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Learning and memory: Regional changes in *N*-methyl-D-aspartate receptors in the chick brain after imprinting

(excitatory amino acid receptors/synaptic transmission/hemispheric asymmetry/chicken)

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ABSTRACT **An extensive series of experiments has implicated a restricted region of the chick forebrain in the learning process of imprinting. The region is the intermediate and medial part of the hyperstriatum ventrale (IMHV). Previous studies have shown that training is associated with an increase in the area of the postsynaptic density of axospinous synapses in the left but not the right IMHV. The postsynaptic density is a site of high receptor density, and at least some axospinous synapses are excitatory. We found that imprinting is associated with a 59% increase in *N*-methyl-D-aspartate-sensitive binding of the excitatory amino acid L-[^{3}H]glutamic acid in the left IMHV. The increase is probably due to an increased number of binding sites. The profile of sensitivity of the sites to a series of amino-, phosphono-substituted carboxylic acids (2-amino-3-phosphonopropionate to 2-amino-8-phosphonooctanoate) is characteristic of *N*-methyl-D-aspartate-type receptors. There were no significant effects of training on binding in the right IMHV. The effect of training on left IMHV binding could not be attributed to light exposure, arousal, or motor activity *per se* but was a function of how much the chicks learned. The changes in the left IMHV could increase the effectiveness of synaptic transmission in a region crucial for information storage and so form a neural basis for recognition memory.**

It has often been proposed that learning in vertebrates leads to changes in synaptic connectivity and that these changes form the neural basis of memory for the acquired information (1, 2). It has not been easy to test this proposition, since any detected change in connectivity could represent some nonspecific consequence of training (3). The comparable problem of interpreting changes in certain aspects of brain biochemistry following learning has largely been overcome in the case of imprinting in domestic chicks. Imprinting is a learning process by which the young of certain species (precocial) come to recognize an object as a result of being exposed to it (4). After young chicks have been trained by exposing them to a visually conspicuous object, there is an increase in the incorporation of [^{3}H]uracil into acid-insoluble substances in the dorsal part, or roof, of the cerebral hemispheres (5). This regional increase was not a side effect of training, because (*i*) when sensory input is restricted to one cerebral hemisphere by commissurotomy and monocular occlusion, incorporation is higher in the forebrain roof of the "trained" hemisphere than in that of the "untrained" hemisphere (6), (*ii*) the magnitude of incorporation is positively correlated with a measure of how much the chicks have learned (7), and (*iii*) the increase associated with training cannot be ascribed to short-lasting effects of sensory stimulation (8).

Autoradiography was used for more refined localization and implicated the intermediate and medial part of the hyperstriatum ventrale (IMHV) in imprinting (9, 10). Lesions to the IMHV impair the acquisition and retention of preferences for an imprinting stimulus (11–13). All the findings taken together strongly suggest that the IMHV is crucially involved in information storage in the learning process. This part of the hyperstriatum ventrale appears also to be involved in memory in at least one form, though not in all forms of associative learning (12, 14–16, *).

Is imprinting associated with a change of synaptic connectivity within the IMHV? Bradley *et al.* (17) demonstrated an increase in the mean length of the postsynaptic density (PSD) profiles of synapses in the left IMHV. The increase was restricted to PSD profiles of axospinous synapses. There was no significant effect of imprinting on the PSD length of synapses on dendritic shafts in the left IMHV, nor on axospinous or dendritic shaft synapses in the right IMHV (18). There is strong evidence that at least some axospinous synapses in the mammalian central nervous system are excitatory and possess receptors for L-glutamate (19, 20). Accordingly we inquired, in experiments 1 and 2, (*i*) whether imprinting is associated with changes in the binding of L-[^{3}H]glutamate to membranes from the IMHV, (*ii*) whether imprinting is associated with changes in the *N*-methyl-D-aspartate (*N*-Me-D-Asp)-sensitive component of L-[^{3}H]glutamate binding, and (*iii*) whether the left and right sides of the IMHV differ in this respect. Changes in *N*-Me-D-Asp-sensitive binding were found only in the left IMHV. In experiment 3, we investigated the link between the learning process and the magnitude of these changes.

