Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11417–11421, November 1994
Neurobiology

Learning-related changes in Fos-like immunoreactivity in the chick forebrain after imprinting

(memory/hyperstriatum ventrale/hippocampus/immediate early genes/visual pathways)

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Communicated by James L. McGaugh, June 24, 1994 (received for review August 27, 1993)

ABSTRACT **The intermediate and medial part of the hyperstriatum ventrale (IMHV) is a part of the chick forebrain that is critical for the learning process of imprinting and may be a site of information storage. Chicks were either trained on an imprinting stimulus or dark-reared. Trained chicks were classified as good or poor learners by their preference score (a measure of the strength of imprinting). A monoclonal antibody against the immediate early gene product Fos was applied to sections through IMHV and other forebrain regions. In the IMHV, significantly more immunopositive nuclei were counted in good learners than in poor learners or dark-reared chicks. There was a positive correlation between counts of labeled nuclei and preference score that was not attributable to sensory activity *per se*, locomotor activity during training, or a predisposition to learn well; rather, the results indicated that the change in Fos immunoreactivity in the IMHV was related to learning. In the hyperstriatum accessorium, significantly fewer immunopositive nuclei were counted in good learners than in poor learners or in dark-reared chicks. In the dorsolateral hippocampal region, more immunopositive nuclei were counted in trained than in dark-reared chicks. No significant effects of training were found in the anterior hyperstriatum ventrale, lobus parolfactorius, neostriatum, medial hippocampal region, or ventrolateral hippocampal region, but counts in this last region were positively correlated with training approach. The results for IMHV implicate Fos or Fos-related proteins in memory processes and pave the way for the identification of the cell types that show the learning-related increase in gene expression.**

Imprinting is a type of learning through which the young of certain species form a preference for an object as a result of being exposed to it (1, 2). Imprinting is readily demonstrated in the domestic chick, and a part of the chick forebrain, the intermediate and medial part of the hyperstriatum ventrale (IMHV), has been shown to be critically important for this type of learning and for memory of features of the imprinting object (3, 4). The available evidence (5–23) strongly suggests that information necessary for the recognition of an imprinting object is stored in the IMHV.

Imprinting leads to a number of changes in the IMHV. These include an increase in net ribonucleic acid synthesis (9), an increase in the mean length of postsynaptic density profiles of axospinous synapses (17, 18), an increase in *N*-methyl-D-aspartate-receptor binding (13, 24), and an increase in endogenous phosphorylation of the myristoylated alanine-rich C-kinase substrate (MARCKS) protein (14).

The c-*fos* gene product Fos contributes to gene regulation by forming one half of the heterodimeric AP-1 transcription factor (25, 26). Activation of certain neurons by a variety of procedures can influence their level of expression of the c-*fos* gene (27–33), and such an effect has been found in the chick forebrain (34, 35). Imprinting affects neuronal activity in the IMHV (20–22), and this ability might lead to c-*fos* expression. If this were so, c-*fos* expression in the IMHV should change in a learning-dependent way. We have sought to test this prediction in the present study. We have also taken the opportunity to investigate c-*fos* expression in the anterior part of the hyperstriatum ventrale and in certain other areas previously investigated in learning studies of the avian brain. These areas are the hyperstriatum accessorium (HA) (see refs. 14, 36, and 37), the posterior neostriatum (see ref. 38), the hippocampus (see ref. 39), and the lobus parolfactorius (see ref. 40).

MATERIALS AND METHODS

Training and Testing. Thirty-two dark-reared chicks (Ross I) were each placed in a running wheel 15–30 hr after hatching and trained by exposure for 1 hr to an imprinting stimulus (a rotating red box) (12); a further 16 chicks remained in darkness. The distance run during training (training approach) was measured (one running wheel revolution = 94 cm). Ten minutes after training, each chick was given a preference test in which it was exposed, in counterbalanced order, twice to the training stimulus and twice to a novel stimulus [a blue box with black, diagonal stripes (41)]; each exposure period lasted 2 min. The preference score—i.e., approach to the training stimulus during the preference test expressed as a percentage of total approach during the test (12)—was used as a measure of the strength of imprinting. When chicks were not in the running wheel, they were kept in individual compartments of a dark incubator maintained at 34°C. Chicks were matched in pairs for training approach, but in each pair, the preference score of one chick ("good learner") was higher than that of the other ("poor learner") (14). The preference scores of the good learners were >65, and those of the poor learners were <65. A third, dark-reared chick was also selected from the same batch; the three chicks from the same batch formed a matched set, the good learner and the poor learner having been treated identically. All subsequent procedures were performed "blind." Chicks were decapitated 50–70 min after the end of training, and their brains were stored at −70°C.

