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Olfactory Associative Conditioning in Infant Rats with Brain Stimulation as Reward:

II. Norepinephrine Mediates a Specific Component of the Bulb Response to Reward

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Abstract

One of the circuits modified by early olfactory learning is in the olfactory bulb. Specifically, response patterns of mitral-tufted cells are modified by associative conditioning during the early postnatal period. In addition, previous work has demonstrated that mitral-tufted cell single units respond to both olfactory conditioned stimuli and rewarding stimulation of the medial forebrain bundle-lateral hypothalamus (MFB-LH). The present study suggests that norepinephrine β -receptor activation is required for early olfactory learning using MFB-LH stimulation as reward. Propranolol injected before odor-MFB-LH pairings blocks the acquisition of conditioned behavioral responses and their neural correlates to the conditioned odor. Furthermore, propranolol blocks a specific class of the mitral-tufted cell responses to MFB-LH reward stimulation. The relationship of this response to reward and early learning is discussed.

The olfactory bulb receives extensive centrifugal inputs conveying information regarding a variety of internal states such as arousal level, hunger, and sexual receptivity. Responses of the bulb to odors can be modulated by these centrifugal inputs (Cattarelli, 1982; Gray, Freeman, & Skinner, 1986; Pager, 1978; Potter & Chorover, 1976). For example, responses of mitral-tufted cells (the primary output neurons of the bulb) to specific odors are dependent on the current level of hunger or behavioral arousal (or both) of the animal (Gervais & Pager, 1979; Scott, 1977).

In addition, noradrenergic (NE) centrifugal input to the olfactory bulb may also modulate neural plasticity in that structure, allowing long-term changes in bulb function after associative conditioning or other memorial events (Cornwell-Jones & Bollers, 1983; Gray et al., 1986; Rosser & Keverne, 1985; Sullivan, McGaugh, & Leon, 1991; Sullivan, Wilson, & Leon, 1989). The role of NE in plasticity is not limited to the olfactory bulb because NE has been implicated in synaptic plasticity in other sensory systems (Kasamatsu & Pettigrew, 1979; Bear & Singer, 1986) as well as hippocampal long-term potentiation (Dahl & Sarvey, 1989; Neuman & Harley, 1983).

NE is present and functional in the neonatal rat olfactory bulb (McLean & Shipley, 1991; Wilson & Leon, 1988a). Indeed, the role of NE in olfactory bulb plasticity appears to manifest itself at least as soon as the first postnatal week. For example, association of an odor and reinforcer during the preweaning period produces a conditioned behavioral response to that odor that is correlated with specific changes in olfactory bulb neural response patterns. These neural changes include enhanced glomerular layer focal 2-deoxyglucose uptake and

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modified mitral-tufted cell single unit responses to the conditioned odor (Coopersmith & Leon, 1984; Sullivan & Leon, 1986; Wilson, Sullivan, & Leon, 1987). As early as the first postnatal week, blockade of NE activity during pairing of odor and reinforcement prevents both the behavioral and neural changes that normally occur with learning (Sullivan et al., 1989, 1991). Conversely, simultaneous pairing of an odor with an NE α -receptor agonist results in long-term modification of bulb physiology and olfactory behaviors in neonates (Sullivan et al., 1989, 1991). Furthermore, NE agonists combine additively with conventional rewards in early olfactory conditioning (Sullivan et al., 1991).

These results suggest that at least one of the loci of odor-reward convergence may occur in the olfactory bulb, which allows associative changes to occur. In support of this hypothesis, we have recently demonstrated that mitral-tufted cells respond to medial forebrain bundle-lateral hypothalamic (MFB-LH) stimulation in young rats (Wilson & Sullivan, 1990). Furthermore, pairing an odor with MFB-LH stimulation produces both a conditioned odor preference and modified mitral-tufted cell responses to subsequent presentations of that odor (Wilson & Sullivan, 1990). Because NE has been shown to be critically involved in olfactory conditioning, the present study examined the role of NE in associative conditioning using MFB-LH stimulation as reward and the role of NE in mitral-tufted cell responses to that reward. We demonstrated that propranolol, a NE antagonist, blocks early olfactory learning with MFB-LH stimulation as reward and that NE may mediate a specific component of mitral-tufted cell response to reward in young rats.