MATERIALS AND METHODS

Rearing and Training. Ross I chicks of either sex were hatched and reared individually in darkness. In experiments 1 and 2, 15–30 hr after hatching the chicks were matched in pairs according to age. One chick in each pair remained in darkness and the other was trained (17) by being placed in a running wheel and exposed to a rotating red box, an effective imprinting stimulus (11, 12), for a total of 140 min. All chicks were then assigned codes and held individually in darkness for 7–8 hr before being decapitated. After coding, all procedures were performed without knowledge of the chick's experimental history. In experiment 3, chicks were trained as before and, immediately before killing, their preferences

Abbreviations: IMHV, intermediate and medial part of the hyperstriatum ventrale; PSD, postsynaptic density; *N*-Me-D-Asp, *N*-methyl-D-aspartate; AP3, 2-amino-3-phosphonopropionic acid; AP4, 2-amino-4-phosphonobutyric acid; AP5, 2-amino-5-phosphonopentanoic acid; AP6, 2-amino-6-phosphonohexanoic acid; AP7, 2-amino-7-phosphonoheptanoic acid; AP8, 2-amino-8-phosphonooctanoic acid; df, degrees of freedom.

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were measured in a test that involved exposing each chick individually in a running wheel to the training object or to a novel object, in a balanced order (12). The chick's approach to each object was measured and a preference score was calculated that expressed the chick's approach to the familiar object as a percentage of its total approach during the test. Chicks were then grouped into sets of three, and the mean preference score was calculated for each set.

Preparation of Membranes. A piece of brain tissue, comprising mainly the IMHV, either left or right, was dissected out and placed in a tube to which was added the corresponding region from the other two chicks in the set. Subsequent analyses were conducted without knowledge of the brain side from which the sample was removed. The tissue was homogenized in 1 ml of ice-cold 0.32 M sucrose with 10 strokes of a motor-driven Teflon-in-glass homogenizer. Samples were maintained at 0–4°C until the start of the binding assay. The homogenate was centrifuged at 1000 × *g* for 10 min, the pellet was discarded, and the supernatant was diluted with 2 ml of 0.32 M sucrose and centrifuged at 14,000 × *g* for 20 min. The resulting supernatant was discarded and the pellet was suspended by sonication in 1 ml of distilled water. The suspension was left to stand for 40 min and then centrifuged at 7000 × *g* for 20 min. The resulting pellet was washed gently with 1 ml of distilled water to remove the buffy layer and the washings were centrifuged at 40,000 × *g* for 30 min. The supernatant was removed and the pellet was suspended by sonication in 0.5 ml of distilled water. The suspension was centrifuged at 40,000 × g for 30 min, after which the supernatant was discarded and the pellet was suspended by sonication in 0.36 ml of 50 mM Tris acetate buffer (pH 7.0). L-Glutamate- and *N*-Me-D-Asp-sensitive binding (see below) was not significantly affected by further washing.

Binding Assay. Membranes were incubated in triplicate at 30°C in 50 mM Tris acetate buffer (pH 7.0) with L-[^{3}H]glutamic acid (Amersham). A parallel series of tubes contained in addition either L-glutamic acid or *N*-Me-D-Asp as displacer. L-Glutamate- and *N*-Me-D-Asp-sensitive binding was determined by subtracting from total binding the amount of radioligand bound in the presence of the appropriate displacer. Binding was characterized in preliminary experiments on dark-reared chicks. For experiments 1, 2, and 3 the concentration of L-[^{3}H]glutamic acid was 1 μM; further details were as follows. In experiment 1, the displacer was L-glutamic acid (1 mM) and the incubation was terminated after 18 min by vacuum filtration through a Millipore HAWP filter, mean pore diameter 0.45 μm. The membranes were washed immediately on the filter with 10 ml of ice-cold buffer, a process that took <8 sec. The material retained on the filter was digested with $NaDodSO_4$ and the radioactivity was determined by liquid scintillation spectrometry. In experiments 2 and 3, the displacer was *N*-Me-D-Asp (0.7 mM) and the incubation was terminated after 18 min by centrifugation at 20,000 × *g* for 4 min at 0–4°C. The supernatant was aspirated and the pellet was washed twice with 1 ml of ice-cold buffer, a process that took <8 sec. The pellet was then digested and subjected to scintillation counting as in experiment 1.

