

Chemical Dependencies of Learning in the Rabbit Olfactory Bulb: Acquisition of the Transient Spatial Pattern Change Depends on Norepinephrine

Charles M. Gray
Department of Neurology
and the Neuroscience Program
Baylor College of Medicine

Walter J. Freeman
Department of Physiology-Anatomy
University of California, Berkeley

James E. Skinner
Department of Neurology and the Neuroscience Program
Baylor College of Medicine

Intracerebral cannulas were implanted in both olfactory bulbs of 6 rabbits. A surface electrode-array (8 × 8) was implanted epidurally on the lateral surface of the left bulb. Each rabbit was conditioned to respond to sniffing to an odor paired with cutaneous shock while receiving continuous intrabulbar infusion of either vehicle or propranolol (100 μM at 1 μl/hr) in vehicle. After two training sessions to the original odor, a response to a new odor was conditioned under the influence of the alternate infusate. Electroencephalographic (EEG) activity was sampled on inspirations before and during odor presentations. During vehicle infusion a transient alteration in the pattern of activity was acquired that occurred during the second and third inspirations following presentation of the reinforced odor. The acquisition did not occur when propranolol was infused. No significant pattern changes occurred with unreinforced odors in either condition. There was no local anesthetic effect of the racemic mixture of propranolol found for any type of electric activity, including antidromic spike activity observed in an independent control group. Intrabulbar norepinephrine injection (100 μM, 10 μl.) resulted in an amplitude increase of the bulbar 40-80-Hz EEG and a potentiation of the transient spatial pattern change to a novel odor, when compared with those observed during vehicle infusion. It is concluded that norepinephrine released under centrifugal control may act to prevent or delay habituation that otherwise occurs rapidly to unreinforced odors.

The coding of olfactory information is thought to depend on the selective sensitivity of the receptor neurons to different odors (Le Gros Clark, 1957; Mackay-Sim, Shaman, & Moulton, 1982; Moulton, 1976; Mozell, 1964). Adrian (1950) first proposed that the central representation of an odor might be detected in distinctive spatial patterns of neuronal activity in the olfactory bulb. This hypothesis is supported by the selective degeneration of mitral cells following prolonged odor exposure (Doving & Pinching, 1973) and the localization of enhanced metabolic activity in the bulb by the use of 2-deoxyglucose (Lancet, Green, Kauer, & Shepherd, 1982). Evidence in support of the hypothesis from single-unit recording has been inconclusive (Holley, Duchamp, Revial, & Juge, 1974); cells in the olfactory mucosa, the bulb, and the olfactory cortex all respond differentially to separate odors, but with extensive overlap of their response profiles (e.g.,

Lettvin & Gesteland, 1965). Previous investigations, however, have demonstrated that a close statistical relation exists between the spatial and temporal distributions of the unit firing rates in mitral cells and of the amplitudes of the bulbar electroencephalogram (EEG) in both control states and during exposure to odors (Freeman, 1975). These findings suggest that a test of Adrian's hypothesis could best be accomplished by analyzing the spatiotemporal patterns of bulbar EEG activity.

The field potentials on the surface of the bulb contain two main oscillatory components: 3-7-Hz activity in phase with respiration and 40-80-Hz oscillatory bursting activity superimposed upon the inspiratory phase of each respiratory wave. These surface potentials are thought to arise from an open-field dipole generated by postsynaptic potentials in the granule cell interneurons (Freeman, 1975; Nakashima, Mori, & Takagi, 1978; Rall & Shepherd, 1968). The amplitude of each 40-80-Hz burst reflects the strength of the coupled granule and mitral cell synaptic interactions (Freeman, 1979). The spatial distribution of burst activity recorded with arrays of 64 electrodes reveals regions of high-amplitude foci that comprise approximately 20% of the bulbar surface area. These foci correspond to the regions of local enhancement of metabolic activity detected through the use of 2-deoxyglucose (e.g., Lancet et al., 1982). The burst pattern and location of the foci fluctuate continually but the average pattern is essen-

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Correspondence concerning this article should be addressed to James E. Skinner, Mail Station F603 One Baylor Plaza, Baylor College of Medicine, Houston, Texas 77030.

tially invariant in the absence of changes in behavioral state (Freeman, 1978). The pattern and location of the foci are different for each rabbit.

Previous studies on odor-related changes in EEG activity patterns revealed no statistically significant differences in burst patterns during odor presentations without reinforcement (Freeman, 1978). Behavioral observations showed that the rabbits would sniff unreinforced odors on more than 90% of all trials in the first session. These response rates would then habituate to background levels by the second or third session (Freeman, Viana Di Prisco, Davis, & Whitney, 1983). During aversive conditioning the pairing of a previously habituated odor with shock as an unconditioned stimulus (UCS) produced a statistically significant difference in the pattern of activity observed in the first and second training sessions between control and odor bursts. By the third session, these differences in bulbar activity were no longer significant, although the rabbits continued to sniff the odors at rates greater than 90%. The control pattern changed to a new form that persisted until or unless the rabbit was trained to respond to a new odor (Freeman & Schneider, 1982). Changes in spatial pattern did not occur with the UCS alone or during conditioning to nonolfactory stimuli.

In another study, water-deprived rabbits were trained to discriminate between a reinforced and a nonreinforced odor introduced in the same session (reinforced with water as a UCS) by sniffing or licking (Viana Di Prisco & Freeman, 1985). A transient spatial pattern difference between control and test bursts for both conditioned stimuli (CSs) emerged after two or three sessions and persisted through to the sixth session of testing with each odor combination. A tendency for burst-amplitude reduction was observed when either CS was given. The rabbits exhibited discriminative licking responses to the CS+ by the second session and responded to both the CS+ and CS- by sniffing. Examination of the spatial amplitudes revealed that the shapes of the foci changed as each new odor combination was introduced.

On the basis of these and other findings, it has been proposed that the spatial modification of bulbar bursting activity manifests a form of synaptic plasticity that may be regulated centrifugally (Freeman, 1983). Fibers projecting to the olfactory bulb can be separated into two categories: (a) those arising from olfactory structures that are thought to mediate mitral cell inhibition by synaptic excitation of the granule cell interneurons (Mori & Kishi, 1982; Nakashima et al., 1978; Price & Powell, 1970a, 1970b) and (b) those arising from basal forebrain and midbrain structures that project diffusely to all layers of the bulb and are thought to have a neuromodulatory influence on mitral cell activity (Halasz & Shepherd, 1983). In the latter group there exists a projection arising from the locus coeruleus, a structure that is known to contain norepinephrine as a neurotransmitter (Fallon & Moore, 1978; Halasz, Ljungdahl, & Hokfelt, 1978; Macrides, Davis, Young, Nadi, & Margolis, 1981). It is this projection that is thought to participate in the centrifugal regulation of the bulbar synaptic plasticity.

The aim of the present experiment was to test the hypothesis that the acquisition of the transient spatial pattern change depends on the action of intrabulbar norepinephrine (NE).

