# Regional Patterns of c-*fos* mRNA Expression in Rat Hippocampus Following Exploration of a Novel Environment versus Performance of a Well-Learned Discrimination

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Previous studies using c-*fos* cRNA *in situ* hybridization demonstrated a differential involvement of hippocampal subfields CA1 and CA3 in the acquisition of an olfactory discrimination (Hess et al., 1995). The present experiments employed the same method to examine changes in neuronal activity associated with two related behaviors: (1) initial exploration of the training apparatus and (2) performance of a well-learned odor discrimination. Rats in the two groups had similar labeling patterns within hippocampus indicating increased expression in all three major subfields with the greatest effect being in CA1. This pattern of "CA1 dominance" was notably different from that produced during early stages of two-odor discrimination learning in prior experiments. Hippocampal labeling in exploration and performance rats differed in that (1) hybridization was greater in CA1, CA3, and dentate gyrus in the former group and (2) a tendency for labeled cells to occur in clusters was more evident in exploration animals. Levels of c-*fos* mRNA in olfactory and visual structures were not predictive of expression patterns within hippocampus although labeling in piriform cortex and dentate gyrus was correlated in rats performing a well-practiced discrimination. Moreover, the *pattern* of hybridization in olfactory bulb was found to be behaviorally dependent. These results, together with those from previous studies, indicate that hippocampus has multiple patterns of regional activation but that one of these is common to very different behavioral circumstances. It is hypothesized that this common pattern emerges whenever the animal responds to distant cues using species-specific or well-learned behaviors and involves coordinated temporal convergence of sensory and septal/brainstem inputs.

[*Key words: gene expression, in situ hybridization, odor discrimination learning, olfactory bulb, piriform cortex, visual system*]

The discovery that depolarization and associated calcium influx can stimulate the expression of the immediate-early gene c-*fos* (Greenberg et al., 1986; Morgan and Curran, 1986) has provided a technique for assessing levels of neuronal activity in brain subdivisions during various behaviors (Morgan et al., 1987; Sagar et al., 1988; Dragunow and Faull, 1989). Experiments using *in situ* hybridization and immunohistochemical techniques have shown that levels of c-*fos* mRNA and protein are dramatically changed in sensory/motor systems of animals exposed to appropriate environmental stimuli with the spatial location of the affected neurons varying according to cue composition (Hunt et al., 1987; Sharp et al., 1989; Ehret and Fischer, 1991; Guthrie et al., 1993). Regionally differentiated patterns of c-*fos* induction have also been observed in studies using shock avoidance training (Nikolaev et al., 1992) and conditioning vs pseudo-conditioning of the nictitating membrane reflex (Irwin et al., 1992).

The present work used c-*fos* expression to investigate the possibility that the subdivisions of hippocampus become differentially active across behavioral circumstances. Neuroanatomical investigations have established that the two pyramidal cell fields of hippocampus (i.e., CA1 and CA3) project to very different target regions (Swanson and Cowan, 1977; Witter et al., 1989; Van Groen and Wyss, 1990; Jay and Witter, 1991). Accordingly, variations in the balance of activity between the two regions could result in variations in hippocampal contributions to behavior. This idea stands in contrast to hypotheses positing a unitary function for the hippocampal formation. Previous work in this laboratory has demonstrated that c-*fos* mRNA expression is elevated in an equivalent and correlated fashion in the hippocampal regions CA3 and CA1 of rats engaged in "nose poking" behavior for rewards in an open field. In contrast, field CA3 exhibited a much larger increase than CA1 when animals were switched to a paradigm which required them to discriminate between two odors before making a nose poke response. Interestingly, induction of c-*fos* mRNA in the dentate gyrus was not observed in either training condition. Thus, the hippocampus did not respond as a unit to changes in task demands and behavior but instead exhibited regionally differentiated increases and decreases in c-*fos* mRNA expression.

The experiments reported here sought to determine if other behavioral circumstances alter the balance of activity in hippocampus in a manner different from those just described. Two conditions quite different from each other but nonetheless related to the nose-poke/olfactory discrimination paradigms were used: (1) initial exploration of the olfactory training apparatus and (2) discrimination performance involving already learned odors.

These two behaviors constitute the beginning and end points of a sequence in which the behaviors studied in the earlier experiment (i.e., nose-poke/respond to odors) are intermediary steps. Levels of c-*fos* expression were also assessed in olfactory and visual structures as measures of the selectivity of any changes occurring in hippocampus, as well as to identify sensory effects that might relate to shifts in hippocampal activity patterns.

## Materials and Methods

### Animals

Male Wistar rats from Hilltop Laboratories (26–38 d old at sacrifice) were used. They were housed in groups of four to six in transparent Plexiglas cages (12" × 22") on a regular 12 hr day–night cycle. In order to decrease cage odor, animals were placed on a fat sufficient test diet (Harlan-Teklad) given ad libitum. Rats were water deprived and only allowed to drink from a glass bowl placed in the home cage for 8 min per day. Animals were handled for ~2 min per day following behavioral training sessions to familiarize them with the experimenter.

### Training apparatus

The odor discrimination training apparatus is an open wedge-shaped chamber with six separate "odor alleys" and an extension at its thin end that serves as a starting compartment (see Hess et al., 1995, for a detailed description). Two holes were placed at the end of each of the odor alleys. One of these holes contained a protruding glass connector and served as an outlet for the odor-carrying tubes. The other, placed directly above the first, was larger and served as a target for nose poke responses. A 1 ml syringe was used to administer 0.025 ml water reward through these larger holes for every correct response. A strobe light was mounted 8 cm above the test apparatus and flashed for 2 sec following incorrect responses. Odor discrimination training was conducted in a dimmed room.

Odors were generated by forcing 7 liters per minute of air through 125 ml flasks containing pure peppermint extract (Schilling; Hunt Valley, MD) or amyl acetate (Fisher; Tustin, CA). A 1:12 dilution of saturated odor vapor in air was used for both odors. These dilutions were chosen to match perceived odor intensities. The outlet of each flask was connected to Tygon tubing (0.5 cm inner diameter) that carried the odorized air and ejected it into the training apparatus through a given odor outlet during trials. Odor release was interrupted between trials through the use of an exhaust valve that was placed in the line ahead of the odor flasks and diverted the air flow.

### Odor discrimination training procedure

Odor training was preceded by two sessions (50 min total in 2 d) during which rats learned to nose poke for water reward in the training apparatus. During these sessions, every nose poke at one of the six holes was rewarded with water, as long as consecutive pokes were not at the same hole. This served to familiarize the animals with the training apparatus and the nose poke response that would be required of them during odor discrimination. Subsequently, over the course of several days, rats were trained on the two-odor discrimination. They learned to nose poke at the positive odor (peppermint), which was reinforced with water, and avoid the negative odor (amyl acetate), poking at which resulted in a mildly aversive 2 sec strobe flash. For each trial, the odors were randomly assigned to two of the six arms. The odors were turned off 2 sec after the nose poke. Nose pokes into unassigned holes had no consequence. However, if no correct or incorrect response occurred within 1 min, the trial was terminated, the odors turned off, and the animal returned to the start chamber.

### Animal treatment groups

*Home cage controls ("naive").* In order to determine baseline levels of c-*fos* mRNA expression, eight rats were killed without undergoing any type of experimental manipulation. They were taken from the home cage, anesthetized, and intracardially perfused with paraformaldehyde, as described below. Cage-controls were not water deprived or handled. Five of these animals were 26–34 d old at sacrifice and served as controls for the exploration group. Three of the rats were 35–38 d old at sacrifice and served as controls for the familiar two-odor discrimination group. These two groups were combined for quantitative analyses.

*Exploration of a novel environment.* Seven animals (31–34 d old at

sacrifice) were water deprived and handled (5 min/d) for 7–10 d. On the day following the last period of handling, the rats were placed in the odor discrimination training apparatus for 30 min and then sacrificed. This environment was novel to the animals and they all actively explored the apparatus for at least part of the 30 min period.

*Performance of a familiar two-odor discrimination ("overtrained").* Five rats (35–38 d old at sacrifice) were trained to criterion (≥80% correct in a block of 10 trials, and active avoidance of the "negative" odor) on the odor discrimination problem. This took an average (±SD) of 200 ± 53 min, and 171 ± 54 trials spread across 3–6 (4.6 ± 1.1) d. Following initial learning of the problem, training was continued for 145 ± 39 min, 274 ± 38 trials, spread across 4–5 (4.2 ± 0.4) d, during which time rats maintained their level of performance. The reason for the variability in training sessions was due to different rates of learning by the animals. In any case, all rats were "overtrained" on the problem as evidenced by their rapid and accurate performance in later sessions. On the last day, animals received 66 ± 5 trials in 30 min, after which time they were sacrificed. This time point was chosen because c-*fos* mRNA levels have been shown to peak at about 30 min following stimulation in a variety of paradigms (Shin et al., 1990; reviewed by Morgan and Curran, 1991). Virtually no errors were made in the last session.