Immunocytochemical Procedure. Coronal 16-μm sections were cut at (*i*) a level centered on the anteroposterior (A/P) coordinate A7.6 of Kuenzel and Masson's atlas (42) and (*ii*) a level centered on A/P coordinate A10.6. For each of the A/P levels, on a given slide there were mounted six sections: two from each of the chicks in a matched set (good learner, poor learner, dark-reared). The slides from each matched set were subsequently processed identically. Sections were fixed

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Abbreviations: HA, hyperstriatum accessorium; IMHV, intermediate and medial part of the hyperstriatum ventrale; A/P, anteroposterior.

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in cold 4% (wt/vol) paraformaldehyde/phosphate-buffered saline, pH 7.4, incubated with a monoclonal antibody (Oncogene Science) against amino acids 127–151 of chicken Fos (43), and treated with biotinylated secondary antibody and avidin/peroxidase complex (Vector Laboratories). 3,3′-Diaminobenzidine was used as the chromagen.

Controls for Antibody Specificity. Omission of the primary antibody or preincubation of the primary antibody with 3 nmol·ml^{-1} of the target peptide resulted in no staining. Normal staining was obtained if the primary antibody was preincubated with the unrelated 25-amino acid peptide valosin (Sigma) at 3 nmol·ml^{-1}. Samples of the target peptide, but not valosin, were labeled by the antibody.

Sampling and Statistical Analysis. Four sections from each brain (two sections at each A/P level) were analyzed. Sections were viewed at ×78.75. Images (512 × 512 pixels) were captured at 256 gray-level resolution using a Seescan Solitaire image analyzing computer, the input stage of which was a monochrome television camera. The computer divided the intensity of light transmitted through the specimen by the incident light intensity, giving a resultant image intensity proportional to antilog (− OD of specimen). Darkly stained nuclei were counted in 0.3 mm × 0.9 mm rectangular sampling frames placed over forebrain regions as shown in Fig. 1. Using the COUNT-DARK command (Seescan plc), the computer searched for small, dark groups of pixels in each sampling frame and counted them if one or more of the pixels in such a group were darker by >50 gray levels than the darkest pixel surrounding the group. The 50 gray levels always corresponded to >0.1 OD unit. This threshold criterion ensured that nuclei were counted rather than background. The results therefore represent numbers of nuclei darker than a consistent threshold criterion. Because all sections (dark-reared, poor learner, good learner) in any one replication of the experiment were processed identically and simultaneously, this procedure did not bias counts in favor of any experimental group.

The raw means from the seven brain regions correlated significantly ($P < 0.01$) with their variances. The data were therefore transformed to square roots (44) for statistical analysis. Residuals from all ANOVAs of the transformed data conformed to the normal distribution (45). The data were subjected to a split-plot ANOVA with the factors training condition (good learners, poor learners, dark-reared), side (left, right), and position (the two frames within each anatomical region). To study the relation between counts of immunopositive nuclei and preference score, these two variables were standardized with respect to the matched sets: from each data value was subtracted the quantity (mean of matched set − overall mean). Partial correlation analyses were also performed between number of labeled nuclei and preference score, holding constant the effect of training approach, all standardized as above. The results of statistical tests have been quoted only if significant.

RESULTS

Behavioral Data. The mean preference score of the good learners (76.5 ± 3.04, mean ± SEM) was significantly higher than that of the poor learners (56.4 ± 2.65) ($F_{1,15} = 48.87$, $P < 0.001$). The means of both groups of chicks were significantly greater than the "no choice" level of 50 (good learners, $t = 8.71$, 15 df, $P < 0.001$; poor learners, $t = 2.43$, 15 df, $P = 0.03$).

The mean values of training approach for good and poor learners were very similar and not significantly different from each other (good learners, 169.2 ± 45.2; poor learners, 164.3 ± 40.6). Approach activity during the preference test was not significantly affected by training condition [good learners, 16.9 revolutions ± 3.86, $n = 16$; poor learners, 27.2 ± 8.07, $n = 16$].

Overview of Regional Effects. The mean numbers of stained nuclei per sampling frame are given in Table 1.