This approach was based on two sets of observations regarding the role of NE in synaptic plasticity: (a) Locus coeruleus neurons are activated by unconditioned stimuli (Aghajanian & Vandermaelen, 1982; Aston-Jones & Bloom, 1981), and (b) several forms of use-dependent synaptic plasticity in cortical tissues require the presence of NE in the tissue (Bliss, Goddard, & Riives, 1983; Daw, Rader, Robertson, & Ariel, 1983; Gibbs, Broyles, & Cohen, 1986; Hopkins & Johnston, 1984; Kasamatsu & Pettigrew, 1979; Kasamatsu, Pettigrew, & Ary, 1981; Kasamatsu, Watabe, Scholler, & Heggelund, 1985). A further aim of the present experiments was to determine whether the observed bulbar plasticity was dependent on the activation of intrabulbar beta-receptors. Recent findings suggest that the postsynaptic action of central norepinephrine is mediated by beta-receptor activation (Minne-man, Dibner, Wolfe, & Molinoff, 1979). In addition, the induction of hippocampal long-term potentiation, a form of synaptic plasticity, can be blocked by perfusion of the tissue with propranolol and/or timolol (Hopkins & Johnston, 1984), drugs known to competitively inhibit beta-receptor activation by norepinephrine (Minne-man, Pittman, & Molinoff, 1981). The present study was focused on the transient response, because it occurs early during acquisition, is evoked by a single novel or conditioned stimulus, and is therefore amenable to chemical investigations limited in time by the capacity of implantable continuous infusion pumps.

The behavioral paradigm used here was to condition the relative frequency of an autoshaped response, sniffing an odor. In a previous study it was demonstrated that the relative frequency of sniffing responses to repeated odor presentations served as a reliable index of habituation, acquisition, and discrimination (Freeman et al., 1983). Under the conditions of that study it was found that rabbits would sniff at high rates (>90%) to novel odors presented without reinforcement. These response rates would habituate within one to three sessions (i.e., 10-30 trials). Habituation could be prevented by classical aversive conditioning of the odor. When reinforced and unreinforced odors (CS+ and CS-) were presented in the same session, the rabbits exhibited discriminative sniffing responses (i.e., reduced sniffing rate to the CS- odor) that developed within two to three sessions (Freeman et al., 1983). Thus although the conditioned and novel odors both evoke the behavioral response initially, relative changes in the magnitude and frequency of sniffing serve as a measure of the acquisition of classical olfactory conditioning. In the present study the relative frequency of sniffing was used to assess the degree of behavioral habituation and discrimination as well as to serve as a reliable indicator of odor detection.

Method

Subjects

Six New Zealand White adult rabbits of either sex (3-4 kg) were used. The animals were obtained from a commercial breeder and were individually caged in a well-ventilated room isolated from other animals. They were given food and water ad lib, and their health was monitored daily by staff under the supervision of the Berkeley Division of Animal Resources.

Surgery

Each rabbit was surgically anesthetized with ketamine (20 mg/kg) and pentobarbital (35 mg/kg, iv) and mounted in a stereotaxic apparatus. A stainless steel cannula (26 ga.) was implanted dorsally in each bulb at a depth of 3 mm from the bulbar surface and at a location 3–4 mm anterior to the olfactory peduncle. Each cannula was sealed with an obturator and fixed to the skull with dental cement. A single platinum electrode (100 μ m in diameter) was implanted in the left bulb 2 mm anterior to the cannula and at a depth of 1 mm to monitor long-latency event-related slow potentials (ERSPs).

A miniature electrode-array (8 \times 8 electrodes with outside dimensions of 3.5 \times 3.5 mm; Eastman, 1975) was implanted epidurally over the lateral bulbar surface (Freeman, 1978). Two stainless steel electrodes and one platinized-platinum reference electrode were placed in the orbit. The orbital cavity and dorsal openings were filled with sterile agar-saline and sealed with dental cement, and the incision was closed around the pedestal with wound clips.

Behavioral Conditioning

After a week of recovery, two conditioning experiments were performed in each animal with one or the other of two intrabulbar infusions in a counterbalanced order. Two days prior to conditioning, osmotic minipumps (Alzet, 1 μ l/hr flow, 1-week in duration) were attached to the intrabulbar cannulas with polyethylene tubing and implanted under the skin of the neck. The first conditioning experiment began with either vehicle infusion (0.9% NaCl, 0.1% Na ascorbate) or vehicle plus *dl*-propranolol infusion (100 μ M). Each rabbit was conditioned to the presentation of amyl acetate (CS+) paired with an aversive unconditioned stimulus (mild cutaneous shock to the neck, 5 pulses, 0.1 ms, 100 HZ, 1–3 mA) sufficient to evoke a twitch but not struggling or vocalization. The interstimulus interval was 2.7 s. The intertrial interval ranged randomly between 1 and 4 min. Ten trials were given during each of two identical sessions 4 days apart. Two rabbits received vehicle infusion for the experiment; the other 4 were given propranolol. After removal of the minipumps, two postinfusion sessions were conducted as a control.

The second experiment was conducted a week later, after surgically replacing the minipumps with the alternate infusate for each rabbit. The response to the previous CS+ (amyl acetate) was first extinguished (i.e., no sniffing occurred after 30–50 trials), and thereafter this odor was presented as the CS-. A new reinforced CS+ (butanol) and the CS- were randomly presented for 10 trials each in two identical sessions 4 days apart. Again, two postinfusion control sessions were conducted following removal of the minipumps.

The procedures for instrumentation and familiarization of the animals and the delivery of odors at controlled concentrations (1:1000 in water) with a dilution olfactometer (Moulton, Turk, & Johnson, 1975) have been described previously (Freeman & Schneider, 1982). Sniffing responses were detected by an on-line computer algorithm (G. Davis & Freeman, 1982; Freeman et al., 1983). Evaluation of sniffing behaviors was accomplished by calculating the percentage of detectable responses for each set of 10 trials to a given odor. In the present experiment, only two conditioning sessions were conducted for each stimulus and infusion condition. The results from these sessions were pooled across animals and sessions for each experiment. Statistical evaluations were made by comparing the mean response rates for each of the odors under each infusion condition. A difference *t* test was used for all comparisons.

Evoked Potentials: Control for Local Anesthesia

In order to examine the possibility that *dl*-propranolol was having a local anesthetic action on the bulbar neurons, a separate control

experiment was conducted. Three rabbits were each surgically implanted with bilateral intrabulbar cannulas (as described above), a bipolar stimulating electrode (stainless steel, 120 μ m) in the left lateral olfactory tract (LOT), and a monopolar recording electrode (stainless steel, 120 μ m) in the granule cell layer of the left dorsal olfactory bulb.

Following recovery two experiments were carried out on each rabbit. In each experiment, three sets of recordings were made of the antidromic compound action potential evoked by stimulation of the LOT. These records were taken before and at 30 and 60 min following the start of the intrabulbar infusion (10 μ l/hr flow rate) of either vehicle or *dl*-propranolol in vehicle (100 μ M). Each recording consisted of four averaged evoked potentials in which each average was composed of 10 responses. The amplitude (in microvolts) and latency (in milliseconds) of the first negative peak were computed for each of the four averages at each time point. Responses during the infusion were compared with the responses recorded prior to infusion by calculating a difference *t* value. The experiment was repeated 3 days later with the alternate infusate.