Data from two groups described in a previous study (Hess et al., 1995) were included in some of the analyses described below for purpose of comparison. The animals in these groups were at intermediate stages of the olfactory discrimination paradigm beginning with "exploration" and ending with "overtraining"; that is, (1) a nose-poke group performing a well-practiced nose-poke response for water reward without odor stimulation, and (2) a group of rats previously trained to nose-poke, but sacrificed in the early stages of learning the two-odor discrimination (see Discussion).
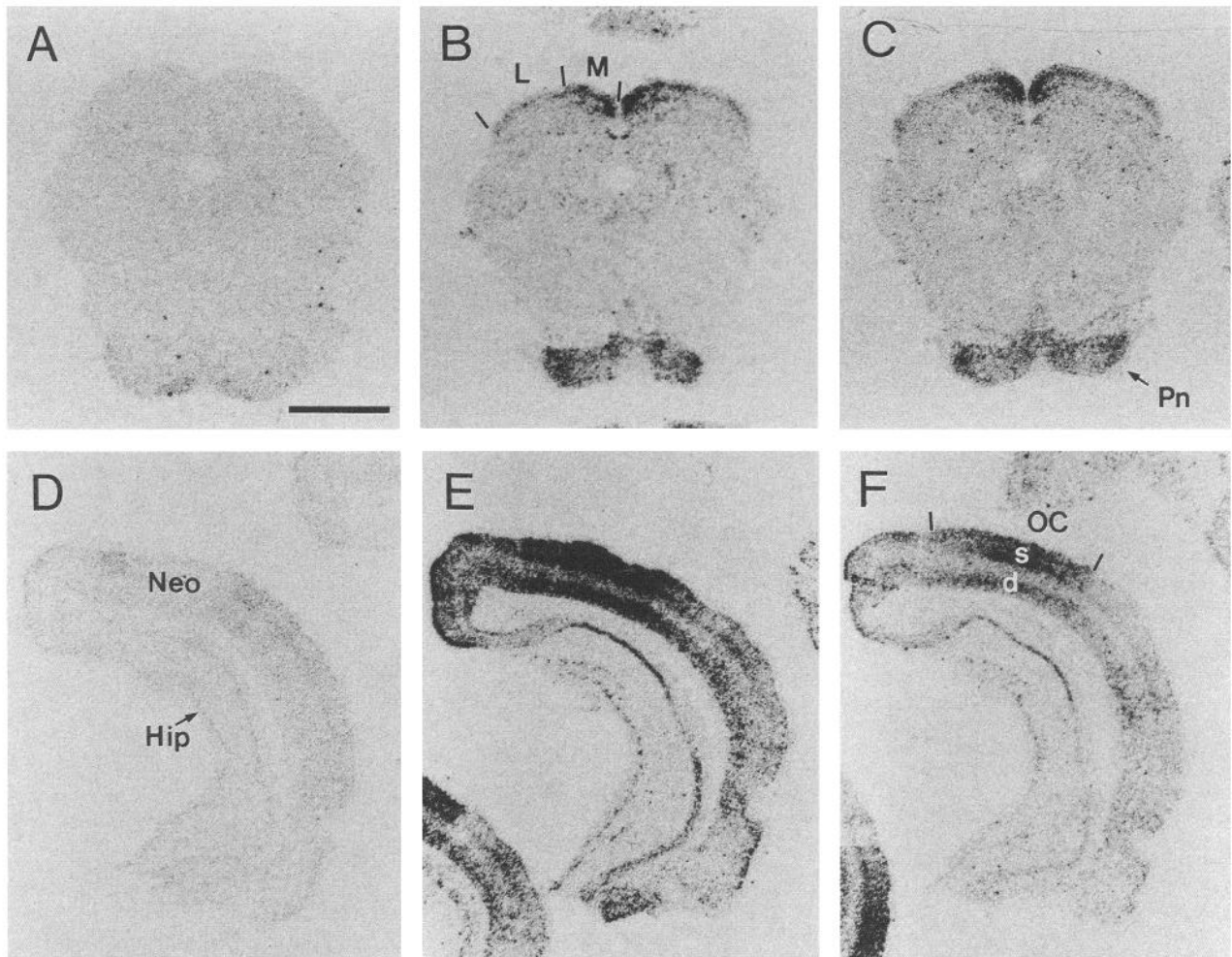
### In situ hybridization

Levels of c-*fos* mRNA were examined in tissue sections using *in situ* hybridization of a ³⁵S-labeled cRNA probe complementary to rat c-*fos* mRNA [i.e., to positions 583–1250 of clone pc-fos (rat)-1 by Curran et al. (1987)]. The antisense cRNA was transcribed from *Pst* I-linearized recombinant clone pBS/ rfos with T7 RNA polymerase in the presence of uridine 5'-[α-[³⁵S]thio]triphosphate. The sense RNA sequence was generated from the same template using T3 RNA polymerase after linearization with *Eco*RI.

Brain tissue was processed for *in situ* hybridization as previously described (Gall et al., 1991; Lauterborn et al., 1993). Briefly, animals were overdosed with sodium pentobarbital and intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Brains were postfixed, cryoprotected, and coronal tissue sections (30 μm thick) cut on a freezing microtome. Tissue was pretreated with proteinase K (1 μg/ml in 0.1 M Tris buffer, pH 8.0, with 50 mM EDTA) for 30 min at 37°C followed by 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Hybridization was conducted at 60°C for 38–40 hr with the ³⁵S-cRNA at 1 × 10⁶ cpm/ 100 μl. Following this incubation, sections were rinsed in 4 × SSC (saline sodium citrate buffer; 1 × SSC = 0.15 M NaCl and 0.015 M Na citrate, pH 7.0), treated with 20 μg/ml ribonuclease A (35 min, 45°C), and washed through descending concentrations of SSC to a final stringency of 0.1 × SSC at 60°C. Sections were mounted and air dried on gelatin coated slides, and the distribution of hybridization was evaluated using Amersham β-max Hyperfilm and Kodak NTB2 emulsion autoradiography with exposure intervals of 1–2 d and 2–3 weeks, respectively. No cellular labeling was seen in tissue hybridized to the sense cRNA sequence.

### Autoradiographic analysis

Densitometric analysis of film autoradiograms was carried out using the Intel based MCID image analysis system (Imaging Research, St. Catherine's, Ontario, Canada). Autoradiographic film optical densities were linearized and calibrated relative to densities obtained from ¹⁴C-labeled brain paste standards exposed to each sheet of film. This allowed the conversion of optical density readings to cpm/25 μg protein (see Gall et al., 1994 for details). For all brain areas analyzed, multiple measures were collected from at least four to five tissue sections at different rostro-caudal levels. Individual section means were averaged to yield a mean and standard deviation for the individual animal. Regional *in situ* hybridization densities were quantified for: (1) intermediate levels of the main olfactory bulb, granule cell layer and periglomerular aspects

*Figure 1.* c-*fos* mRNA expression in the visual system. Bright-field photomicrographs of film autoradiograms showing levels of c-*fos* cRNA hybridization in sections through the superior colliculus (*A–C*) [medial (*M*) and lateral (*L*) sectors are bracketed] and the occipital cortex (*OC*) (*D–F*) for the three groups of animals. Basal levels of expression were generally low in cage-controls (*A, D*), but greater in neocortical (*Neo*) than in brainstem regions. Labeling was dramatically increased above these control levels in the exploration group (*B, E*) in most regions, particularly in the medial superior colliculus (*M*) and superficial (*s*) and deep (*d*) layers of occipital cortex. Also note dense hybridization in the pontine nuclei (*Pn*) and hippocampus (*Hip*) of these rats. Overtrained animals (*C, F*) showed intermediate levels of hybridization in occipital cortex, and denser labeling in medial superior colliculus than exploration rats. Scale bar, 2 mm.

of the glomerular layer [where focal points of c-*fos* mRNA expression have been noted following extended peppermint or amyl acetate odor exposure] (Guthrie et al., 1993; Hess and Gall, unpublished observations); (2) medial and lateral aspects of the superficial gray layer of superior colliculus; (3) layer II of rostral piriform cortex (through the rostro-caudal levels of septum); (4) superficial (layers II/ III) and deep (layers V/ VI) rostral occipital cortical areas 2MM, 2ML, and 2L as defined by Paxinos and Watson (1986), which lie dorsal to the hippocampus and lateral to retrosplenial cortex; and (5) dorsal hippocampus: stratum pyramidale (fields CA1b and CA3) and the suprapyramidal blade of stratum granulosum. Since the exact boundaries of field CA1 are difficult to distinguish on film autoradiograms, only the central portion, overlying the dorsal blade of the dentate gyrus, was measured. As described in the Results, regional c-*fos* mRNA expression ratios were sometimes calculated in order to determine the relative balance of labeling densities between areas within the same animal. This sort of analysis also served as an "internal control," in that it helped reduce inter-animal variability for purpose of comparison. One-way analyses of variance (ANOVA), two-tailed *t* tests, and regression analyses were used for statistical comparisons. The Student-Newmann-Keuls test was used for post-hoc comparisons. A 95% confidence level ($p \leq 0.05$) had to be reached for differences to be considered significant.