In all regions except the lateral hippocampus, the number of counted nuclei per frame did not depend significantly on

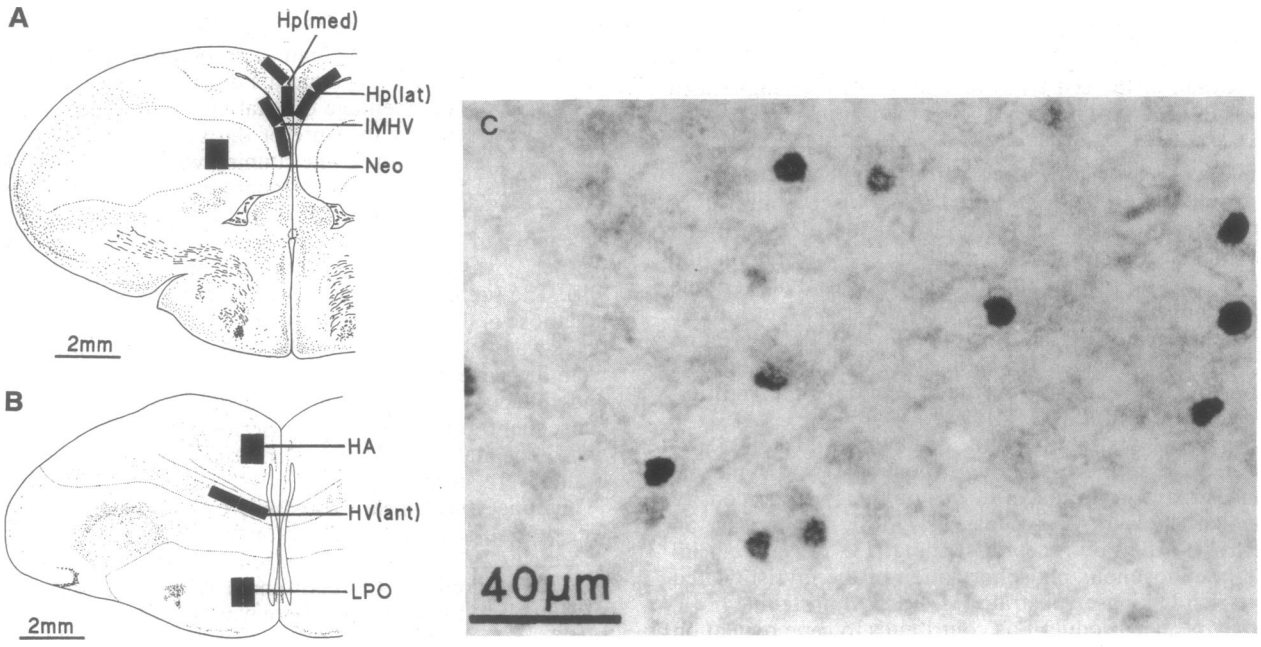

FIG. 1. (*A*) Diagram of a coronal section through the chick forebrain showing the sampling sites in the IMHV, medial and lateral hippocampal regions [Hp(med) and Hp(lat), respectively], and neostriatum (Neo) (modified from ref. 42, A/P coordinate A7.6). Two rectangular sampling frames, each measuring 0.3 mm × 0.9 mm, were placed over each anatomical region studied, in both hemispheres. Here, the positions of the frames are shown as shaded areas on one side of the brain only. The sampling frames over the medial and lateral hippocampal regions are shown in opposite hemispheres because the ventral sampling frames in these two regions partially overlapped. (*B*) Same as for *A* but showing the sampling sites in the HA, anterior hyperstriatum ventrale [HV (ant)], and lobus parolfactorius (LPO) (A/P coordinate A10.6). (*C*) Micrograph of a section through the IMHV of a good learner. Nuclei expressing Fos-like immunoreactivity are darkly stained.

Table 1. Mean numbers of counted nuclei per sampling frame, together with results of ANOVAs and levels of significance for the main effect of training condition

Brain region	Nuclei, no.			ANOVA	
	Dark-reared	Poor learners	Good learners	$F(2,30)$	P
IMHV	30.2	36.2	44.9	7.01	0.003
HA	8.1	9.2	3.7	6.88	0.003
Lateral hippocampus*					
Dorsal	19.9	34.5	29.0	5.51	0.009
Ventral	20.0	24.0	25.0	0.78	NS
Medial hippocampus	22.0	28.2	25.2	0.51	NS
Neostriatum	25.7	30.6	35.5	1.06	NS
Anterior HV	15.2	17.1	20.5	1.06	NS
LPO	9.5	8.5	7.5	0.73	NS

LPO, lobus parolfactorius; HV, hyperstriatum ventrale; NS, not significant.