Norepinephrine Injection

After the propranolol studies were completed, 5 of the rabbits were used in a subsequent study to determine the effects of intrabulbar norepinephrine (NE) on the phasic pattern response to novel odors. Because of a technical failure, 1 animal could not be studied. Two experiments were conducted with each rabbit. In the first experiment, either the vehicle alone or NE (100 μ M) in vehicle was injected (10 μ l, infused during 2 min) into both bulbs. Immediately following the injection, 10 presentations of a novel odor (methylsalicylate) were given, with an intertrial interval of approximately 1 min. Three days later a similar experiment was carried out in each rabbit with the alternate infusate and a new novel odor (methyl-ethyl-ketone). A final control recording was made 3 days later.

Tissue Distribution of [³H]Norepinephrine

To determine whether the NE injected in the above experiment was perfusing the interstitial spaces throughout the bulb, we undertook a final study in the same rabbits. Tritium-labeled NE (0.5 Ci/mM) was injected into both bulbs with a concentration, volume, and infusion time identical to those in the earlier experiment. After 10 min, the animal was anesthetized with iv pentobarbital. Twenty minutes after the injection, the animal was decapitated, and the cranium, with soft tissue removed, was immersed in liquid nitrogen and stored at -70 °C.

The anterior forebrain was removed from each skull while the tissue remained frozen. The olfactory bulbs and anterior olfactory nuclei were dissected, and each was sectioned into two halves in the sagittal plane. Each half was then sectioned in coronal planes at 1.0–1.5-mm intervals, which resulted in five pieces, with the cannula track being between the second and the third. Each individual sample (20 samples/animal) was then weighed, solubilized (under heat, in 0.2 ml of 1 N NaOH), and counted for tritium.

Two of the samples from each bulb lying adjacent to the intrabulbar cannulas were subjected to thin-layer chromatography (TLC), by the method of Schneider and Gillis (1965), to determine the percentage of radio-label comprising unchanged NE. Results from the tritium counts and the TLC were used to determine the amount of unchanged NE in each sample expressed as picomole of NE/milligram of wet tissue weight. Two separate olfactory bulbs were weighed to determine their wet tissue weight. This information was used to predict the concentration of NE that would exist in the bulb following the injection, assuming no metabolic breakdown of the NE and assuming its uniform distribution throughout the bulb. By this technique we

were able to account for differences in the observed distribution of NE from that expected under ideal conditions.

Data Acquisition and Analysis

All electric activity was recorded monopolarly with respect to one of the orbital references. Activity from the array was amplified (10 K gain) filtered (10–300-Hz bandpass, 3-dB falloff), and digitized (500 samples/s). Data were acquired serially by multiplexing at 10 μ s/sample (12-bit resolution). For each trial (CS+, CS-, air), data were acquired 3 s prior to (control period) and 3 s immediately following (test period) odor delivery into the nose cone. From each 6-s interval of data, six 80-ms periods of 40–80-Hz burst activity were selected by visual inspection. These samples were taken from the three inspirations before (C1, C2, C3) and the three (T1, T2, T3) immediately following odor arrival.

Activity recorded from the dorsal depth electrode was referenced to the platinum-platinum electrode in the enucleated orbit. The signal was amplified (1–10 K), filtered (0.1–300 Hz, 3-dB falloff), and displayed on a polygraph for visual examination.

Computer-generated amplitude-density plots were made for each 80-ms C and T sample by computing the root mean square (rms) amplitude for each of the 64 bursts recorded by the array. The range was determined for the distribution of values and divided into seven equal intervals. Each interval was represented by the following symbols in each density plot, from higher to lower amplitude, respectively: # > • > + > = > - > . > .

A chi-square test was used to quantify the differences between patterns before and after odor presentation (Freeman & Schneider, 1982). The 6 patterns (i.e., 3 Cs and 3 Ts) sampled, during each of 10 trials, resulted in 10 replications of 6 patterns in which 64 rms values depicted each pattern. A small-sample paired *t* test was applied to the 10 rms values recorded at each electrode site before (C) and after (T) odor delivery. The 64 *t* values were assembled into a one-tailed histogram (i.e., to form the observed *t* distribution) in which the number of cases in each of 8 equal *t* intervals, ranging between 0 and greater than 3.5, were plotted; these 8 *t* intervals correspond to the alpha probability ranges of $0 < .5 < .3 < .2 < .1 < .05 < .02 < .01$. Using the same *p* ranges and $df = 7$, we constructed a one-tailed histogram from the theoretical *t* distribution (i.e., to form the expected *t* distribution). The comparison of each C and T pattern was evaluated by calculating the Pearson goodness of fit chi-square value for the observed and expected *t* histograms. Each 10-trial session had 15 possible pattern comparisons that could be used under the assumption that all patterns were the same (i.e., 9 C-T, 3 T-T, and 3 C-C). The chi-square calculations were made for each stimulus type (i.e., CS+, CS-, air) in the various experiments. Because the chi-square values ranged over four orders of magnitude, they were expressed as \log_{10} chi-square.

All chi-square values were positive, but in our calculations they arose in two ways: In one there was a deficit of large *t* values in the observed *t* distribution compared with those in the expected one; in the other there was a relative excess of large *t* values in the observed *t* distribution. For chi-square > 1, we flagged these cases of \log_{10} chi-square values as (-) and (+), respectively. For each experimental condition (i.e., infusion condition and stimulus type), a histogram of the flagged chi-square values was made. This histogram contained all the pooled data from all animals collected during the same condition.

The chi-square histogram constructed from the pooled data collected during the familiarization condition established that chi-square value above which only 5% of the values occurred (in this condition to the experimental *t* distribution for control bursts had been shown to conform to the theoretical *t* distribution; Viana Di Prisco & Freeman, 1985). The \log_{10} chi-square values equal to or greater than the criterion score represented pattern comparisons having a signifi-

cant difference. The percentage of significant scores was then determined for each histogram obtained from each experimental condition (i.e., nine separate conditions in the propranolol experiment, three conditions for the NE experiment). For the propranolol infusion experiments, the nine percentage values were pooled to determine the mean and standard deviation, and a confidence interval for $p < .01$ was determined from the normal *t* distribution (i.e., mean \pm SD $\times 1/n^{1/2}$). The null hypothesis was tested: The scores in the experimental conditions were from the same population as the control ones. In the case of the NE experiment, the percentage-of-criterion scores were determined for each animal separately. These scores were then pooled for each of the three conditions to determine the mean and standard deviation. Differences between the means were compared by a difference *t* test.

Results

Propranolol and Vehicle Infusions

Representative data are shown in Figure 1 to illustrate the state of familiarization. Respiration, shown in the top trace (inspiration down), was stable at a rate of 2–3 Hz. The initial respiratory rate of this animal declined from 5–7 Hz during the first familiarization session to 1–3 Hz during the sixth. Spontaneous sniffing (i.e., sudden but brief increase in respiratory rate) was present in less than 20% of the trials after completion of familiarization. Conditioning was deferred until both respiration and spontaneous sniffing rates met the criterion levels of 1–3 Hz and less than 20%, respectively.