The protein concentration of each membrane suspension was measured (21) in duplicate. Mean protein concentration was 66 μg/ml. L-Glutamate-sensitive binding and *N*-Me-D-Asp-sensitive binding were linearly related to protein concentration and were not affected significantly by the method (filtration or centrifugation) of assay termination; the incubations were of sufficient duration for a steady state to be attained.

Statistical Analysis. Data from trained and dark-reared animals were compared by a matched-pairs *t* test. In experiment 2, a split-plot analysis of variance was performed, the factors being experimental treatment (trained/dark-reared) and side (left/right). In experiment 3, a correlation analysis was performed between *N*-Me-D-Asp-sensitive binding and preference score. A partial correlation analysis (22) was also performed on these two variables, holding constant the effect of approach activity during training. This procedure produced values (Fig. 3) for binding and preference score that were corrected for approach during training. If present, any correlation would independently be predicted to be positive (7); a one-tailed test of significance was therefore used in this instance. All other tests of significance were two-tailed. Except where stated otherwise, values of *n* denote the number of tissue samples, each sample comprising material from three IMHVs. All errors are standard errors of the mean (SEM).

RESULTS

Experiment 1. L-Glutamate-sensitive binding in membranes from pooled left and right IMHVs was saturable, with apparent K_d = 0.5 ± 0.09 μM (*n* = 4 experiments). The effect of training on L-glutamate-sensitive binding in the left IMHV is shown in Fig. 1. This measure was significantly (20 ± 6.7%) greater in trained chicks than in dark-reared chicks.

Characterization of *N*-Me-D-Asp-Sensitive Binding Sites. The IC_{50} for *N*-Me-D-Asp-sensitive binding was determined by incubating pooled left and right IMHV membranes with 1 μM L-[^{3}H]glutamic acid and a range of 10 concentrations of *N*-Me-D-Asp between 0 and 800 μM. The IC_{50}, determined by iteratively fitting a logistic function to the data (23), was 11.1 ± 1.8 μM (*n* = 3 experiments). Radioligand displace-

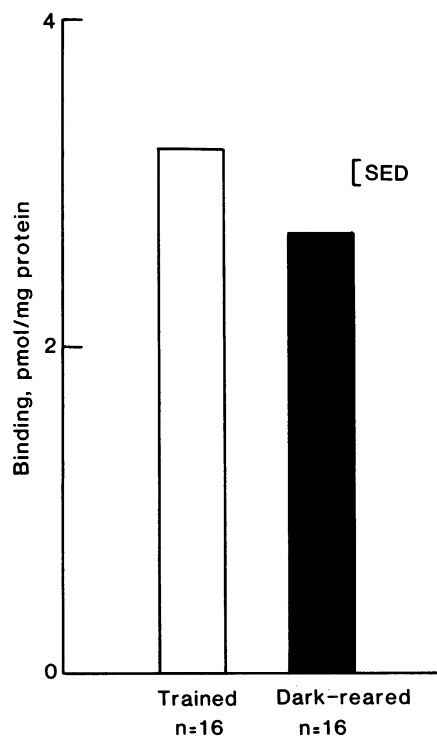

FIG. 1. Experiment 1. The effect of training on L-glutamate-sensitive binding in membranes from the left IMHV. Mean values are shown; SED = standard error of the difference between the two means; *n* = number of samples, each sample comprising material from three chicks. Samples were arranged in 16 pairs: each pair contained samples from chicks in the same batch, selected so that the post-hatch ages of the trained and dark-reared chicks were matched. Binding in the trained group was significantly higher than that in the dark-reared group [matched-pairs *t* = 3.27; 15 degrees of freedom (df); *P* < 0.01]. This L-glutamate-sensitive binding accounted for 61 ± 1.7% (*n* = 32) of total radioactivity.

ment by 800 μM N-Me-D-Asp was not significantly different from the displacement by 400 μM N-Me-D-Asp.