Method

Subjects

The subjects were 49 male and female postnatal day 11 (PN11) Wistar rat pups born to females obtained from Hilltop Lab Animals (Scottsdale, PA). The day of birth was considered PNO, and litters were culled to 8 pups on PN1. No more than 1 male, 1 female, or both were used from a litter for a treatment-test condition. Lights were on at 7:00 a.m. and off at 7:00 p.m. All procedures were in accordance with National Institutes of Health guidelines.

Electrode Implantation

On PN11, pups were anesthetized with a 1:1 mixture of ketamine and xylazine (25 mg/kg ip) and placed in a stereotaxic apparatus. A bipolar, stainless steel, Teflon-coated electrode (twisted 0.13-mm-diameter strands, 0.5 mm tip separation) was implanted in the MFB-LH (0.5 mm posterior to bregma, 1.5 mm lateral, and 7.5 mm ventral to skull surface) and attached to the skull with dental acrylic. The pups were kept warm during recovery from the anesthesia, which generally took 2-4 hr.

Drugs and Conditioning

At least 1 h after return to normal behavioral activity, the pups were injected subcutaneously with either saline or 20 mg/kg DL-propranolol hydrochloride (Sigma Chemical Co., St. Louis, MO). This dose of propranolol has been shown to be maximally effective at disrupting early olfactory learning (Sullivan et al., 1989, 1991). Thirty-five minutes later the pups were placed in glass training chambers (1-L beakers) for a 10-min habituation period, which was followed by olfactory conditioning. Pups were randomly assigned to receive either *paired* presentations of odor and MFB-LH stimulation or *backward* presentations of odor and MFB-LH stimulation. (Half the pups in each group were saline treated, and the other half were propranolol treated.) For paired presentations, the odor was delivered for 15 min, with the MFB-LH stimulation delivered every 30 s (total of 30 MFB-LH stimuli concurrent with the odor stimulus). Pups in the backward group received 30 MFB-LH stimuli (1/30 s) and then 15 min of odor (nonconcurrent MFB-LH-odor presentations). The odor was peppermint (Shilling) that was

presented through a flow-dilution olfactometer at 2 L/min and at a 1:10 concentration of saturated vapor. The saturated vapor was obtained by circulating air over a pool of peppermint extract in a 25 ml filtering flask. For MFB-LH stimulation, the pups were connected to a constant-current stimulator via lightweight leads. Stimulation consisted of a 200-Hz, 300-ms-duration train of 0.1-ms-duration monophasic pulses at 100-1,300 $\hat{\text{A}}$. Stimulus intensity for training was set to reliably evoke behavioral activation. If a pup did not behaviorally respond to the stimulus, then intensity was set at 1,000 $\hat{\text{A}}$. Pups were tested for behavioral odor preference or mitral-tufted cell response patterns 18-24 h later.

Odor Preference Test

Testing was performed in the absence of any drugs. The odor preference test was a two-odor choice test consisting of an opaque Plexiglas arena (20 $\hat{\text{A}}$ 24 $\hat{\text{A}}$ 34 cm) with a stainless steel grate floor (7-mm-diameter holes). The floor was divided in half, perpendicular to the long side of the arena, by a neutral zone (2 cm). Under the floor, on each side of the neutral zone, was a tray filled with either clean pine wood chips or pine wood chips mixed with peppermint odor. The pup was placed on the central neutral zone, and the amount of time the pup spent over each odor was recorded. The test lasted 60 s and was repeated three times. Between tests, the floor was washed with water and dried. The direction the pup was placed in the test apparatus was counterbalanced between trials. All observations were made by observers who were unaware of the subjects' treatment.

Neurophysiology

Details of the recording procedure have been presented previously (Wilson & Sullivan, 1990). All recordings were taken from urethane-anesthetized, normally respiring pups using an electrode positioned ipsilateral to the MFB-LH stimulating electrode. A bipolar stimulating electrode was placed in the lateral olfactory tract (LOT), and mitral-tufted cell single unit responses were recorded in the olfactory bulb with a glass microelectrode filled with 2 M NaCl.

Test odors were delivered to the external nares through glass tubing from a flow dilution olfactometer. Pups respired normally from a continuous flow (500 ml/min) of clean air to which a 1:10 dilution of saturated vapor could be added with a Harvard syringe pump controlled by a Zenith 286 computer. Mitral-tufted cell responses to repeated 4-s odor pulses were monitored. Stimuli were repeated three times, and responses were analysed with peristimulus time histograms (250-ms bin widths). At least 60 s elapsed between stimuli. Responses were categorized as either excitatory, suppressive, or no response as compared with a 5-s preodor baseline, as previously described (Wilson & Leon, 1988b; Wilson & Sullivan, 1990). Excitatory responses were characterized by an increase in firing rate during the 4-s stimulus above the variability of the 5-s baseline, whereas suppressive responses were characterized by a decrease in firing rate below baseline variability. The ratio (R) of the percent of excitatory responses to the total response rate (excitatory + suppressive) was determined ($R = \% \text{ excitatory} / \text{total } \% \text{ responding}$), and comparisons of this ratio were made between groups. All cells were recorded ipsilateral to the MFB-LH electrode.