The electrical activities recorded from the depth electrode and from 1 of the 64 in the array are also shown in Figure 1 (middle and lower traces). Activity from the depth electrode exhibited a clear high-amplitude respiratory wave with a superimposed 40–80-Hz oscillatory burst on the inspiratory phase. The activity shown in the lower trace was selected from an electrode in the array that was in the center of the focus of high-amplitude 40–80-Hz activity. This activity was filtered to eliminate the respiratory wave. The high-amplitude bursting activity was consistent with a state of alert and attentive behavior by the animal (Freeman, 1975, 1978).

In each animal a focus of high-amplitude bursting activity was recorded within the array. Across animals, the relative size and location of the focus varied. As shown in Figure 1, the spatial amplitude pattern was somewhat irregular in shape and varied slightly with each inspiration. After familiarization, we assessed in each animal the direct influences on bulbar electrical activity (EEG) of both the vehicle and the propranolol infusion. Each animal was presented with several air and novel odor stimuli, just before the first conditioning session, to rule out the possibility that the nonspecific influences produced by surgery, infusion pressure, or local anesthesia by propranolol had not disrupted the functioning of the olfactory bulbs. Under both infusion conditions, there was no loss of the animal's ability to detect an odor, as indicated by sniffing behavior, nor was the EEG activity detectably altered.

Figure 2, taken from the same rabbit shown in Figure 1, illustrates that vehicle infusion did not produce a change in olfactory behavior or the bulbar EEG. A typical set of responses is displayed that was recorded before and after the presentation of a novel odor. On odor arrival, the animal

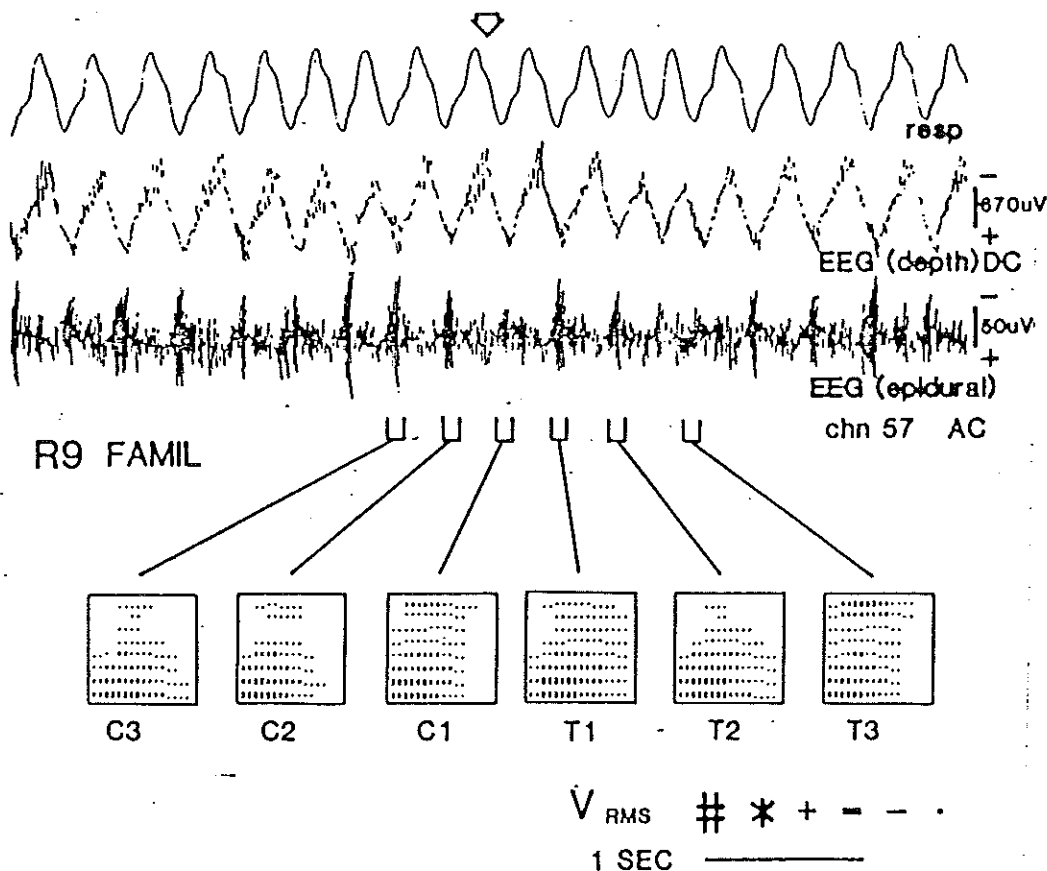


Figure 1. Example of data obtained from a single 6-s trial during the sixth session of familiarization. (Three bursts were sampled for spatial pattern analysis in the control and test periods. The root mean square voltage was calculated for each channel, and the result is displayed as an amplitude-density plot for each burst. The number symbols represent the highest amplitudes; blank spaces, the lowest. Each frame was scaled individually to its extrema. The plots show a high-amplitude focus and the relative stability of the spatial pattern. Small differences can be seen in the spatial pattern from burst to burst. There was a weak respiratory [resp] response during the air delivery. The arrow marks the opening of the solenoid valve for odor delivery. EEG = electroencephalographic activity; C = control; T = test.)

initiated sniffing behavior associated with changes in the EEG. The amplitudes of both the respiratory wave and the bursting activity decreased, as observed before infusion, and were superimposed upon a depth-negative slow potential. The latencies of the electrical and behavioral responses were normal and generally ranged between one and two respiratory cycles after the arrival of the odor in the nose cone.

Similarly, propranolol infusion had no detectable effect on the background respiratory-wave, burst-activity, slow potential, or sniffing behavior to a novel odor. In the control experiment performed in 3 additional rabbits, propranolol infusion at the same concentration (100 μ M) but at a tenfold greater flow rate (10 μ l/hr) had no detectable effect on the short-latency antidromic responses of the bulbar neurons (Table 1).

Neither propranolol nor vehicle infusion altered the amplitude of the initial antidromic responses compared with those responses observed immediately prior to infusion. The data, shown in Table 1, would be expected to show an amplitude

reduction if the racemic mixture were sufficiently concentrated to produce the local anesthetic effect.

The pooled chi-square histogram obtained during familiarization is shown in Figure 3. This empirical distribution of scores showed that 4.7% of the values exceeded 2.20. We chose this value as our criterion for determining significant pattern differences. In addition, the greater proportion of chi-square values by 2:1 lay in the "similar" distribution (i.e., ratio of positive to negative values [P/N]; Table 2). This proportion was not changed significantly by the infusion of either vehicle or propranolol (i.e., Vehicle(Air), Vehicle(Post), Propranolol(Air), Propranolol(Post)). Moreover, there was no significant change in the percentage of values greater than 2.2 for any of the control conditions. This finding confirms the results obtained from visual inspection of rms-density plots and of polygraph records, which showed that neither the amplitudes nor the spatial locations of the 40-80-Hz activities were altered in any animal by the infusion of either vehicle or propranolol.