The sensitivity of L-[^{3}H]glutamate binding to a series of amino-, phosphono-substituted carboxylic acids is given in Table 1, part A. At a concentration of 10 μM, both D-2-amino-5-phosphonopentanoic acid (D-AP5) and DL-2-amino-7-phosphonoheptanoic acid (DL-AP7) showed significant displacement of bound L-[^{3}H]glutamate. There was no detectable displacement by the remaining members of the series, including the L isomer of AP5. The results in Table 1, part B, show that the quantity of radioactivity displaced by 0.7 mM N-Me-D-Asp was very similar to that displaced by 10 μM D-AP5 in the same experiment. If these two compounds bind to different sites, the amount displaced by a mixture of them would be expected to be greater than the amounts displaced separately. However, if the D-AP5 and N-Me-D-Asp separately occupied all the available sites, displacement by the mixture should not be greater than displacement by either compound alone. The mixture did not displace more than either compound alone (Table 1, part B), indicating that D-AP5 and N-Me-D-Asp were binding to the same sites.

By varying the concentration of L-[^{3}H]glutamic acid in the incubation medium, N-Me-D-Asp-sensitive binding in the left IMHV of dark-reared chicks was found to be saturable with apparent $K_d = 0.1 \pm 0.03$ μM and $B_{max} = 0.9 \pm 0.18$ pmol/mg of protein (n = 6 experiments). N-Me-D-Asp-sensitive binding was not affected by freezing and thawing, and accounted for 29 ± 3.2% (n = 32) of L-glutamate-sensitive binding in fresh tissue (see also Table 1, part B). Steady-state levels of binding had been attained after 5 min of incubation and this binding had decreased by >95% when measured 5 min after the concentration of nonradioactive L-glutamic acid was raised from 0 to 1 mM.

Experiment 2. N-Me-D-Asp-sensitive binding in the left IMHV was significantly (59 ± 18%) greater in trained chicks than in dark-reared chicks. For 44 of the 65 samples summarized in Fig. 2*A*, samples from the right IMHV were also available and N-Me-D-Asp-sensitive binding was measured in these samples. Half of the samples were taken from trained chicks and half from dark-reared chicks. An analysis of variance showed there to be a significant interaction between experimental treatment and side ($F_{1,41} = 5.82$; $P < 0.025$). That is, the two sides differed significantly with respect to the influence of training. As in the larger parent data set, N-Me-D-Asp-sensitive binding in the left IMHV samples from trained chicks was significantly greater than that in the left IMHV samples from dark-reared chicks. There was no such effect of training in the right IMHV (Fig. 2*B*).

Table 1. Sensitivity of L-[^{3}H]glutamate binding to amino-, phosphono-substituted carboxylic acids (10 μM) and N-Me-D-Asp (0.7 mM) in membranes pooled from right and left IMHV

Displacer	% displacement	Significance
	*Part A**	
DL-AP3	3.3 ± 3.7	NS
DL-AP4	6.9 ± 4.4	NS
D-AP5	28.5 ± 2.1	$P < 0.0001$
L-AP5	0.73 ± 6.8	NS
DL-AP6	0.09 ± 5.2	NS
DL-AP7	13.1 ± 5.2	$P < 0.05$
DL-AP8	6.4 ± 6.6	NS
	Part B†	
D-AP5	24.8 ± 2.1	$P < 0.0001$
N-Me-D-Asp	25.9 ± 2.5	$P < 0.0001$
D-AP5 + N-Me-D-Asp	23.4 ± 2.8	$P < 0.0001$

The concentration of L-[^{3}H]glutamic acid was 1 μM in all experiments. Values are counts (± SEM) displaced by the stated concentration of each compound, expressed as a percentage of counts displaced by 1 mM nonradioactive L-glutamic acid. AP*n* denotes the prefix 2-amino-*n*-phosphono-, and *n* denotes the length of the carbon chain thus: 3, propionate; 4, butyrate; 5, pentanoate; 6, hexanoate; 7, heptanoate; 8, octanoate. Also given is the level of significance when comparing the stated mean with zero; NS, not significant. See text for further explanation.