*In the lateral hippocampus, there was a significant interaction between training condition and sampling position within anatomical region ($F_{2,282} = 5.55$, $P = 0.004$). Data from the dorsal and ventral sampling positions in this region were therefore analyzed separately.

the position of the sampling frame within a hemisphere or on the side of the brain. Therefore, data from all positions in these regions, within and between hemispheres, were combined. In the lateral hippocampus there was a significant interaction between training condition and position of the sampling frame; data from the dorsal and ventral sampling frames in this region were therefore analyzed separately (see below).

IMHV. There was a significant effect of training condition on counts of labeled nuclei per frame ($F_{2,30} = 7.01$, $P = 0.003$). The mean value for the good learners was significantly greater than that in the poor learners ($t = 2.68$, 30 df, $P = 0.012$), and there was no significant difference between the poor learners and the dark-reared chicks (Table 1).

On examination of the residuals from the ANOVA, it was found that neither the residual variance in good learners (0.48) nor that in poor learners (0.40) differed significantly from that (0.27) in the dark-reared chicks. If the trained chicks were analyzed as a single group, their variance was 0.96, a value significantly greater than that of the dark-reared chicks ($F_{16,15} = 3.56$, $P = 0.009$).

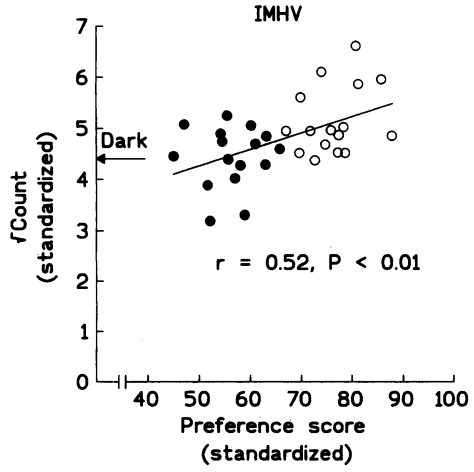

FIG. 2. Counts (transformed to square roots) of labeled nuclei per sampling frame in the IMHV of chicks that were exposed to the training stimulus, plotted against preference score. Data have been standardized (*Materials and Methods*) and corrected to a constant value of training approach by partial correlation analysis. The mean of the transformed counts in dark-reared chicks is indicated by the arrow. Open circles, good learners; closed circles; poor learners.

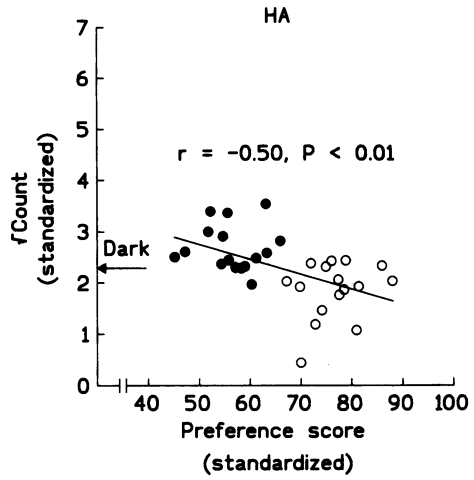

FIG. 3. Plot, as for Fig. 2, of data from HA. The mean of the transformed counts in dark-reared chicks is indicated by the arrow.

A correlation analysis was performed on the square-root-transformed counts and the corresponding preference scores, both standardized as described in *Materials and Methods*, for the trained chicks. A significant correlation was found ($r = 0.51$, 30 df, $P = 0.003$). Fig. 2 shows the results of a partial correlation analysis, holding training approach constant; the partial correlation coefficient was also significant ($r = 0.52$, 29 df, $P = 0.003$).

HA. There was a significant effect of training condition on numbers of labeled nuclei per frame ($F_{2,30} = 6.88$, $P = 0.003$). Significantly ($t = 3.68$, 30 df, $P = 0.001$) fewer nuclei were counted in good learners than in poor learners (Table 1). The mean count in the good learners was significantly lower than that in the dark-reared chicks ($t = 2.20$, 30 df, $P = 0.04$).

Counts of labeled nuclei were standardized as for the IMHV. The correlation between counts of labeled nuclei per frame and preference score of the trained chicks was significant ($r = -0.51$, 30 df, $P = 0.003$); the corresponding partial correlation coefficient, holding training approach constant, was also significant (−0.50, 29 df, $P = 0.004$); see Fig. 3.