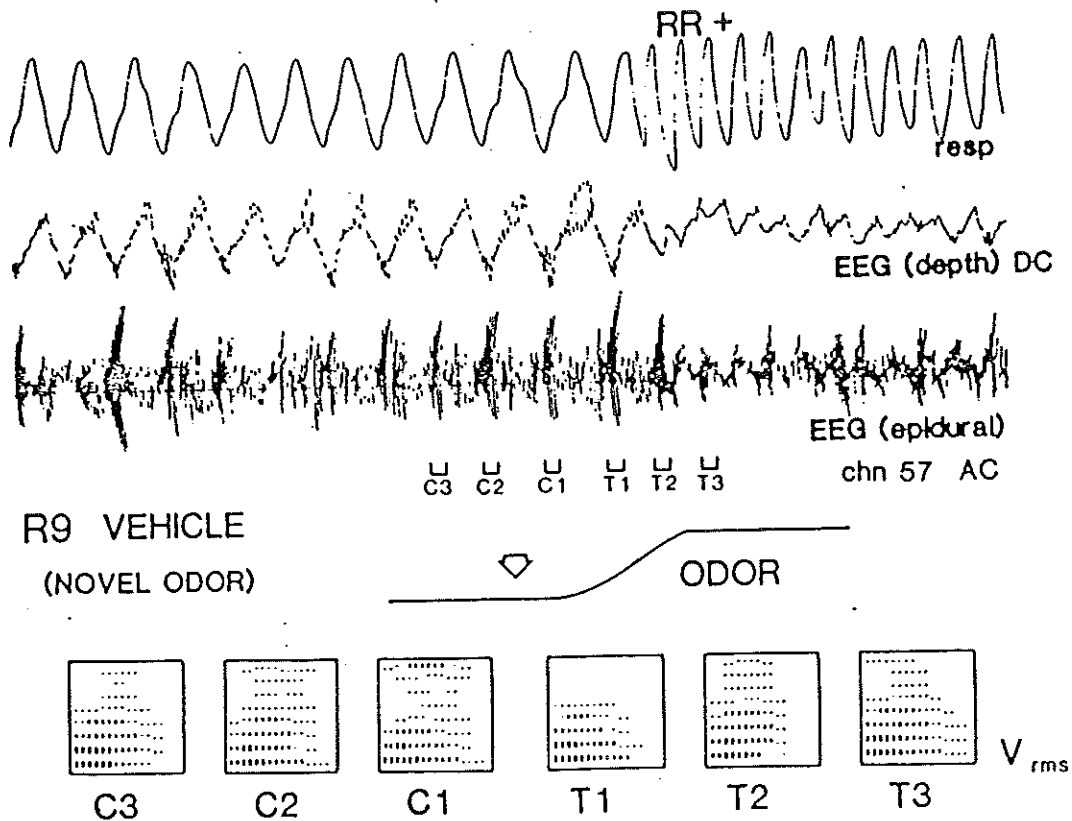


Figure 2. Illustration of event-related changes in bulbar activity that accompany the presentation of a novel odor. (Odor arrival into the nose cone was calibrated previously with dry ice and a CO_2 meter. The rabbit initiated a sniffing response to the novel odor with a latency of 0.39 s. The diminished respiratory [resp] wave was superimposed on an initially depth-negative slow potential of several seconds duration. The amplitude of bursting activity increased at T1 and decreased on the subsequent four or five inspirations. The variation of the focus about a stable mean is apparent. The key question for analysis is exemplified by the difference shown between C1 and T1: Did it exceed the range of variation shown by C1 to C3? On the average, for unreinforced odors it did not. Voltage and time scales are identical to those in Figure 1. The arrow marks the opening of the solenoid valve for odor delivery.

During vehicle infusion, the C-T comparisons made for the presentation of the reinforced odor (CS+) showed statistically significant changes in the pattern (Table 2). Both the P/N and %>2.2 measures were altered by the CS+ ($p < .005$). Although the P/N measure was altered following the nonrein-

forced odor (CS-), no change in the %>2.2 parameter was detected. During propranolol infusion, the CS+ did not evoke the transient pattern alteration. Neither histogram parameter showed any significant change. That the same phenomenon is observed in individual subjects is illustrated by the obser-

Table 1
Means (\pm SD) of the Amplitude (in Negative μV) of the Antidromic Compound Action Potentials Recorded From the Olfactory Bulbs in 3 Rabbits Before and During Intrabulbar Infusion of Vehicle and *dl*-Propranolol (100 μM , 10 $\mu\text{l/hr}$)

Subject	Preinfusion	During infusion	
		30 min	60 min
Vehicle			
1	69.5 \pm 14.1	62.9 \pm 11.1	69.5 \pm 10.0
2	38.0 \pm 4.9	33.9 \pm 11.3	40.6 \pm 4.1
3	35.8 \pm 6.4	36.3 \pm 5.2	37.5 \pm 11.2
Propranolol			
1	72.1 \pm 4.5	70.2 \pm 10.8	64.8 \pm 8.6
2	41.9 \pm 3.0	42.4 \pm 4.1	42.8 \pm 14.6
3	74.7 \pm 2.8	72.8 \pm 3.3	72.1 \pm 9.7

Note. Each of the infusion means in each animal was compared to its preinfusion control; no statistically significant differences (difference t tests) were observed in any animal. The latencies associated with these responses all remained constant at around 2.5 ms.

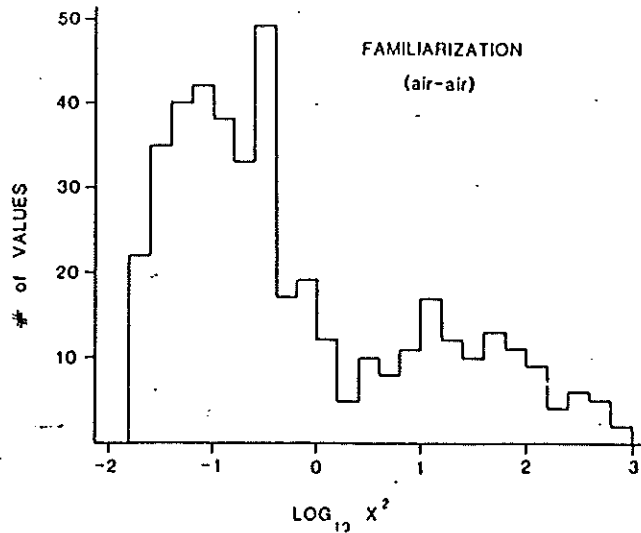


Figure 3. A bimodal chi-square histogram obtained from pooling all control-test (C-T) and test-test (T-T) flagged values of \log_{10} chi-square from 6 animals over six familiarization sessions. (Bin widths = 0.2; $N = 420$. The majority of negative values indicated a preponderance of spatial patterns that were more similar than expected by chance. The percentage of values greater than 2.2 was 4.7%.)

vation that for all 6 subjects the $\%>2.2$ value evoked by the CS+ during vehicle was larger than that evoked during propranolol ($p < .015$, binomial distribution, assuming equal probability for each score).