## Results

### Visual system

Figure 1 shows regions of the visual system selected for quantitative analysis and illustrates representative cases from each of the three groups. In both "exploration" and "overtrained" rats, the expression of c-*fos* mRNA was increased above "naive cage control" levels in superior colliculus and occipital cortex. Densitometric measures of these fields are presented in Figure 2. As shown, c-*fos* mRNA levels in control rats were low but slightly greater in the occipital cortex than in superior colliculus. There were no reliable differences between medial versus lateral colliculus; however, values for the superficial versus deep layers of cortex were highly correlated ($r = 0.99$) across rats with the former region having slightly more labeling ($p = 0.02$, paired *t* test). In the exploration group (Fig. 1*B,E*), hybridization was greatly elevated above control levels in all four regions sampled ($p < 0.0001$ ANOVA, $p < 0.001$ Student-Newmann-Keuls) with the cortex showing a greater increase than the colliculus
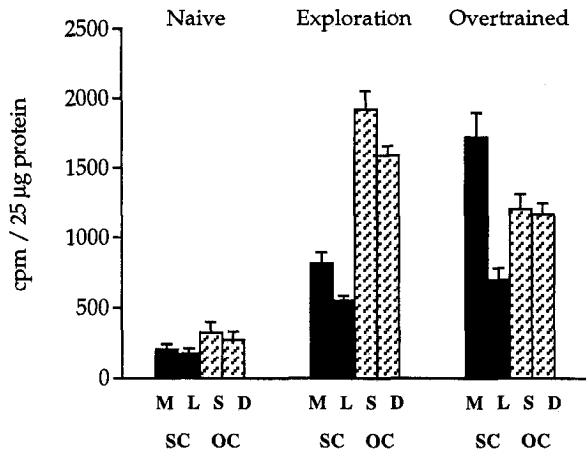
*Figure 2.* Bar graph summarizing densities of hybridization to c-*fos* mRNA in superior colliculus and visual cortex in the three groups of animals. Regions measured include: medial (*M*) and lateral (*L*) aspects of superficial superior colliculus (*SC*), and superficial (*S*) and deep (*D*) layers of occipital cortex (*OC*) (see Fig. 1 for fields measured). Bars indicate group mean values ($\pm$SE). Expression of c-*fos* mRNA was low in naive rats but markedly increased, with regional differentiation, in both the exploration and overtrained groups. Exploration rats had significantly more hybridization in superficial and deep visual cortex than overtrained rats ($p < 0.0001$ ANOVA; $p < 0.001$ and $p < 0.01$, respectively, Student-Newmann-Keuls), while overtrained rats had greater labeling in medial, but not lateral superior colliculus ($p < 0.0001$ ANOVA, $p < 0.001$ Student-Newmann-Keuls). The relative difference between labeling of medial and lateral superior colliculus was greater for overtrained than for exploration rats ($p = 0.0019$ ANOVA, $p < 0.01$ Student-Newmann-Keuls).

(Fig. 2). For these rats, labeling was greater in the medial than in the lateral superior colliculus ($p < 0.01$, unpaired *t* test) and greater in superficial than deep cortex ($p = 0.001$, paired *t* test); however, the laminar ratio was not significantly different from controls.

Hybridization to c-*fos* mRNA in visual regions was also greatly elevated above control levels in rats performing a familiar odor discrimination, but the pattern of this increase was different than that for "exploration" rats. First, the magnitude of the increase was significantly lower for visual cortex but larger for superior colliculus (Fig. 2). As a result, the pronounced regional difference in expression found in the exploration group was not present in the odor discrimination group. Second, within structure distributions were not the same: the discrepancy between medial versus lateral colliculus (150 $\pm$ 41%) was significantly greater for the overtrained rats while the discrepancy between superficial versus deep cortex was smaller and not statistically significant (Fig. 2). Thus, exploration had a greater effect on visual cortex than did responding to familiar odors while the reverse was true for the superior colliculus.

*Olfactory system*

Olfactory structures were among those regions exhibiting the highest levels of expression in cage-control rats. Levels of c-*fos* mRNA in olfactory bulb and cortex relative to other telencephalic structures are shown in the pseudocolor images of film autoradiograms in Figure 3. Figure 4 summarizes the quantitative results for the three groups. In the olfactory bulb of control rats, granule and glomerular layer expression were highly correlated ($r = 0.98$) and not detectably different. As seen in both Figures 3 and 4 there was a large increase in hybridization in

both granule cell and glomerular layers in tissue from the exploration and overtrained groups. For the exploration group, this was accompanied by a pronounced change in the balance of expression between the glomerular versus granule cells; that is, in contrast to the control group, expression was much greater in the granule cell layer than in the glomerular layer (56 $\pm$ 16%; $p < 0.0001$ ANOVA, $p < 0.001$ Student-Newmann-Keuls; Fig. 4*A*). There was also a change in relative labeling density between medial and lateral aspects of the bulb. While there was no reliable side-to-side difference in the glomerular layer in cage-controls, exploration rats had significantly greater hybridization medially than laterally (Fig. 4*B*). Control rats did have more labeling in the medial, as compared to the lateral, sector of the granule cell layer but this bias was more pronounced in the exploration group.
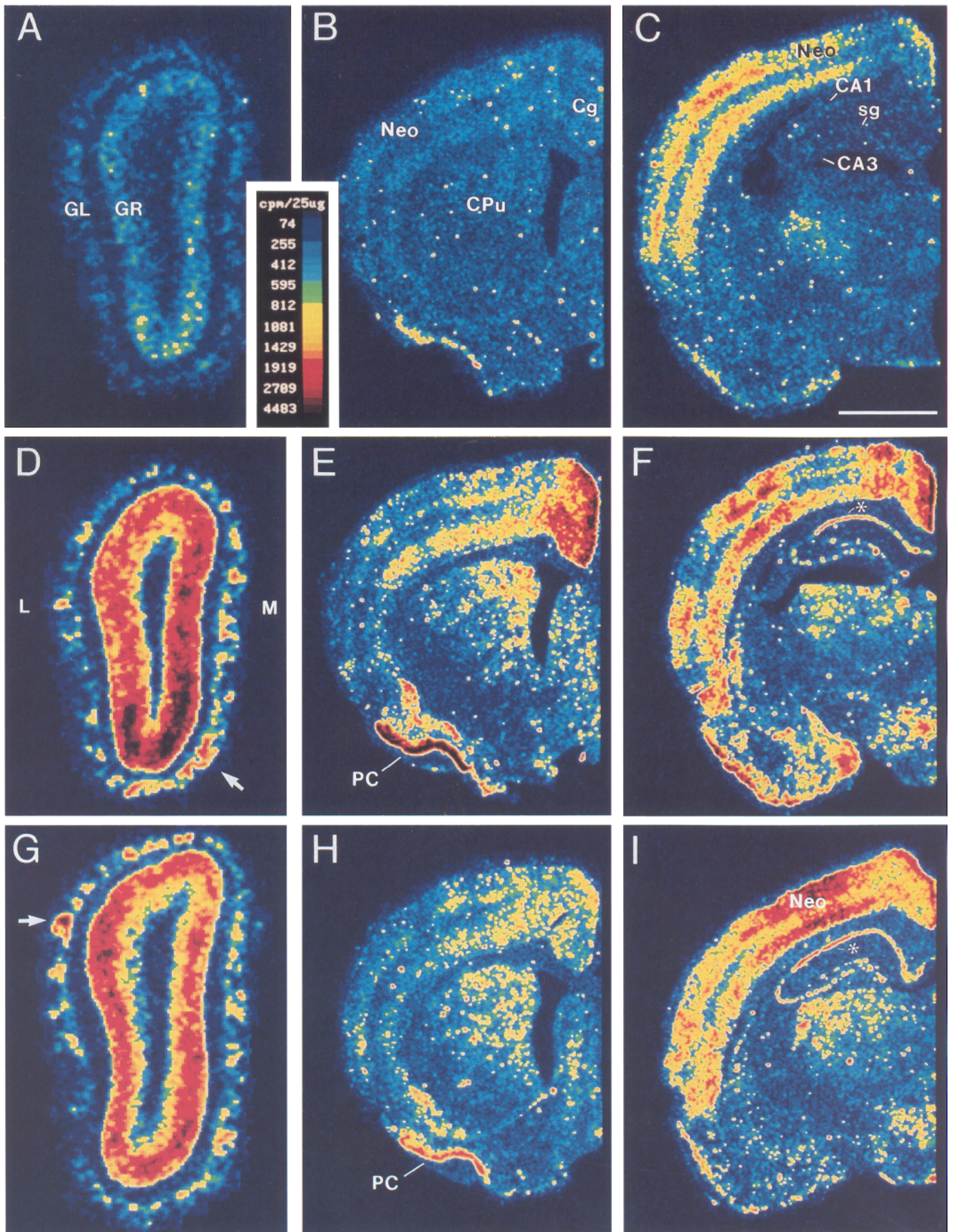
Expression of c-*fos* mRNA was clearly increased above control levels in the olfactory bulbs of rats performing a familiar odor discrimination but, as was the case for visual structures, there were differences from the results obtained for the exploration group. First, the variation in labeling between glomerular layer and granule cell layer for overtrained rats was less pronounced ($p < 0.0001$ ANOVA, $p < 0.01$ Student-Newmann-Keuls; Fig. 4*A*). Second, differences in the labeling of medial versus lateral aspects of the bulb were not significant. The latter point is illustrated in graphs of Figure 4, *B* and *C*, which plot within-subject ratios of labeling densities in medial versus lateral olfactory bulb for the three groups. Finally, while expression in the medial and lateral segments of the glomerular layer was highly correlated in the control and exploration rats ($r > 0.87$), this was not the case for the overtrained animals ($r = 0.21$).