*Standard error of difference for comparing two means = 6.4; n = 10 experiments.

†Standard error of difference for comparing two means = 2.1; n = 5 experiments.

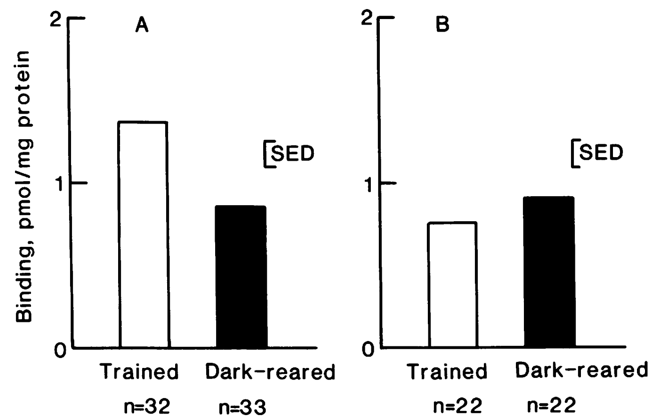

FIG. 2. Experiment 2. The effect of training on N-Me-D-Asp-sensitive binding of L-[^{3}H]glutamic acid to membranes from the IMHV. Mean values are shown; SED = standard error of the difference between the two means; n = number of samples, each sample comprising material from three chicks. Samples were matched in pairs as in Fig. 1. (*A*) Left IMHV; binding in the trained group was significantly higher than in the dark-reared group (matched-pairs $t = 3.46$; 30 df; $P < 0.005$); assays were completed on 65 of the original 68 samples. (*B*) Right IMHV; the samples were taken from chicks that also contributed samples summarized in *A*. There was no significant effect of training ($t = 0.91$). N-Me-D-Asp-sensitive binding in the corresponding left IMHV samples was significantly greater in trained chicks than in dark-reared chicks ($t = 2.58$; 41 df; $P < 0.025$).

Experiment 3. There was a significant positive correlation between preference score and N-Me-D-Asp-sensitive binding in the left IMHV ($r = 0.38$; 34 df; $P < 0.012$, one-tailed significance test). The corresponding correlation coefficient for N-Me-D-Asp-sensitive binding in the right IMHV was not significant ($r = 0.10$; 30 df).

Approach during training is weakly correlated with preference score (24). It is therefore possible that the observed correlation between N-Me-D-Asp-sensitive binding and preference score reflects a relation between binding and locomotor activity during training. We therefore corrected binding and preference score for training approach activity, using the method of partial correlation. The training approach activity was measured as the number of revolutions made by the running wheel as a chick attempted to approach the red box during training. There was a positive partial correlation between N-Me-D-Asp-sensitive binding in the left IMHV and preference score ($r_{xy \cdot z} = 0.41$; 34 df; $P < 0.01$, one-tailed test). The 36 values that contributed to this correlation were divided into three groups of equal size according to their ranked corrected preference scores. The mean corrected binding and preference scores together with the standard errors were calculated for each group of 12 values and are plotted in Fig. 3. The lowest mean corrected preference score (for group 1) is not significantly different from 50, and the corresponding mean binding (0.69 ± 0.15) for this group is not significantly different from that (0.87 ± 0.17) in the left IMHV of the dark-reared chicks of experiment 2 (see Fig. 3). The mean preference score of a comparable group (n = 12) of dark-reared chicks was 50.2 ± 5.7 (unpublished observation). The highest mean corrected preference score (for