To examine the time course of the response, we pooled the chi-square values over both sessions for each experimental condition according to sets T1, T2, and T3 (Table 2). As shown in the footnote, the change in $\%>2.2$ evoked by the CS+ occurred during the second and third inspirations (T2 and T3), and not during the first (T1). The respiratory-response latency following the CS+ indicated detection of the

Table 2
Parameters of Chi-Square Histograms During the Vehicle and Propranolol Infusion Experiments

Condition	P/N	$\%>2.2$	$\%CR$
Familiarization	0.46	4.71	
Vehicle			
Air	0.47	6.56	31
CS-	1.17**	6.35	72*
CS+	1.20**	13.10***	91*
Post	0.86	6.35	
Propranolol			
Air	0.50	6.43	40
CS-	0.74	7.07	86
CS+	0.99	7.02 ^b	97
Post	0.54	4.27	

^a These values distributed over time, that is, during T1, T2, and T3 inspirations after odor delivery are, respectively, 6.06, 18.18%, and 15.15%. ^b These values distributed over the same intervals are, respectively 7.02, 8.77, and 5.26.

* $p < .10$. ** $p < .005$, assuming the null hypothesis that all values are from the same population.

odor during the first inspiration after odor arrival in the nose cone (T1). No significant change in $\%>2.2$ occurred at T2 and T3 for odor presentations under propranolol. The transient pattern change that occurred at T2 and T3 was accompanied by similar long-latency alterations in the bulbar electrical activity, as shown in Figure 2 (i.e., amplitude suppression of both the 3-7-Hz respiratory waves and the 40-80-Hz oscillatory bursts, and the onset of the negative slow potential).

A measure of the acquisition of the transient pattern change was obtained by calculating separately the $\%>2.2$ values for Conditioning Sessions 1 and 2. For presentations of the CS+ during vehicle infusion, there was an increase from 8.89% in Session 1 to 22.2% in Session 2 ($p < .01$ when the 22.2% score evoked by the CS+ was compared with the other eight obtained during the second session). This trial-dependent increase in the $\%>2.2$ measure was not observed under propranolol.

Observations of sniffing behavior revealed that the propranolol and vehicle infusions did not impair odor detection. In the first conditioning stage, in which a single novel odor was introduced with reinforcement, each animal exhibited a sniffing frequency of greater than 90% for both Sessions 1 and 2 under the influence of either infusate. In the second stage, odor discrimination was evaluated by comparing the sniffing rates to both CS- and CS+ under each of the two infusion conditions (Table 2). Animals receiving vehicle or propranolol infusions exhibited significant increases in sniffing rates above control levels for both the CS- and CS+ odors. There was, however, no clear evidence at this point in the behavioral acquisition curve for behavioral discrimination between the CS+ and CS- in either condition (Table 2).

Norepinephrine Injection

This experiment was designed to determine the effects of intrabulbar norepinephrine (NE) on the transient spatial pattern change following the presentation of a novel odor. The results are shown in Table 3. In the control condition (i.e. air, no injection), 4.0% of the chi-square values were above 2.2. Under the influence of intrabulbar NE, the novel odor produced a large increase in $\%>2.2$ without a change in variance, whereas during vehicle the novel odor produced a smaller

Table 3
Parameters of Chi-Square Histograms During the Norepinephrine-Injection Experiments

Condition	$\%N$	P/N	$\%>2.2^a$
Air	68.3	0.46	3.7 ± 5.2
Novel odor			
Vehicle	35.6	1.81	15.6 ± 23.1 ^b
Norepinephrine	27.8	2.60	38.9 ± 6.4 ^c

^a These values are means ($\pm SD$) of the individual subject's contributions to the pooled histogram. ^b These values distributed over time, that is, during T1, T2, and T3 inspirations after odor delivery are, respectively, 6.7, 26.7, and 20.0. ^c These values distributed over the same intervals are, respectively, 25.0, 50.0, and 58.3.

* $p < .05$, Hartley F_{max} test, compared with air and norepinephrine conditions.

† $p < .01$, t test, compared with air condition.

increase in $\% > 2.2$ which was associated with a large variance. As in the previous experiment, the major contribution to the $\% > 2.2$ value is derived from the C-T comparisons made during the second and third inspirations (T2 and T3).

Polygraph records of activity in the focus revealed in each animal that norepinephrine injections resulted in a significant increase in the amplitude of the 40-80-Hz activity. This increase was sustained throughout the 10-trial session (i.e., 10-20 min). Following novel odor presentation, in the presence of injected NE, the usual amplitude suppression of the 40-80-Hz activity occurred subsequent to the T1 inspiration (Figure 4). Vehicle injection produced an initial increase in 40-80-Hz amplitude, but this effect subsided in 2-5 min.

To investigate the possibility that NE had a nonspecific effect on C-T pattern differences, we performed the following analyses. The data recorded from all animals during novel

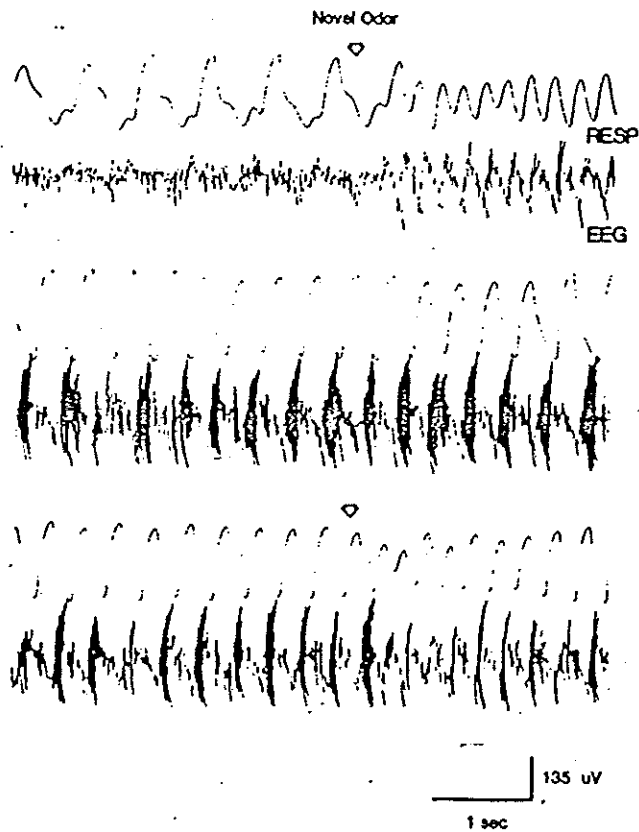


Figure 4. Polygraph records of respiration and bulbar electroencephalographic (EEG) activity taken over the course of a single session of the norepinephrine (NE)-injection experiment in one animal. (The EEG was sampled from an electrode in the array overlying the epicenter of the focus. The upper pair of traces were taken during novel odor presentation prior to injection. The middle pair show activity 30 s following the injection of NE. Note the large increase in burst amplitude and the change in the depth and rate of respiration. The bottom pair of traces were sampled on the eighth presentation of the same novel odor [i.e., approximately 8 min following the injection]. Bursting amplitude is still large relative to control, and the odor presentation evokes a burst amplitude reduction beginning with the second inspiration after odor arrival.)

odor presentation under intrabulbar NE showed that none of 12 C-C comparisons (i.e., the ones obtained just prior to the novel odor) was greater than 2.2. In contrast, 5 out of 12 of the T-T comparisons (made after the novel odor) were greater than 2.2. In other words, the patterns recorded during the precodor interval did not differ significantly, whereas those recorded after the arrival of the odor changed repeatedly (Figure 5). This finding was supported by visual inspection of the averaged rms-density plots. In 1 animal a particularly marked change was observed in the pattern following odor presentation under NE (Figure 6). The surround activity changed somewhat in the control period from one session to the next, but the focus remained in a stable position during each of the three observations. It was only after novel odor arrival, and in the presence of NE, that the position of the focus and its surround activity significantly changed. Statistical verification of this result is shown in Figure 5 and Table 3.