Hippocampus. There was a significant interaction between training condition and sampling position within the lateral hippocampus (Table 1). When the data from the dorsal and ventral positions in this region were analyzed separately, a significant effect of training was found only in the dorsal sampling frame ($F_{2,30} = 5.49$, $P = 0.009$). Significantly more stained nuclei were counted in both good and poor learners than in the dark-reared chicks ($t = 2.13$, $P = 0.042$ and $t = 3.26$, $P = 0.003$, respectively). Good and poor learners did not differ significantly from each other.

When training approach was included as a covariate in the analysis of data from the ventral sampling frame in the lateral hippocampus, it was found to be significantly ($r = 0.68$, 14 df, $P = 0.004$) correlated with counts of nuclei.

Neostriatum, Anterior Hyperstriatum Ventrale, and Lobus Parolfactorius. No significant main effects, no significant interactions, and no significant correlations or partial correlations between counts and preference score were found in these brain regions (Table 1).

DISCUSSION

Training with an imprinting stimulus was found to increase the number of Fos-stained nuclei counted in the IMHV. This increase cannot be attributed solely to exposure to the training stimulus or to nonspecific effects of training (e.g.,

arousal, stress) because the mean number of labeled nuclei counted in the IMHV of the poor learners was not significantly different from that in the dark-reared chicks. Only in the good learners were there significantly more counts than in the dark-reared birds. There were also significantly more labeled nuclei counted in good learners than in poor learners. The latter result cannot be explained by differences in the amount of locomotor activity exhibited during training because the approach activities of the good and poor learners were very similar; rather, the increase is attributable to learning.

Although the labeling in the poor learners was not significantly different from that in the dark-reared chicks, the mean level was numerically higher than the dark-reared mean (Table 1). The mean preference score of the poor learners (56.4 ± 2.65) was significantly ($P = 0.03$) greater than the "no preference" level of 50, suggesting that these chicks exhibited a low level of learning. It is thus possible that the nonsignificant increase, relative to the dark-reared level, in the mean counts of labeled nuclei in the poor learners is due to the same process that underlies the significant increase in the good learners. There was a significant partial correlation between transformed counts and preference score (Fig. 2). The value of the ordinate that corresponds to the no-preference score of 50 is 4.23. This number is very close to (and is not significantly different from) the mean value (4.38) found for dark-reared chicks. These results, together, suggest that, starting from the base level found in the dark-reared birds, the number of labeled nuclei increases with the amount chicks learn about the training object.

It could be argued that chicks hatched with higher numbers of Fos-expressing cells will learn better than chicks hatched with fewer Fos-expressing cells. On this "predispositions" hypothesis there are no effects of learning. If the hypothesis were correct, the dark-reared chicks should include good and poor learners having high and low numbers of Fos-expressing cells, respectively. Hence the variance of cell numbers found in good learners (hatched with high numbers of these cells) should be significantly smaller than that in the dark-reared chicks. This was not the case. Nor was it the case for the poor learners. Hence, the predispositions hypothesis may be rejected in favor of an effect attributable to learning (46).

There was no evidence of a hemispheric asymmetry in Fos expression either in dark-reared birds or in trained birds, in contrast with the results of several studies in which asymmetrical effects of training on the IMHV region have been observed. Thus, imprinting on a red box is associated with (*i*) a change in the rate of spontaneous impulse activity in the left IMHV relative to the right (5), (*ii*) an increase in myristoylated alanine-rich C-kinase substrate protein phosphorylation (14), and (*iii*) an increase in *N*-methyl-D-aspartate-receptor binding (13, 24). Both (*ii*) and (*iii*) occur in the left IMHV but not in the right. In the present experiment the training period was shorter than in the others, in which training lasted between 90 min (14) and 140 min (13, 24). The time between the beginning of training and killing also differed between experiments. Thus, the changes in Fos expression in this study occurred ≈2 hr after the beginning of training. The change in myristoylated alanine-rich C-kinase substrate protein phosphorylation was present ≈3 hr after the onset of training, the asymmetry in spontaneous impulse activity after ≈9 hr (but not ≈3 hr), and the change in *N*-methyl-D-aspartate receptor binding after ≈13 hr but not ≈10 hr (24). Diverse as these measures of neuronal activity are, these results taken together show that bilateral changes in neural function in IMHV are demonstrable shortly after imprinting, but that in the hours that follow, the functional activities of the two sides diverge.