These data demonstrate that exploration produces a bulb response that is differentiated between layers (granule > glomerular) and within layers (medial > lateral). Performance of a discrimination involving familiar odors also increases expression but in a more homogeneous fashion.

In piriform cortex, c-*fos* cRNA hybridization was about fivefold greater in the exploration group than in cage-controls (Fig. 5). Overtrained odor discrimination rats had intermediate c-*fos* mRNA levels in the piriform at about two-fold control values. The exploration and overtrained group values were significantly different from one another ($p < 0.0001$ ANOVA, $p < 0.001$, Student-Newmann-Keuls). Interestingly, hybridization was greater in the olfactory cortex than in the glomerular and granule cell layers of the olfactory bulb in the exploration group, while the reverse was true in overtrained rats (compare Figs. 4 and 5; see Fig. 3). Thus, in both absolute and relative terms, performance involving familiar odors by experienced animals had only modest effects on piriform cortex.

*Hippocampus*

As can be seen in the pseudocolor images of film autoradiograms presented in Figure 3, hybridization within hippocampus was uniformly low in controls (Fig. 3*C*) and markedly greater in exploration (Fig. 3*F*) and odor discrimination (Fig. 3*I*) rats. In the latter groups, the largest increase above control appeared to occur in field CA1 (asterisks, Fig. 3*F,I*). The quantitative measures of hybridization densities presented in Figure 6 agree with these observations. There were no statistical differences between labeling in the three principal hippocampal subfields in the control group but regional mRNA levels were correlated in within rat comparisons (CA1 vs CA3, $r = 0.89$; CA3 vs stratum granulosum, $r = 0.86$). In the exploration group, c-*fos* mRNA

A

GL  GR

| cpm/25ug |
|----------|
| 74       |
| 255      |
| 412      |
| 595      |
| 812      |
| 1081     |
| 1429     |
| 1919     |
| 2709     |
| 4483     |

B

Neo

Cg

CPu

C

Neo

CA1

sg

CA3

D

L                    M

E

PC

F

*

G

H

PC

I

Neo

*

levels were significantly greater than in control rats for all three subdivisions with the greatest elevation being in field CA1; hybridization was 74 ± 37% (mean ± SD) greater in field CA1 than in CA3 (Fig. 6B), and 94 ± 25% greater than in stratum granulosum ($p < 0.0001$ ANOVA, $p < 0.001$ Student-Newmann-Keuls). Hybridization in CA3 and stratum granulosum did not differ significantly. Thus, exploration not only increased c-fos expression but led to a marked change in the balance of c-fos mRNA between the three subdivisions. Additionally, the correlations between hippocampal subdivisions observed in the control group were absent in animals exploring the new environment (CA1 vs CA3, $r = 0.32$; CA3 vs stratum granulosum, $r = 0.32$).

Animals performing a well-learned odor discrimination had a similar hippocampal c-fos expression profile as rats exploring a novel environment. All three subdivisions demonstrated a significant increase in expression above control levels (Fig. 6A). Levels of hybridization in the exploration group were significantly greater than in the overtrained group in field CA1, stratum granulosum, and field CA3 ($p < 0.0001$ ANOVA; $p < 0.001$ for CA1 and stratum granulosum, $p < 0.05$ for CA3 Student-Newmann-Keuls). As in the novel environment group, overtraining led to a change in the balance of expression between the subdivisions. As shown in Figure 6B, hybridization in region CA1 was 53 ± 24% greater than in CA3 and 99 ± 14% greater than in stratum granulosum ($p = 0.0001$ repeated measures ANOVA, $p < 0.001$ Student-Newmann-Keuls). However, in contrast to the exploration group, expression was significantly greater in CA3 than in stratum granulosum ($p = 0.0001$ repeated measures ANOVA, $p < 0.05$ Student-Newmann-Keuls) and labeling in the three subdivisions was highly correlated (CA1 vs CA3, $r = 0.84$; CA3 vs stratum granulosum, $r = 0.86$).

Comparison of the results for hippocampus with those for other brain regions revealed further and interesting differences between the control, exploration, and overtrained groups. The values for all three hippocampal subdivisions were correlated with those for visual and piriform cortices in the home cage controls ($r > 0.87$), but not in the exploration group ($r \leq 0.4$ for five of six comparisons). In overtrained rats, c-fos expression in the three hippocampal subfields remained highly correlated with that in piriform cortex ($r > 0.85$), but much less so with that in visual cortex ($r = 0.44$–$0.76$).

Figure 7 presents high magnification bright-field photomicrographs showing the cellular localization of hybridization in the three hippocampal subdivisions (stratum granulosum and stratum pyramidale fields CA3 and CA1) as observed in the three groups. Included for comparison are two treatment groups that were described in an earlier paper (Hess et al., 1995): (1) animals performing a well-learned nose-poke response for water reward in the absence of olfactory cues, and (2) rats in the early stage of learning the two-odor discrimination in a familiar apparatus (see Discussion). Expression of c-fos mRNA in the dentate gyrus of exploration (Fig. 7D) and overtrained (Fig. 7M) rats appeared patchy with pairs or small groups of well-labeled granule cells separated some distance from one another by unlabeled cells. The granule cells were particularly heavily labeled in animals exploring a novel environment (Fig. 7D). Labeling of the granule cells appeared comparable to that in control rats for the remaining two behavioral groups (Fig. 7G,J). Cells in field CA3 were not intensely labeled but clusters of neurons with higher grain densities were evident; there were no apparent differences between the four experimental groups, although exploration rats had statistically significantly more labeling than overtrained rats. Hybridization in field CA1 varied considerably across the four experimental groups being greatest in the exploration (Fig. 7F) and overtrained (Fig. 7O) animals, at intermediate levels in rats that performed a practiced nose-poke response (Fig. 7I), and only slightly above background in animals that had recently acquired a two-odor discrimination (Fig. 7L). The exploration group again had clusters of intensely labeled pyramidal cells (Fig. 7F), while hybridization in the rats responding to well-learned odors was more diffuse (Fig. 7O).

## Discussion

Previous work in this laboratory showed that changes in behavior are associated with subfield-specific changes in c-fos expression within hippocampus (Hess et al., 1995). The present experiments demonstrate the converse and somewhat surprising result, that is, that quite different behaviors are accompanied by similar relative levels or patterns of expression in this structure. These points are illustrated by Table 1 which summarizes the relative levels of c-fos mRNA across hippocampal subdivisions in four of several stages in the sequence leading to rapid acquisition of novel odor discriminations. Photomicrographs showing the intrahippocampal distribution of c-fos expression in representative cases for these four experimental groups, and the home cage control group, are included in Figure 8.