Tissue Distribution of [3 H]Norepinephrine

On the basis of the measurements of wet weight from two bulbs (average weight was 110 mg), the 1.1 nmol injection of NE was predicted to produce a tissue concentration of 10-pmol NE/mg of wet weight tissue (assuming no metabolic breakdown and uniform dispersion in the bulb). Results from the thin-layer chromatogram revealed that $65\% \pm 15\%$ of the tritium counts represented activity from unchanged NE. On the basis of this finding, the tritium counts from all samples were multiplied by a correction factor of 0.65.

No clear difference was observed for tritium counts between left and right bulbs or between medial and lateral halves of the same bulbs at the same rostrocaudal locations. Therefore we pooled all corrected tritium count values according to the rostrocaudal locations

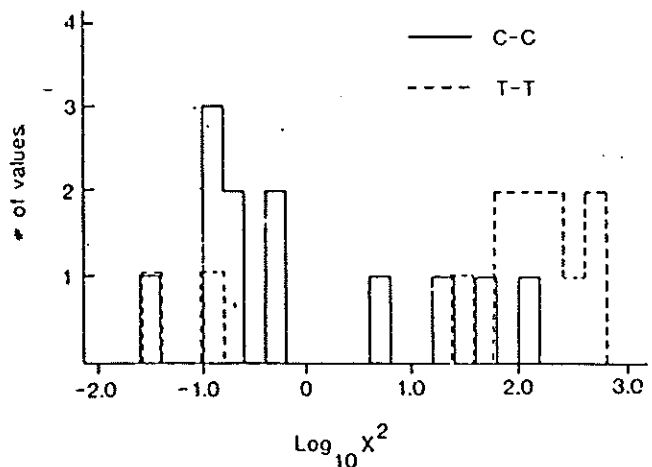


Figure 5. Two pooled chi-square histograms of the C-C and T-T comparisons made during novel odor presentations in the presence of injected norepinephrine in 4 subjects. (In the control condition [C-C] prior to odor arrival, the majority of values are flagged negatively, which indicates pattern similarity. None of the values in this condition are greater than 2.2. During the arrival of the odor [T-T], the distribution was shifted, which indicates pattern change. Of the 12 values obtained, 5 were greater than 2.2.)

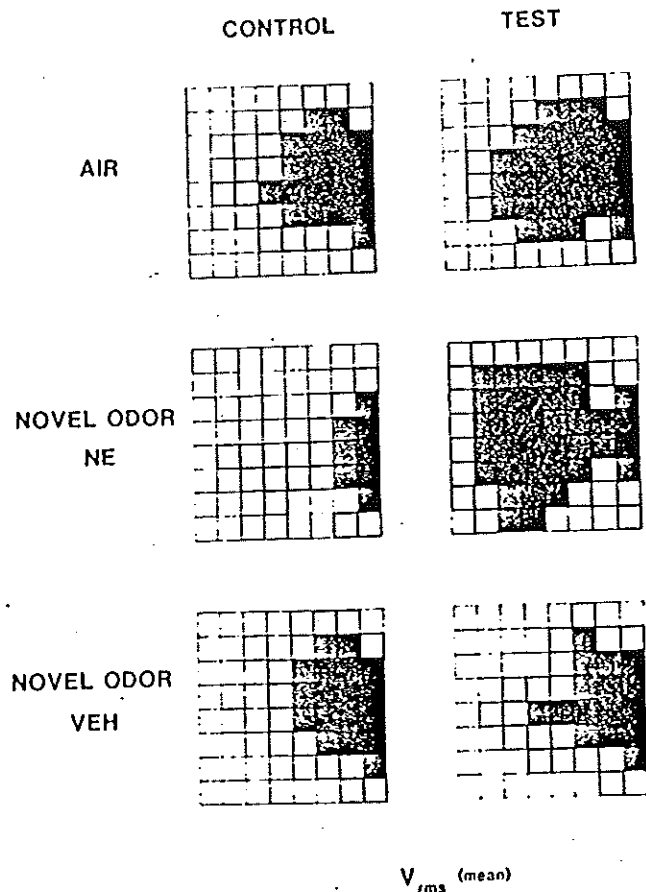


Figure 6. A sequence of average amplitude-density plots obtained from a single rabbit over the course of the norepinephrine (NE)-injection experiment. (Each map is a mean of root mean square voltage taken from the 30-hurst sample in each session shown. The scale shows increasing amplitude from light to dark. The patterns in the top row were taken just prior to NE injection on 10 trials to an air stimulus. The patterns in the middle row were taken immediately following the intrabulbar injection of 10 μ l of NE [100 μ M] in vehicle during presentation of a non-reinforced novel odor. The bottom patterns were obtained 3 days later immediately following vehicle injection during the presentation of a second novel odor. The spatial pattern shifted significantly only during the presentation of the novel odor paired with NE injection.)

1-5 and obtained a grand mean and standard deviation at each location. The results shown in Figure 7 are expressed as picomoles of NE/milligram of wet weight tissue 20 min following the bolus injection of 1.1 nmol of NE.

The highest concentration of injected NE was found in the tissue samples adjacent to the cannulas (i.e., sample position 2 and 3). This concentration was 23%-30% of that expected under ideal conditions. At locations 1 and 4, the concentration decreased to approximately 12% and 15%, respectively, of the expected ideal value. The mean concentration of NE observed in samples from the anterior olfactory nuclei was found to be approximately tenfold less than that observed at locations 2 and 3 in the bulb. These findings indicate that approximately 20% of the injected NE was detected in the bulb as unchanged NE 20 min following the injection. On the basis of previous observations of the NE content in the rat olfactory bulb (3.0 ± 0.1 pmol/mg of tissue; Haubrich & Denzer, 1973), this value represents an

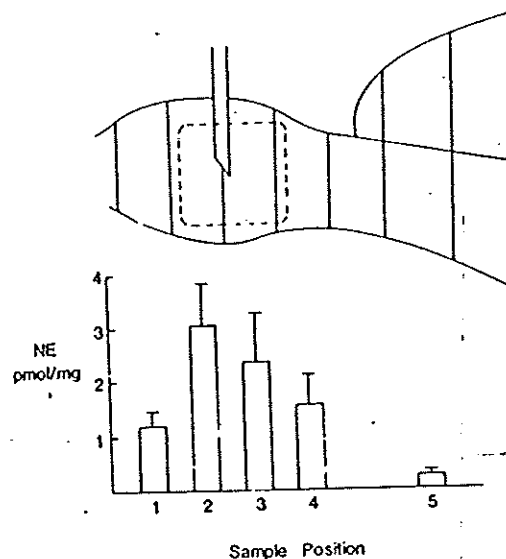


Figure 7. Results obtained from the tissue distribution study of injected [3 H]norepinephrine. Each value in the bar graph represents the mean and SD of 20 samples taken at each position. Above the graph is a schematic representation of the bulb and forebrain, showing the relative locations of the samples, the intrabulbar cannula, and the recording array. (The concentration of norepinephrine [NE] 20 min following the injection was highest within the boundaries of the electrode array.)

approximate twofold increase in the tissue NE within a distance of 2.0 mm from the cannula.