Responses to MFB-LH Stimuli

An additional 10 naive pups (11-12 days old) were used to examine mitral-tufted cell single unit responses to MFB-LH stimulation alone (no odor presented). These animals were prepared as just described for single unit recording with a stimulating electrode in the MFB-LH and one in the LOT. MFB-LH stimulation was the same as that used in the conditioning procedure: 200 Hz, 300 ms, and 500-1,000 $\hat{\text{A}}$. Responses to MFB-LH stimulation were analyzed as for odor responses, by comparing poststimulus firing rate to a 5-s prestimulus baseline. Responses were either excitatory, suppressive, or null, as previously described (Wilson & Sullivan, 1990). After 2-5 cells were analyzed in a given animal, the pup was injected subcutaneously with 20 mg/

kg DL-propranolol. Recording was resumed 45 min after the injection; the cells were localized, and the responses were characterized for an additional 60 min. Our previous research suggests that the effects of systemic propranolol injections on bulb physiology peaks approximately 60 min after injection and returns to baseline by 120 min at this age (Wilson & Leon, 1988a). Additional pups ($n = 2$) were injected with saline rather than propranolol. No difference was observed between responses during the preinjection period and postsaline period, thus these saline data are included in the preinjection baseline data for statistical analysis. Comparisons were made between mitral-tufted cell response patterns to MFB-LH stimulation pre- and postpropranolol.

Results

Stimulation of the MFB-LH produced behavioral activation in both saline- and propranolol-treated groups. This activation ranged from mouthing to probing to generalized activity, as previously reported (Moran, Schwartz, & Blass, 1983; Wilson & Sullivan, 1990). Stimulus intensity required to produce this activation varied across animals, from 200 to 1,300 $\hat{\text{A}}$. Using an analysis of variance, no significant difference was found in stimulus intensity used between the conditioning and drug conditions, $F(1, 34) = 0.6$ *ns* (mean stimulus intensity for all pups = 643 $\hat{\text{A}}$).

Temporal association of an odor and MFB-LH stimulation produced a relative odor preference in pups trained with saline (Figure 1A). Propranolol during training blocked acquisition of this learned response, Drug $\times$ Training interaction, $F(2, 27) = 3.9$, $p < .05$; post hoc Tukey tests revealed that the paired-saline group was significantly different from all other groups ($p < .05$). Pups in both saline-backward and propranolol-backward groups were not significantly different from naive pups.

In addition to the learned behavioral response, mitral-tufted cell single unit responses to the conditioned odor were also modified (Figure 1B). A total of 97 cells from 12 pups were tested (3 pups/group, at least 24 cells/group). The naive group was not included in this analysis to reduce the total number of pups tested. Previous studies (Sullivan & Leon, 1986; Wilson et al., 1987) have shown that single unit responses in naive pups do not differ from other control groups. Mitral-tufted cells in the paired-saline group showed more suppressive and fewer excitatory responses to the conditioned odor ($R = 0.42$) than pups in the backward-saline group ($R = 0.68$). Propranolol during training blocked the acquisition of this modified mitral-tufted cell response ($\hat{\text{I}}^2 = 3.0$, $p < .05$). There was no significant difference in overall response rates between the groups (paired-saline = 42%; paired-propranolol = 42%; backward-saline = 46%; backward-propranolol = 48%).

These results suggest that NE is required during training for acquisition of both learned behavioral responses and their neural correlates in young rats. To further examine the role that NE plays in odor-reward association in young rats, 10 additional naive PN11-12 rats were used to monitor mitral-tufted cell responses to MFB-LH stimulation before and after propranolol injections.

A total of 72 cells were tested, 45 before propranolol injection and 27 between 45 min and 105 min after injection (20 mg/kg sc). As previously reported (Wilson & Sullivan, 1990), cells recorded during the baseline period responded to MFB-LH stimulation by either increasing their firing rate (Type E; Figure 2), decreasing their firing rate (Type I), or did not demonstrate a significant response (null response). These different response types could be obtained with a single stimulation site and at a variety of stimuli intensities and did not appear to be dependent on recording location within the mitral cell body layer (Wilson & Sullivan, 1990).