One can see that exploration of a novel environment (the olfactory training apparatus) and performance of a well-learned odor discrimination elicit a common pattern of c-fos expression / neuronal activation in the hippocampus (CA1 > CA3 ≥ SG) (Fig. 8A,D). Since these two behaviors are in many respects
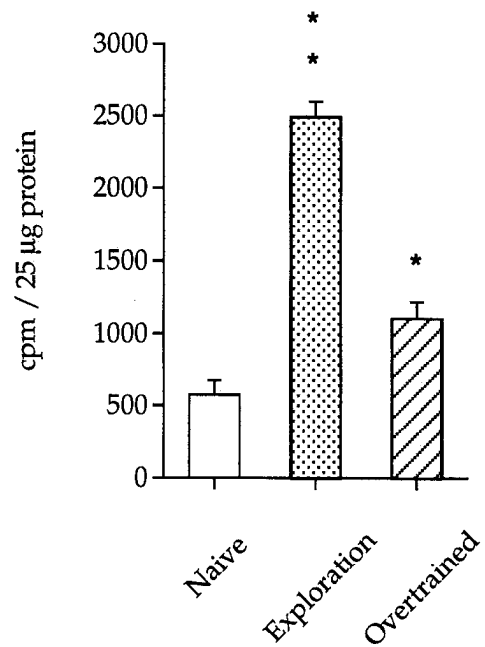
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*Figure 3.* c-fos mRNA expression in the olfactory system and hippocampus. Calibrated pseudocolor images of film autoradiograms showing densities of hybridization at the level of the main olfactory bulb (A, D, G), rostral piriform cortex (B, E, H), and rostral hippocampus (C, F, I) for individual rats from each of three groups. In naive rats (A–C), c-fos mRNA levels were generally low, with greater expression in the piriform cortex and portions of neocortex. In the exploration group (D–F), hybridization densities were dramatically increased above control levels. Particularly dense labeling was observed in olfactory bulb, piriform cortex (PC), cingulate cortex (Cg), and portions of neocortex (Neo). Note that c-fos expression was significantly greater in medial (M) versus lateral (L) regions of the granule (GR) and glomerular layers (GL) of olfactory bulb (D; particularly dense labeling was observed in the ventro-medial sector, arrow). In hippocampus of exploration rats, pyramidal cells in subfield CA1 (F; see asterisk) were more densely labeled than those in field CA3 or the granule cells of the dentate gyrus (sg). Animals overtrained on the two-odor discrimination (G–I) also showed c-fos mRNA levels that were significantly greater than control, but the regional distribution of labeling was different from that found in the exploration group. Portions of neocortex, piriform cortex, and the olfactory bulb showed particularly dense hybridization, but there was no significant difference in labeling in the granule cell layer between medial versus lateral bulb and there was a trend opposite to that obtained in the exploration group with regard to the glomerular layer (note dorso-lateral focus of activation, arrow). There was substantially less labeling in the piriform cortex compared to that seen in the exploration group while cingulate cortex had intermediate levels of expression. The regional pattern of hybridization in hippocampus of overtrained animals was similar to that in the exploration group, with CA1 pyramidal cells (I, asterisk) having greater levels of labeling than CA3 neurons or the granule cells. The values on the color calibration bar are in cpm/25 μg protein. Scale bars: B–C, E–F, H–I, 2162 μm; A, D, G, 1039 μm. Abbreviation: CPu, caudate-putamen.
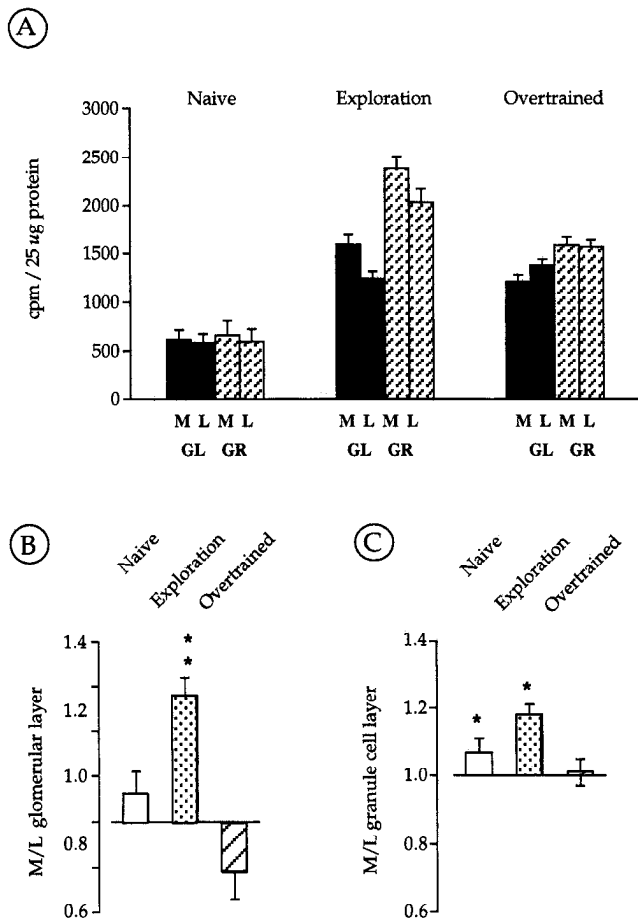
*Figure 4.* Bar graphs illustrating (*A*) mean levels of c-*fos* cRNA hybridization in the main olfactory bulb for each of the three groups, and (*B, C*) medial to lateral field ratios for the granule and glomerular layers. Regions measured include the medial (*M*) and lateral (*L*) glomerular layer (*GL*) and the medial and lateral granule cell layer (*GR*). *A*, In the olfactory bulb of "naive" rats, granule and glomerular layer labeling were both low and not detectably different. Exploration and overtrained rats had significantly increased levels of hybridization in medial and lateral portions of both laminae (*p* < 0.0001 ANOVA, *p* < 0.001 Student-Newmann-Keuls) but differed in the laminar distribution of labeling. *B* and *C*, Medial versus lateral field labeling ratios demonstrate a significant medial bias for the glomerular layer (*B*) and granule cell layer (*C*) of the exploration group and a smaller medial bias for the control group, but no reliable differences between sides in the overtrained group. The trend toward greater labeling in the lateral versus the medial sector of the glomerular layer in the overtrained animals (*B*) did not reach statistical significance (**, *p* ≤ 0.0005; *, *p* < 0.04; paired *t* tests).

*Figure 5.* Bar graph showing levels of c-*fos* cRNA hybridization in piriform cortex for the three animal treatment groups. Bars indicate group mean values (±SE) (*p* < 0.0001 ANOVA; *, *p* < 0.01; **, *p* < 0.001 Student-Newmann-Keuls).

disparate, this may represent a "common mode" of hippocampal functioning, that is, one which emerges during a wide variety of circumstances and that makes a generalized contribution to behavior. Two features are noteworthy about the pattern. *First*, it includes modest induction in the granule cells of the dentate gyrus, a structure that had proven to be singularly unresponsive in prior experiments. *Second*, it is associated with a response in field CA1 that is decidedly greater than that of the other two subdivisions. It is possible that these events are related. Field CA1, particularly in the rostral regions evaluated in the present experiments, receives the great majority of its afferents from the CA3 pyramidal neurons (reviewed by Amaral and Witter, 1989). Thus, a modest change in aggregate activity or, perhaps more importantly, in the degree of moment to moment synchrony of

cell spiking in CA3, are likely to have substantial impact on the number of CA1 neurons that discharge during a behavioral episode. Elevated input from the dentate gyrus could produce either or both types of effects in CA3. In any event, the pattern of c-*fos* expression observed in the present experiments is of a type that would be expected under conditions in which the full trisynaptic, intrahippocampal circuit (i.e., SG to CA3 to CA1) had been repeatedly activated. It should be noted that the direct projections from the entorhinal and perirhinal cortices to field CA1 (reviewed by Witter et al., 1989, and Amaral and Witter, 1989) may also modulate activity in this hippocampal subfield under certain behavioral conditions.