Discussion

The principal findings of this study demonstrate the following: (a) Local intrabulbar infusion of propranolol prevents the trial-dependent acquisition of a transient change in the spatial pattern of bulbar 40-80-Hz activity that normally occurs in response to a reinforced odor. The pattern change occurs maximally on the second and third inspirations following odor detection and is associated with amplitude decrease of both the respiratory and 40-80-Hz rhythms and the onset of a negative slow potential. No abnormality of the EEG activity was apparent following the propranolol administration, nor was the ability to detect an odor impaired. No local anesthetic effect of the *d/l* mixture was detected, with the antidromic response of the mitral cells used as the criterion. (b) Local intrabulbar injection of NE potentiated the transient pattern change observed following the presentation of a novel odor. The maximal pattern change occurred during the T2 and T3 inspirations, in parallel with the amplitude decrease of the 40-80-Hz activity. NE injection also produced an increase in amplitude of the bulbar 40-80-Hz EEG activity. (c) The concentration of exogenous NE in the tissue 20 min following injection into the bulbar ventricle indicated an approximate doubling of the endogenous NE within a 2.0-mm distance from the cannula. Samples taken from the anterior olfactory nuclei showed that there was not a large fraction of NE transported posteriorly through the ventricular system into the forebrain. On the average, 65% of the detected NE re-

mained in the tissue as unchanged NE, a value consistent with previous findings in the cat visual cortex (Kasamatsu, Itakura, & Jonsson, 1981).

Other findings confirm previous observations. There was a spatial pattern of 40–80-Hz activity unique to each rabbit (Freeman, 1978). Individual patterns continually fluctuated in shape about a stable average pattern (Freeman, 1978). The spatial pattern during T1, the inspiration in which odor detection occurred, showed no reliable change from the patterns observed in the preodor control period (Viana Di Prisco & Freeman, 1985).

The time limit for drug infusion imposed the scheduling of sessions every 3–4 days instead of 7 or more days. Previous experience has shown that rabbits undergoing aversive olfactory conditioning at such frequent intervals become restless and hyperirritable. This behavior was reflected in the background rates of sniffing, which were nearly twice those found with rabbits under less stress (Freeman et al., 1983). The need for repeated surgery under anesthesia to replace the minipumps may have exacerbated this tendency. It has also been determined that rabbits given a CS– odor on trials interspersed with trials to a CS+ odor, both introduced in the same session, respond preferentially to the CS+ but also respond to the CS– (Viana Di Prisco & Freeman, 1985) at lower rates found with pseudoconditioning (Freeman et al., 1983). These two factors may have contributed to the failure of the animals to show clear-cut discrimination behavior between the CS– and CS+ under the influence of either infusate.

The transient spatial pattern change always occurs in response to the CS+ during early acquisition of olfactory conditioning to a single odor (Freeman & Schneider, 1982). As acquisition becomes complete, around the third 10-trial conditioning session, the transient change becomes less apparent (i.e., is not always detectable), and the change of the focus to a new pattern becomes discernible (Freeman & Schneider, 1982). If a CS– is interspersed among the trials in discriminative conditioning, the transient pattern change in response to both the CS+ and CS– persists, at least until the sixth session (Viana Di Prisco & Freeman, 1985).

Our experience with bulbar EEG has been that long-term differences require several weeks either to become large enough or to persist long enough to give an adequate sample over the intersession variation. Because we have not yet defined that variation, we do not have an effective statistical treatment such as that for transient within-trials differences. Thus both NE and propranolol may have affected long-term changes in the spatial patterns, but our experiments were not structured to detect them.

An unresolved question is whether a significant transient pattern change normally occurs with a novel odor that leads to burst suppression, sniffing, and an ERSP. When an odor is not reinforced directly or in context, this response configuration abates and vanishes within a few trials; the chi-square test over 10 trials shows no difference in spatial pattern for a CS– introduced without interspersed CS+ trials (Freeman & Schneider, 1982). We believe that this abatement manifests habituation and propose that NE may act to prevent or delay habituation by postsynaptic activation of beta-receptors. Evidence from the studies of M. Davis, Cedarbaum, Aghajanian, and Gendelman (1977) and M. Davis (1980) supports this

conclusion. They found that clonidine, an alpha-2 agonist, which may act presynaptically to suppress NE release, significantly increased the rate and magnitude of habituation of acoustic startle in rats. This effect was independent of any influence on sensitization and indicated that the animals were able to respond normally. Potter and Chorover (1976) found in hamsters that habituation of bulbar responses to odors was accelerated following bulbar transection. In the present study, NE also increased the amplitude of bursting activity, perhaps by reducing local inhibitory input to mitral cells (Jahr & Nicoll, 1982) as an aspect of countering habituation. Transection of the bulbar stalk in hamsters (Potter & Chorover, 1976) and cats (Becker & Freeman, 1968) increased rather than decreased the amplitude of background bulbar activity.

Norepinephrine might be directly involved in long-term synaptic changes, but it is conceivable that those changes might merely be enabled by the prolongation of sensory-induced activity, owing to NE suppression of habituation. This effect could account for the well-established requirement of temporal contiguity needed between CS and UCS to support acquisition of conditioning. Hopkins and Johnston (1984) found that the induction and maintenance of hippocampal long-term potentiation depended on a beta-receptor NE mechanism only during the period of tetanic stimulation. Gibbs et al. (1986) demonstrated that temporal pairing of a conditioned stimulus with locus coeruleus stimulation as a substitute for an unconditioned stimulus sufficed to produce learning-dependent changes in the activity of lateral geniculate neurons in the pigeon. NE depletion in the visual cortex by 6-hydroxydopamine prevented the change in receptive field properties of visual cortical neurons that normally occurs during maturation (Daw et al., 1983), and local repletion of NE restored the cortical plasticity (Kasamatsu, Pettigrew, & Ary, 1981; Kasamatsu, Watabe, Scholler, & Heggelund, 1985). These and other changes requiring the release of NE (Nelson, Schwartz, & Daniels, 1985) in cerebral tissue might at least in part be accounted for by NE block of habituation during synaptic change. The advantage of our method is the access it gives to very early changes in activity during learning.

In conclusion, blockade of the bulbar beta-receptors prevents the acquisition of the transient pattern change, with no significant change in any of the chi-square parameters. This result suggests a dependency on beta-receptor activation for acquisition of new learning to occur (Kasamatsu, & Shirokawa, 1985) and not a norepinephrine dependency for the maintenance of the spatial pattern that has resulted from previous learning. Intrabulbar injection of NE increases the magnitude of the transient pattern change observed during presentation of a novel odor. Taken together, these findings are consistent with the hypothesis that intrabulbar beta-receptor activation acts to prevent or delay olfactory habituation. The temporal pairing of NE release coupled to beta-receptor activation with the odor-evoked synaptic activities that occur during inspiration may provide the basis for acquisition of olfactory discrimination behavior.

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