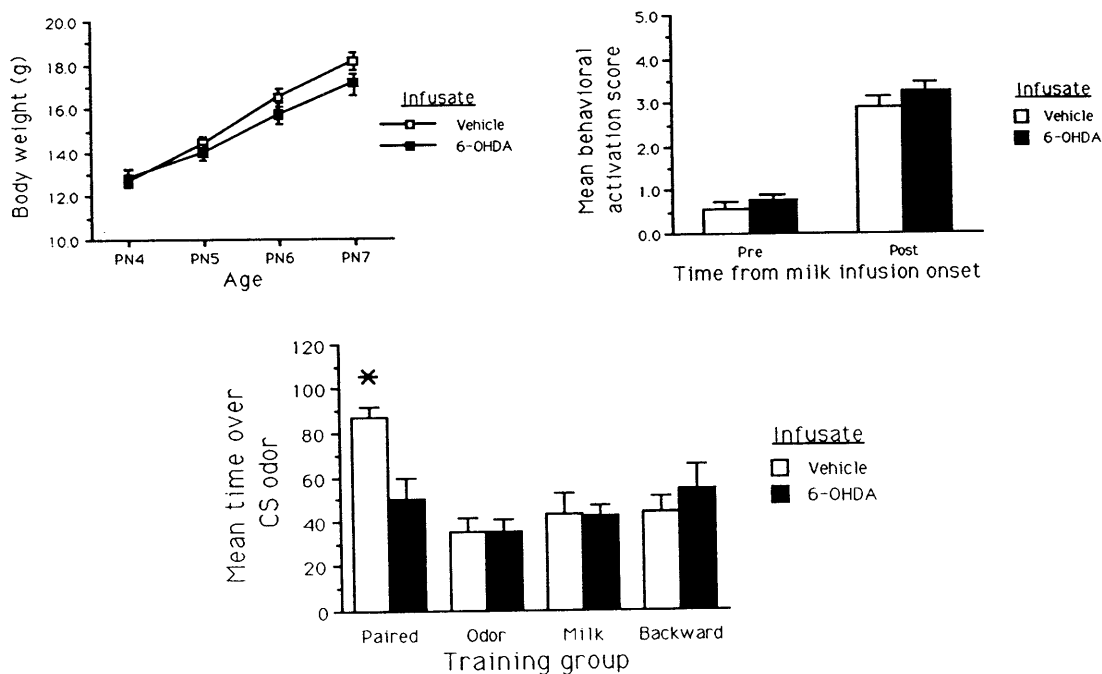


Fig. 1. Top left: mean body weight (\pm S.E.M.) for lesioned and vehicle-infused pups from the day of surgery (PN4) through testing (PN7). Top right: behavioral response of pups before and during intra-oral milk infusions. Lesioned and vehicle-infused pups were activated to a similar extent by the milk. Bottom: mean (\pm S.E.M.) time spent over citral in the two odor choice test. Only vehicle-infused, Paired trained pups acquired a relative odor preference. Asterisk represents significantly different from all other groups, $P < 0.05$.

present results suggest that the locus coeruleus and NE are necessary for acquisition and/or post-training consolidation [12] of early olfactory memories.

These results further define the neural substrates of early olfactory learning. Although the lesions performed here presumably reduced NE throughout the forebrain, previous work has demonstrated the olfactory bulb to be a critical target for NE in early learning. In fact, there is functional convergence of olfactory conditioned stimuli and NE-mediated UCS information within the olfactory bulb itself [16]. Thus, we speculate that the NE projection from the brainstem nucleus locus coeruleus to the most rostral aspect of the forebrain, the olfactory bulb, is required for olfactory learning in newborns. Blockade of NE, either at the target by infusing propranolol directly into the bulb [14], or at the source by lesions of the locus coeruleus (present report), prevents acquisition of early olfactory memories.

In newborn rats, the physiology of the locus coeruleus appears to make it particularly sensitive to sensory stimuli which support odor preference conditioning in pups [5,7]. For example, tactile stimulation functions as a UCS to produce a learned odor preference in neonates but not older pups [10,18]. Similarly, tactile stimulation activates the locus coeruleus in neonates but not in older pups or in adults [7]. Furthermore, locus coeruleus neurons are electrotonically coupled in newborns but not in adults [1]. This coupling may serve to amplify locus coeruleus responsiveness in the neonate by recruiting additional neurons and increasing NE release at target sites following stimulation. Together, the enhanced sensitivity and amplified response may increase the probability that odors experienced by the neonate will be associated with NE release in the bulb creating a memory for that odor.

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