The distinguishing characteristics of the "common mode" are suggestive of conditions that elicit it and of the type of contributions it may make to brain operations. Concerning the former, stratum granulosum receives its dominant input from the entorhinal cortex (Witter et al., 1986, 1989; Amaral and Witter, 1989), a structure that itself is innervated by sensory systems (Witter et al., 1986, 1989; Insausti et al., 1987; Witter, 1993). The granule cells are also targets of a complex collection of excitatory (associational) and inhibitory neurons located in the hilus (Amaral and Witter, 1989; Ribak and Seress, 1983; Ribak et al., 1985; Halasy and Somogyi, 1993; Han et al., 1993) which receive input from the septum/diagonal bands and the ascending monoaminergic systems (Lynch et al., 1977; Swanson, 1977; Nyakas et al., 1987; Loy and Moore, 1979; Freund et al., 1990; Miettinen and Freund, 1992). This collection of anatomical features encourages the hypothesis that intense activation of (and c-*fos* induction in) the granule cells may result from repeated sampling of environmental stimuli in association with some pattern of septal/brainstem activation. Circumstances of this type do in fact occur during the performance of a two-odor discrimination by well-trained rats. Recording studies indicate that the animals engage in a stereotyped sniffing pattern when presented with odors and that this behavior synchronizes activity in the
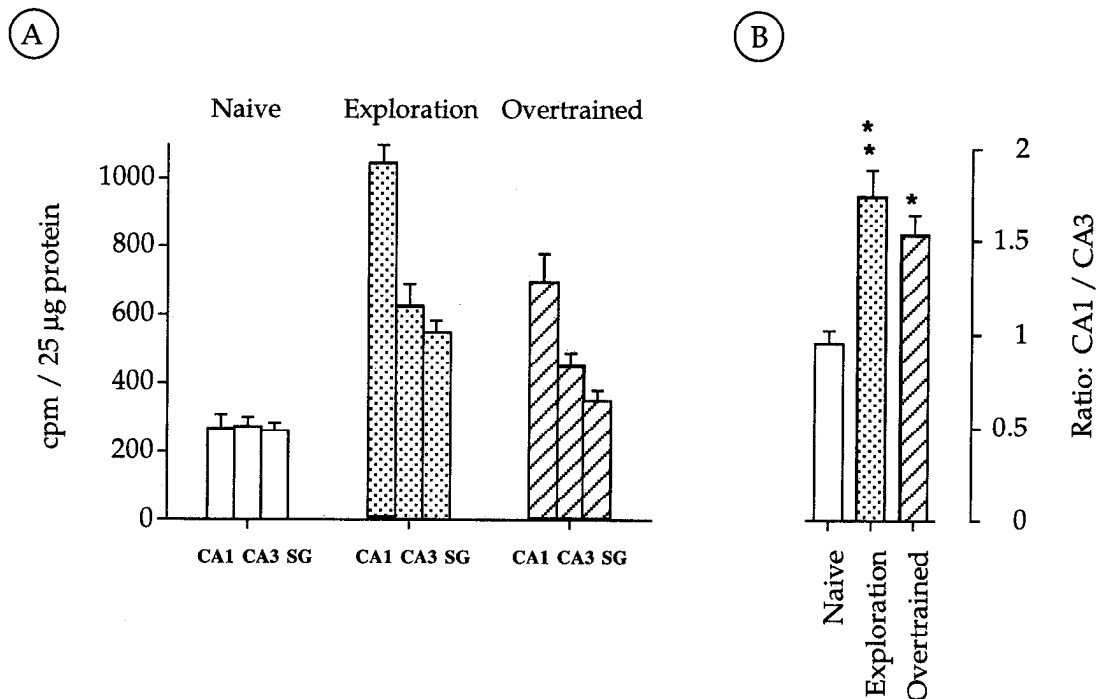
*Figure 6.* Bar graphs showing (*A*) densities of hybridization to c-*fos* mRNA in dorsal hippocampus and (*B*) ratios of hybridization in CA1/CA3 for the three groups. *A*, Hybridization was significantly increased above control levels in the three hippocampal subdivisions in both exploration and overtrained rats ($p < 0.0001$ ANOVA; $p < 0.001$ and $p < 0.05$, respectively, Student-Newmann-Keuls), with similar relative levels of expression across subfields evident in the two groups (i.e., CA1 > CA3 $\geq$ SG). For statistical comparisons of hybridization levels in stratum granulosum of overtrained versus naive rats, one of the eight naive rats was excluded as an outlier since measures from this rat fell greater than 2 SDs from the group mean. *B*, CA1:CA3 ratios show that the relative distribution of hybridization across these subfields is similar in the exploration and overtrained groups even though absolute expression levels differed between the treatments ($p = 0.0001$ ANOVA; *, $p < 0.01$; **, $p < 0.001$ Student-Newmann-Keuls test for CA1:CA3 ratio comparisons to the naive group).

sequence of connections leading from the olfactory bulb through cortex and into hippocampus (Komisaruk, 1970; Macrides, 1975; Macrides et al., 1982). Such experiments have also shown that cells in field CA1, at least, fire with short latencies and in synchronized bursts in response to odors used as discriminative cues (Eichenbaum et al., 1987; Wiener et al., 1989), indicating that events in olfactory bulb and cortex drive hippocampal circuitry during sampling. The septum plays a prominent role in organizing rhythmic activity in hippocampus (Nicoll, 1985; see Bland, 1986, for review) and hence it can be assumed that the septal projections to the infragranular dentate gyrus make a significant contribution to the synchronization that emerges in performance of a discrimination. Thus, the coordinated temporal convergence of sensory and septal inputs, an event certain to strongly affect granule cell firing, provides a reasonable explanation for the induction of c-*fos* in these neurons during the performance of an already learned two-odor discrimination.

Exploration also involves sampling of cues that, like odors, are associated with distant objects. Electrophysiological studies have shown that rhythmic activity occurs in hippocampus during exploration and is accompanied by synchronized bursts of cell spiking in CA1 that are coded to particular spatial cues (Hill, 1978; Muller et al., 1987; Eichenbaum et al., 1989). There are thus reasons to assume that septal (synchronizing) and sensory (spatial) inputs converge repeatedly in the dentate gyrus during exploration. The observations of Buzsaki (1989) that the activity of granule cells is significantly greater during theta-concurrent exploratory activities as opposed to consummatory behaviors are in accord with this idea. In all, then, exploration and perfor-

mance of odor discrimination, while very different in most regards, both involve an attentive sampling of distal cues, rhythmic activity, and short latency responses in hippocampus.

The observation that levels of c-*fos* mRNA in the piriform cortex and dentate gyrus were highly correlated across rats performing a well-learned discrimination but not in the other groups (Table 2) further supports the idea that neuronal activity in the two regions is linked during active sampling of pertinent cues. The piriform is the source of a large projection to the lateral entorhinal cortex which in turn densely innervates the granule cells of the dentate gyrus (Zimmer, 1971; Wyss, 1981; Price, 1987; reviewed by Switzer et al., 1985). This disynaptic circuit provides a means whereby extensive training using odors as discriminative cues results in a coordination between cortical and hippocampal activity. A similar result was not obtained for visual cortex in the exploration animals (Table 2). The links between the visual system and dentate gyrus are likely to be more complex than those for olfactory cortex and indeed may include circuitries in addition to those passing through neocortex (Thompson and Robertson, 1987a,b). It will be of considerable interest in future studies to determine if c-*fos* expression in other forebrain regions related to vision correlate with activities in dentate gyrus during exploration; areas that exhibit such an effect would be candidates for the relays that communicate spatial information to hippocampus, an issue that is of some importance in its own right.

The pattern of c-*fos* expression associated with exploration and odor discrimination was also characterized by differential labeling of the two output segments of hippocampal circuitry,
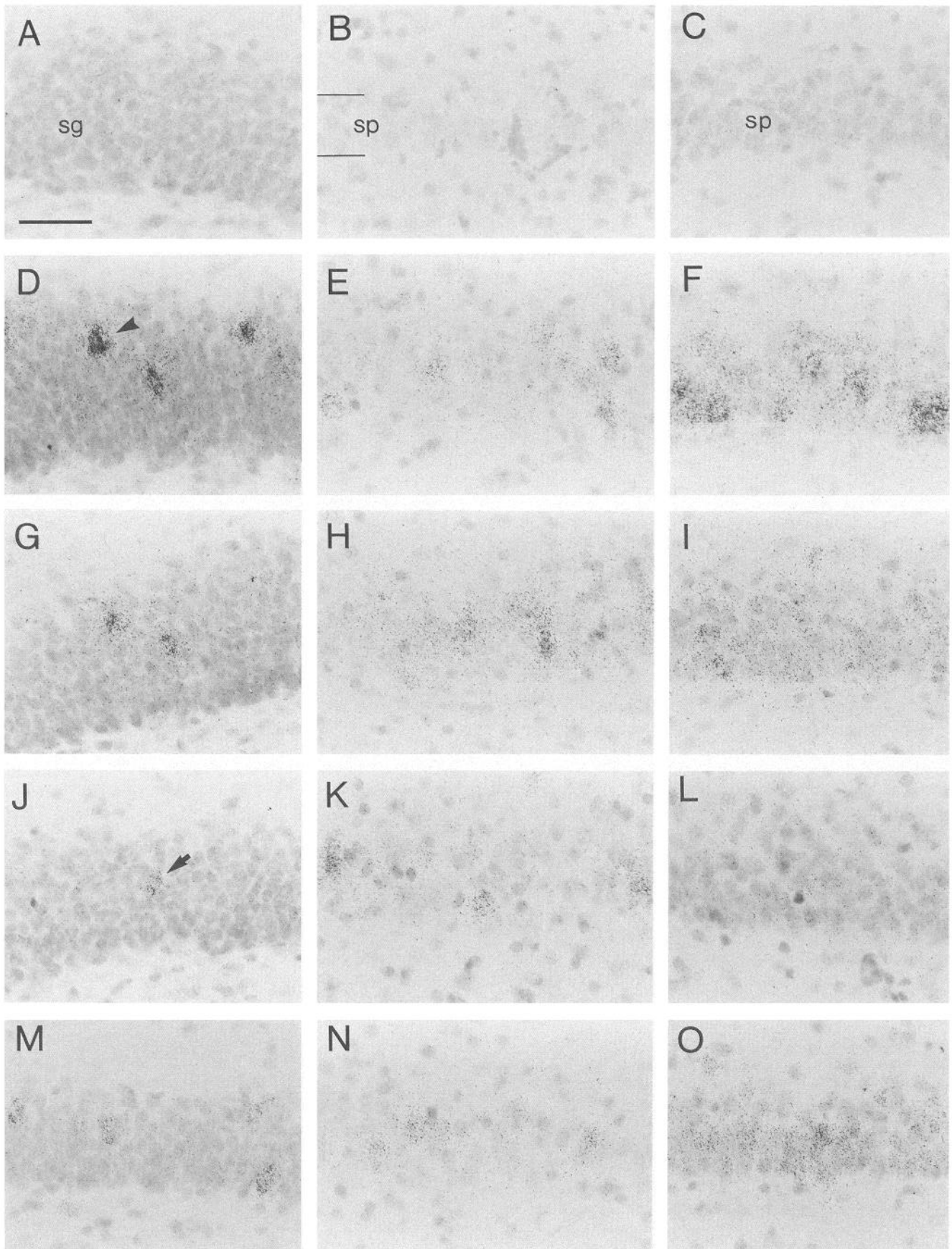
*Figure 7.* High magnification photomicrographs showing the autoradiographic localization of hybridization within stratum granulosum (*sg*) (*left column*), CA3 stratum pyramidale (*sp*) (*middle column*), and CA1 stratum pyramidale (*sp*) (*right column*) for the various groups: *A–C*, home cage