Type E cells accounted for 54.6% of the cells sampled during the baseline period, and Type I cells accounted for 31.9% (Figure 2). Propranolol, however, greatly reduced Type E responses while leaving Type I responses unaffected, Drug $\times$ Response Type interaction, $F(2, 45) = 13.6$, $p < .001$; post hoc Tukey tests revealed a significant difference between saline and propranolol groups in Type E and null-response categories ($p < .05$). Altering MFB-LH stimulus intensity did not reinstate Type E responses. It should be noted that the stimulus intensities used here were sufficient to evoke behavioral activation in both saline- and propranolol-injected awake pups, as already described.

In addition to response patterns to MFB-LH stimulation, mitral-tufted cell spontaneous activity rates were significantly depressed by propranolol. Baseline firing rate before propranolol was 4.6 Hz (± 0.6) and was reduced to 2.3 Hz (± 0.5) after injection, $t(49) = 2.7$, $p < .02$. Antidromic response latencies to LOT stimulation were not significantly affected by propranolol (baseline = 8.1 $\pm$ 0.7 ms; postinjection = 7.6 $\pm$ 0.5 ms). The relatively low spontaneous activity rate and long antidromic latencies in the predrug conditions are characteristic of the bulb at this age (Wilson & Leon, 1986).

Discussion

The present results suggest that NE may be required for early olfactory learning with MFB-LH stimulation as reward. Blockade of NE α -receptors with propranolol during training prevents acquisition of behavioral and neural conditioned responses to the odor. These results extend our previous findings on the role of NE in early olfactory learning with tactile stimulation as reward (Sullivan et al., 1989, 1991) and correspond to the findings of others on NE and olfactory learning in mature animals (Gray et al., 1986; Rosser & Keverne, 1985).

Of importance, the present results suggest that the propranolol-induced deficit in early learning is correlated with a specific change in the olfactory bulb response to reward. Type E mitral-tufted cell responses to MFB-LH stimulation are greatly reduced by a dose of NE α -receptor antagonist that prevents learning. The occurrence of Type I responses is unaffected. A causal relationship between Type E responses and olfactory bulb modification cannot be determined from these data. However, the possibility remains that association of olfactory receptor neuron activation of mitral-tufted cells concurrent with NE-mediated mitral-tufted cell Type E responses to reward may be critical for synaptic modifications correlated with early learning. NE has also been implicated as an important mediator of reward information in the pigeon lateral geniculate nucleus during visual conditioning (Cohen, Gibbs, Siegelman, Gamlin, & Broyles, 1982).

It has previously been shown the olfactory bulb NE depletion does not affect olfactory thresholds (Doty, Ferguson-Segall, Lucki, & Kreider, 1988). Furthermore, NE antagonists have limited effects on the magnitude of MFB-LH stimulation-induced reward (Stellar & Rice, 1989). In fact, in the present study, no difference in stimulus thresholds for MFB-LH-induced behavioral activation was observed between saline and propranolol pups. Thus, in the paired condition, both saline and propranolol pups were similarly aroused. Furthermore, we believe these results do not represent a state-dependent or context effect, but rather the failure of propranolol pups to express an odor preference when tested without propranolol is due to a disruption of acquisition. We have recently demonstrated that pups acquire and express normal olfactory memory after being trained in the absence of propranolol but tested in the presence of propranolol (Sullivan & Wilson, in press). Thus, despite being in different propranolol-induced states, pups can express an acquired odor preference. Together, these findings suggest that propranolol directly or indirectly affects neural plasticity underlying early learning rather than reducing responsiveness to olfactory stimuli or modifying the hedonic value of the MFB-

LH stimulation. The role of other neurotransmitters-neuromodulators in early learning is currently being investigated.

Mitral-Tufted Cell Responses to Reward

The majority of centrifugal inputs to the olfactory bulb terminate on inhibitory interneurons, granule cells. Granule cells, in turn, form reciprocal dendrodendritic synapses with mitral-tufted cells. NE modulates granule cell-mediated inhibition in both the mature and immature bulb, apparently by inhibiting or suppressing granule cells (Jahr & Nicoll, 1982; Wilson & Leon, 1988a). Therefore, activation of the NE input to the bulb would suppress granule cell activity, thereby releasing mitral-tufted cells from inhibition. The Type E responses to MFB-LH rewarding stimulation, thus, may represent a disinhibition of mitral-tufted cells by direct or indirect activation of NE inputs to the bulb (Figure 3). Type I responses, on the other hand, may represent excitatory input to granule cells, perhaps via acetylcholine, serotonin, or other centrifugal inputs (Macrides & Davis, 1983).