The HA is a visual projection area (47–51). The results of several experiments demonstrate that this region is affected by exposure to an imprinting stimulus. Electrophysiological studies of the HA have suggested that, compared with dark-reared chicks, training reduces the tendency of responses to repeated light flashes to wane (habituate) (37, 52, 53). Other observations suggest that there are, within the HA, additional changes that are closely related to imprinting (14). However, whereas lesions of the IMHV impair the acquisition and retention of a preference acquired through imprinting (11, 12, 15, 16), lesions of the HA have no such effects (11, 16, 54, 55). There are reciprocal connections between the IMHV and the HA (56). Taken together, the experimental observations suggest that neuronal activity in this visual projection area may be modulated by the IMHV. Such an interaction between the two regions would be plausible if, as the evidence suggests, the IMHV region is a storage system for recognition memory. Various aspects of visual performance are affected by prior experience (57–60), and these functions may involve interaction between those brain regions involved in retaining information about prior experience and the visual sensory pathways (61).

The avian hippocampus composes part of the dorsal and medial component of the telencephalon and, in the chick, projects to the IMHV (56). Cells within the medial part of the hippocampus form two columns arranged to form a V, the apex of which lies inferior to the open end (Fig. 1*A*). The present study suggests that the medial and lateral components of the hippocampus have different functions and that there is a differentiation of function within the lateral component. The numbers of labeled nuclei counted in the medial column were not related to any of the behavioral measures used in this study. This was not the case for the remaining components that were differentially affected by training. In the dorsolateral sampling frame, the counts of labeled nuclei in the two trained groups of chicks were very similar but were significantly higher than in the dark-reared chicks. These results suggest that the number of nuclei counted is not related to imprinting but to some other consequence of the training procedure, such as, for example, sensory experience. Locomotor activity was not an important determining variable because the number of labeled cells was not significantly correlated with training-approach activity. In recent studies of neuronal activity designed primarily to study the effects of imprinting on the responses of neurons in the left IMHV, the recording electrode occasionally passed along the lateral hippocampal column of cells. Effects of training were found in this part of the hippocampus (M. N. Brown and G. Horn, unpublished data). The present results for the dorsolateral sampling frame contrast with those for the ventrolateral component. In the region covered by this sampling frame there were no significant effects of training on nuclear counts. However, these numbers were strongly correlated ($r = 0.68$, $P = 0.004$) with training approach.

Changes have been found in the incorporation of 2-[^{14}C]deoxyglucose in a region referred to as medial neostriatum/hyperstriatum ventrale after auditory imprinting training (36, 62; see ref. 3). The dorsal component of medial neostriatum/hyperstriatum ventrale appears to correspond to the anterior hyperstriatum ventrale of the present study (3, 63). The absence of changes in this region in the present study may reflect the different kinds of imprinting (visual or auditory) between the two studies or the frequently observed dissociation between studies of 2-deoxyglucose uptake and c-*fos* expression (see refs. 64 and 65).

Nuclei were counted if they were darker than a threshold value. An increase in counts therefore reflects an increase in the amount of reaction product and therefore of gene expression, in at least some of the nuclei in the sampling frame.

The c-*fos* gene is activated in some cell types by the action of protein kinase C (66), and the possibility therefore arises that some of the increase in counts of immunopositive nuclei

in the IMHV found in the present study may be linked to protein kinase C activity. Part of the power of the immunocytochemical technique is that it permits the cells expressing gene product to be characterized by labeling with a second antibody. Ambalavanar *et al.* (67) found that at least 96% of cells with nuclear Fos-like immunoreactivity in the IMHV of trained and dark-reared chicks also contained protein kinase C γ. It is likely that these cells were neurons (68). Cells with Fos-like nuclear immunoreactivity in the IMHV of trained and dark-reared chicks have been found also to contain calmodulin-like and parvalbumin-like immunoreactivity (69). A few of the Fos-positive cells were also labeled by an antibody raised against calbindin D-28k, but it has been found that the increase in Fos-like immunoreactivity after training does not occur in these cells (70). Of particular interest is the observation that, in the right and left IMHV, almost all (95.2 $\pm$ 1.17%) cells that stained for Fos also stained for γ-aminobutyric acid (GABA) (71). If these cells are GABAergic and inhibitory (but see refs. 72 and 73), then the results described in this paper for IMHV have implications for the kind of neuronal circuitry that underlies recognition memory.

We are grateful to C. Bond for assistance and to the Biotechnology and Biological Sciences Research Council for financial support.

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