that is, field CA1 had substantially higher levels of mRNA than did field CA3. The CA1 region, via connections with subiculum and retrohippocampal cortex, as well as through direct projections, densely innervates the ventral striatum (nucleus accumbens) and anterior olfactory nucleus (Van Groen and Wyss, 1990; Brog et al., 1993). The ventral striatum appears to organize locomotor activities of a general type (Evarts et al., 1984; Koob et al., 1991; Mogenson and Yang, 1991; Salamone, 1992) and has been suggested to play a role in exploratory behavior (Mogenson and Nielson, 1984; Schacter et al., 1989). The anterior olfactory nucleus is a large structure that sends its axons to the granule cell layer of the ipsilateral and contralateral olfactory bulb (reviewed by Switzer et al., 1985) and thus constitutes a major contributor to the regulation of bulbar output to cortex. These observations suggest the hypothesis that the hippocampal pattern of c-*fos* expression obtained with the behaviors used in the present studies reflects an operation whereby the three subdivisions link sampled distant cues to (1) cellular machinery that organizes movement through space and (2) the flow of olfactory information into forebrain networks.

Higher resolution analysis of tissue autoradiograms indicated that hippocampal c-*fos* expression was detectably different in the exploration versus performance groups at the cellular level. These differences corresponded with quantitative regional differences indicating greater levels of c-*fos* mRNA in CA1 and dentate gyrus of exploration rats. In stratum granulosum, labeled neurons occurred in pairs or small clusters in both behavioral situations, but hybridization to individual cells appeared to be more intense in exploration animals. Exploration animals also had clusters of labeled cells in field CA1, a pattern that was easily distinguished from the more diffuse hybridization seen in the overtrained group. These differences might be due to increased levels of arousal in rats placed in a novel environment; it is noteworthy in this regard that the diffuse noradrenergic projections from the locus coeruleus are proposed to increase levels of neuronal activity in the forebrain (Loughlin et al., 1986). It is also conceivable that the differences are related to one of the more prominent behavioral distinctions between the two groups, namely, that exploration involves encoding while performance makes use of already learned information. A considerable body of evidence has implicated long-term potentiation (LTP) as a substrate for several varieties of memory (see Lynch et al., 1991; Lynch and Granger, 1992, for reviews) and it is known that induction of LTP requires depolarization of a degree and duration greater than that needed to trigger action potentials (Bliss and Collingridge, 1993). Possibly, the denser hybridization seen in single neurons in the exploration groups is due to the occurrence of postsynaptic responses of a type required for the development of LTP. With regard to this idea, it would be interesting to compare labeling in well-trained rats that have recently acquired a *novel* odor discrimination with that obtained in animals tested with a well-learned discrimination.

The occurrence of the above noted small neuronal clusters in

**Table 1. Relative levels of c-*fos* mRNA in three hippocampal subdivisions during various stages of the odor discrimination paradigm**

|  | SG | CA3 | CA1 |
|---|---|---|---|
| 1. Exploration of novel apparatus | 2 | 2 | 4 |
| 2. Learn nose poke response |  |  |  |
| 3. Perform nose poke response | ø | 2 | 2 |
| 4. Acquire two-odor discrimination | ø | 2 | 1 |
| 5. Perform known discriminations ("Overtrained") | 1.5 | 2 | 3 |
| 6. Rapid acquisition of novel discriminations |  |  |  |

SG, stratum granulosum. ø = control levels; 1 = significantly increased above control by ~16%; 1.5 = ~33% greater than control; 2 = 194 ± 24% (mean ± SD) greater than control; 3 = ~260% greater than control; 4 = ~390% greater than control. In step 4, the CA3/CA1 ratio is 1.82 ± 0.18. In step 5, the CA1/CA3 ratio is 1.53 ± 0.11, and the CA1/SG ratio is 1.99 ± 0.06.

the dentate gyrus indicates that a subpopulation of cells was activated repeatedly during behavior and that these cells tended to be neighbors. Repeated activation probably reflects the presence of a limited number of salient cues in the environment. The proximity effect is not unexpected assuming (1) that activation requires convergence of active afferents and (2) some degree of intermingling of dendritic trees (Barnes et al., 1987). It might be noted that the spatial granularity of hybridization, as seen in tissue autoradiograms, is in accord with the general hypothesis that c-*fos* induction during exploration and discrimination is driven by discrete influences (e.g., sampled cues).

### c-fos *mRNA expression in sensory systems*

Results for the sensory systems are summarized in Table 3. Note that the exploration animals had generally greater expression in olfactory regions than did other groups, and were unusual in having much higher levels of c-*fos* mRNA in the granular than in the glomerular layer of the olfactory bulb. The glomerular cells are thought to regulate the initial stages of processing of sensory input from the olfactory nerve (reviewed by Switzer et al., 1985) while neurons in the granular cell layer and piriform cortex are tightly connected with the output (mitral) cells of the bulb (Haberly and Price, 1977; Switzer et al., 1985). It is thus possible that the pattern seen in the exploration rats reflects the continuous sampling of odors exhibited by these animals.

Also of interest is the observation that the bulbar c-*fos* expression profile evoked during discrimination of familiar odors differed from that observed during initial learning of the same odors. We have previously reported that c-*fos* mRNA is induced during task acquisition in three distinct glomerular and underlying granule cell fields located laterally at intermediate levels of the bulb (Hess et al., 1995). This topographic response corresponds well with that obtained by controlled exposure to peppermint odor (the "positive," predominantly sampled odor in