If this is correct, then blockade of NE $\hat{I}$ -receptors should release granule cells from inhibition, thereby suppressing mitral-tufted cell activity. This is evident from the decrease in mitral-tufted cell spontaneous activity shown here and from an increase in LOT-induced paired-pulse inhibition (Wilson & Leon, 1988a). Furthermore, as shown in the present study, NE antagonists should prevent the disinhibitory response of mitral-tufted cells to MFB-LH stimulation (termed here Type E responses).

NE and Early Learning

The information required for expressing a learned odor preference is almost certainly stored in a variety of brain circuits in the newborn (Kucharski & Hall, 1987; Sullivan & Wilson, 1991). Our investigation has focused on elucidating the mechanisms underlying changes in one of those circuits—the olfactory bulb. We have demonstrated that early olfactory learning, using a variety of reinforcers, modifies an odor-specific group of mitral-tufted cells in a unique way. That is, after associative conditioning, mitral-tufted cells show an increase in suppressive responses and a decrease in excitatory responses to the conditioned odor (Wilson & Leon, 1988b; Wilson et al., 1987). The change is odor specific, lasts at least 24–48 hr and is NE-dependent for its acquisition (Sullivan et al., 1989, 1991) but not expression (Sullivan & Wilson, in press).

Mitral-tufted cells receive direct excitatory input from olfactory receptor axons (Wellis, Scott, & Harrison, 1989; Hamilton & Kauer, 1988) and respond to reward stimuli (Wilson & Sullivan, 1990; present study), presumably via centrifugal inputs to the bulb. Given these response characteristics, it is hypothesized that excitatory olfactory nerve input concurrent with NE-mediated disinhibition produced by reward allows long-term changes in synaptic strength that are subsequently expressed as an increase in inhibitory responses to olfactory nerve input alone. NE receptor antagonists during training, however, should enhance inhibition and decrease input from MFB-LH afferents, which may prevent associative changes from occurring in the olfactory bulb.

Interestingly, the requirement for multiple coactive inputs and the NE dependence of olfactory learning are reminiscent of hippocampal long-term potentiation (LTP), as has been previously noted (Brennan, Kaba, & Keverne, 1990; Wilson & Sullivan, 1990). Although LTP does not develop in the hippocampus or neocortex until several days after PN11–12 in the rat (Wilson, 1984), we are currently examining LTP and LTP-like events in the neonatal olfactory bulb.

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Figure 1.

Neurobehavioral response to the conditioned stimulus (CS) odor. (No drugs were used during the test. A: Behavioral response in a two-odor choice test between a familiar odor [pine wood chips] and the CS odor. B: Mitral-tufted cell single unit response patterns to the CS odor.

Excitation ratios above 50 correspond to more excitatory responses than suppressive responses to the odor, whereas ratios below 50 correspond to more suppressive responses than excitatory. Values are means $\pm$ SE. Asterisks in A and B signify significantly different from saline controls and propranolol groups, $p < .05$. UCS = unconditioned stimulus; MFB/LH = medial forebrain bundle-lateral hypothalamus.)

Figure 2. Mitral-tufted cell single unit responses to medial forebrain bundle-lateral hypothalamus (MFB/LH) stimulation. (A: Examples of Type E [top] and Type I [bottom] responses in control pups. Stimulus artifact produced by the MFB/LH stimulus is marked by an asterisk. Calibration bar is 0.5 s. B: Proportion of Type E, Type I, and null responses during baseline and after propranolol injection. Asterisks signify significantly different from saline, $p < .05$.)

Figure 3. Hypothesized circuitry underlying modification of bulb responses to learned odors in neonates using olfactory associative conditioning with brain stimulation. (It should be emphasized that expression of the learned behavioral response may involve plasticity in additional brain structures. CS = conditioned stimulus; ON = olfactory nerve; M/T = mitral-tufted cell; GC = granule cell; LOT = lateral olfactory tract; UCS = unconditioned stimulus; NE = norepinephrine; ACh = acetylcholine; 5HT = serotonin; MFB/LH = medial forebrain bundle-lateral hypothalamus.)