control; *D–F*, exploration; *G–I*, nose-poke; *J–L*, initial learning; and *M–O*, overtrained. As seen in *D* and *M*, c-*fos* mRNA expression in granule cells of exploration and overtrained rats was "spotty" with spaced clusters of well-labeled cells. The granule cells were particularly densely labeled in animals exploring a novel environment (*D, arrowhead*). Very low to background levels of labeling were present in the dentate gyrus of rats in the nose-poke group (*G*) and in rats initially learning the discrimination (*J, arrow*). Labeling in field CA3 was similar for the four behavioral groups (*E, H, K, N*), with apparent clusters of more intensely labeled cells. In CA1 stratum pyramidale (*sp*) hybridization density varied considerably across groups. Labeling was most dense, and most variable across neurons, in exploration rats (*F*). Overtrained animals (*O*) had the next highest levels of hybridization with somewhat lower labeling in the rats performing a familiar nose-poke response (*I*). Expression in rats initially acquiring the odor discrimination (*L*) was only slightly above control levels. Scale bar: 55 μm.
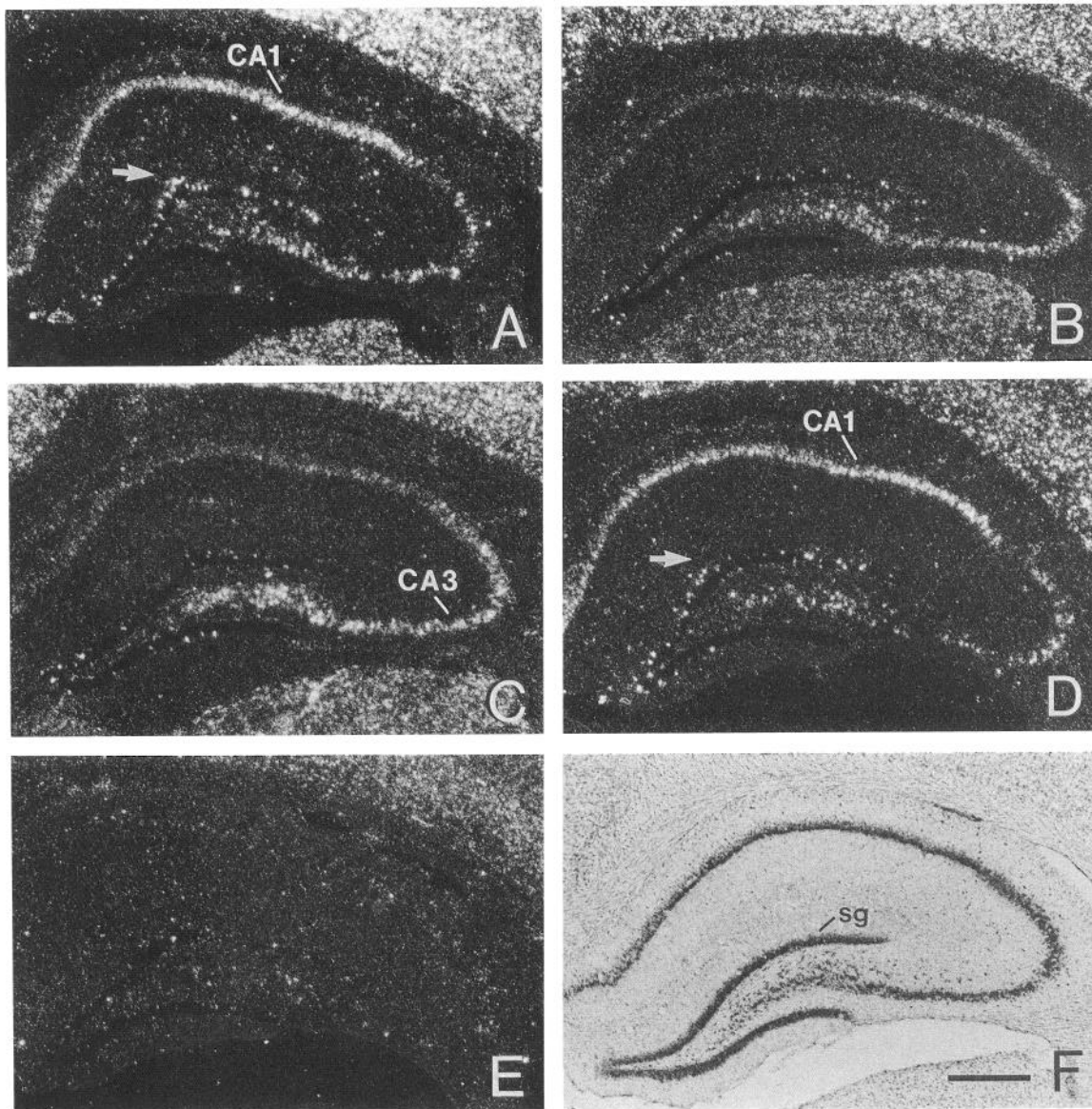
*Figure 8.* Dark-field photomicrographs of tissue autoradiograms showing differential patterns of c-*fos* mRNA expression in the hippocampus of animals performing at different stages in the olfactory learning paradigm. Exploration of the novel training apparatus (*A*) resulted in a pattern of "CA1 dominance" with significantly greater labeling in CA1 than in field CA3 or the granule cells. Performance of the nose-poke response (*B*) increased hybridization in CA1b and CA3 with no appreciable difference between them. It was noted that labeling density tended to be lower in the portion of CA1 closest to the subiculum (CA1a) than in CA1b in the nose poke group. However, CA1a was not measured. Acquisition of the discrimination task (*C*) induced a pattern of "CA3 dominance"; densities of hybridization were notably greater in field CA3 than CA1. Overtrained animals (*D*) performing on a familiar odor pair exhibited a similar pattern of increased c-*fos* expression as observed in exploration rats (*A*) (i.e., "CA1 dominance") but lower absolute hybridization densities. Note that c-*fos* expression in the suprapyramidal stratum granulosum (*sg*) was induced above basal levels only in the animals exploring the training apparatus (*A*; see *arrow*) and in those performing a well-learned odor discrimination (*D, arrow*). Naive rats (*E*) had uniformly low levels of hybridization across the three hippocampal subfields. A Nissl stained hippocampal section (*F*) is shown to provide anatomical detail. Scale bar, 500 μm.

**Table 2. Correlation coefficients for c-*fos* mRNA levels in stratum granulosum versus sensory cortical regions for the various treatment groups**

|  | Naive | Exploration | Nose poke | Acquisition of odor pair | Overtrained |
|---|---|---|---|---|---|
| Piriform cortex vs SG | 0.88 | 0.06 | 0.06 | 0.00 | 0.91 |
| Visual cortex vs SG | 0.90 | 0.40 | 0.32 | 0.23 | 0.44 |
| Piriform vs visual cortex | 0.99 | 0.70 | 0.70 | 0.85 | 0.44 |

Values represent correlation coefficients (*r*). SG, Stratum granulosum.

**Table 3. c-*fos* mRNA expression in olfactory and visual regions in four of several stages of the olfactory discrimination paradigm**

| | Olfactory bulb | | Piriform cortex | Superior colliculus | | Occipital cortex, superficial |
| --- | --- | --- | --- | --- | --- | --- |
| | Medial GL | Lateral GL | | Medial | Lateral | |
| Exploration | 1591 ± 100 | 1243 ± 72 | 2495 ± 102 | 820 ± 79 | 548 ± 38 | 1922 ± 126 |
| Perform nose poke | 1236 ± 86 | 1044 ± 107 | 1358 ± 154 | 1653 ± 155 | 730 ± 80 | 1379 ± 53 |
| Acquire discrimination | 866 ± 57 | 1007 ± 58 | 1039 ± 166 | 1166 ± 158 | 653 ± 55 | 806 ± 92 |
| Overtrained on discrimination | 1209 ± 69 | 1374 ± 63 | 1099 ± 117 | 1722 ± 174 | 705 ± 82 | 1206 ± 103 |

Values represent means ± SE and are in cpm/25 μg protein. GL, Glomerular layer.

the discrimination paradigm) (Guthrie et al., 1993). In overtrained rats, there were no significant differences in expression between medial and lateral aspects of the olfactory bulb, although there was a trend for more labeling in the lateral versus the medial glomerular layer. Thus, it appears that novel and well-learned odors elicit different responses even at primary stages of sensory processing. This may be accounted for by ACh, NE, and/or 5-HT centrifugal projections (see Macrides and Davis, 1983, for review; Zaborszky et al., 1986; McLean and Shipley, 1987; Zheng et al., 1987; Gervais et al., 1988; McLean et al., 1989; Le Jeune and Jourdan, 1993) differentially shaping and enhancing bulbar response patterns across behavioral situations.

The exploration group had very high levels of c-*fos* mRNA in the visual cortex but not in the superior colliculus; indeed, the exploration animals had lower levels of c-*fos* mRNA in medial superior colliculus than the other groups. This suggests that arousal and stress, which are presumably high in animals placed in a novel environment, have greater effects on cortex than on lower brain regions. That each of the remaining groups had strong responses in the medial colliculus may relate to the nose poking responses they used to obtain rewards.

Animals overtrained on the two-odor discrimination were quite similar to the nose-poke group with regard to levels and patterns of expression in the visual system. They were unusual, however, in that levels of c-*fos* mRNA in piriform cortex were low relative to those in olfactory bulb or visual cortex. Chronic recording studies have shown that a very small percentage of the neurons in olfactory cortex respond to odors in the two-odor discrimination paradigm (McCollum et al., 1991). Moreover, only a few samples (i.e., high-frequency sniffs) may be required by well-trained rats to make a correct choice (Coopersmith and Leon, 1984; Coopersmith et al., 1986). This amount of sampling apparently does not cause aggregate activity to be increased above the levels seen in alert rats not engaged in odor directed behaviors. However, expression in the piriform cortex did not correlate with that in visual cortex for rats in the overtrained group but did for the other groups (Table 2). This suggests that individual variations in performance of the discrimination were sufficient to obscure the generalized effects of arousal, and other state variables, on the piriform cortex. It will be of considerable interest to determine if hybridization as seen at the level of single cells is in some way different in the overtrained discrimination animals than in the other groups.

In summary, absolute and relative levels of c-*fos* mRNA across olfactory and visual structures are not in any obvious way predictive of observed variations in hippocampal patterns of expression. Studies are now in progress to identify structures that exhibit high levels of expression in the exploration and overtrained animals but not in the two other groups thus far analysed. Information of this kind would likely be of considerable value

in developing explanations of how disparate behaviors elicit a common hippocampal response. It will also be of interest in future studies to determine whether the pattern of expression of other immediate early genes (e.g., zif/268 and tissue-plasminogen activator) (Qian et al., 1993; Worley et al., 1993) is comparable in behavioral conditions evoking a similar c-*fos* mRNA response.

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