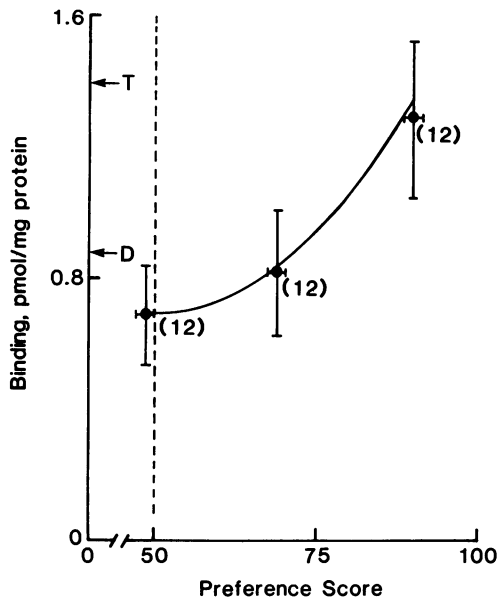

FIG. 3. Relation between N-Me-D-Asp-sensitive binding in the left IMHV and preference score, both corrected by linear regression to constant approach during training as a result of the partial correlation analysis. The higher the preference score, the greater the chick's preference for the training stimulus and the greater the strength of imprinting. Preference scores of 100 and 0 indicate that in the preference test all activity was directed to the imprinting and novel objects, respectively. The "no preference" score of 50 is indicated by the vertical broken line. Arrow D indicates the mean binding in the left IMHV of dark-reared chicks in experiment 2, and arrow T the mean binding in trained chicks in that experiment. Each point represents the mean of 12 values. The standard error bars for binding (vertical) and for preference score (horizontal) are given. The curve is a polynomial, fitted to all 36 points by least-squares regression. For further discussion, see text.

group 3) is significantly greater than 50 (t = 52.8; $P << 0.001$); the corresponding mean binding (1.29 ± 0.24) of this group is not significantly different from that (1.39 ± 0.17) in the left IMHV of the trained birds of experiment 2 (see Fig. 3). Finally, the values of mean corrected binding of groups 1 and 3 are significantly different from each other (t = 2.14; $P < 0.05$).

There was no significant partial correlation between N-Me-D-Asp-sensitive binding in the right IMHV and preference score ($r_{xy \cdot z}$ = 0.09; 29 df).

DISCUSSION

There is strong evidence that, under the incubation conditions used in this study (50 mM Tris acetate buffer without Na^+, Cl^-, and Ca^{2+}), the N-Me-D-Asp-sensitive binding sites correspond to physiological receptors (25–27). Consistent with this view is the finding of rapid association and dissociation of L-[^{3}H]glutamic acid binding at the N-Me-D-Asp-sensitive sites, as well as the pharmacological profile reported in Table 1 (see ref. 28). If the K_d of the N-Me-D-Asp-sensitive binding of L-glutamate is 0.1 μM, the binding would be expected to be virtually saturated at the 1 μM radioligand concentration used in experiments 1–3. This expectation is supported by the similarity between the N-Me-D-Asp-sensitive binding in the left IMHV of dark-reared chicks from Fig. 2 (0.87 ± 0.17 pmol/mg of protein) and the B_{max} for this class of binding sites in the left IMHV of dark-reared chicks (0.9 ± 0.18 pmol/mg). The increase in binding in the left IMHV is therefore likely to be due to an increase in the number of available binding sites. To be sure of this point it would have been necessary to incubate membranes from each sample with a range of ligand concentrations and thereby estimate K_d and B_{max} for each sample; there was insufficient tissue available to do this. Changes have been reported in L-[^{3}H]glutamate binding to hippocampal membranes following eyelid conditioning in rabbits (29). In that study, binding was measured in Tris·HCl buffer and is likely to have revealed binding sites different from those described here (25).

We have shown that imprinting is associated with an increase in N-Me-D-Asp-sensitive binding in the left but not the right IMHV. The effects of imprinting on N-Me-D-Asp-sensitive binding follow a similar pattern to the effects of imprinting on the PSDs of axospinous synapses in the left IMHV (18), suggesting that the increase in area of the PSDs may be associated with an increase in the number of N-Me-D-Asp-type receptors. The magnitude of these increases also appears to be similar. Thus Fagg and Matus (25), using the same incubation medium as us, found that the proportions of N-Me-DL-Asp- and non-N-Me-DL-Asp-sensitive binding to PSDs isolated from rat brains were 58% and 42%, respectively. The mean increase in area of spine PSDs in the left IMHV was 37% after 140 min of training (18). If the increased area led to a corresponding increase in L-glutamate binding sites, and both classes of receptors were equally affected, each would be expected to increase by 37%. If, however, only the N-Me-D-Asp-sensitive binding sites were affected, these would be expected to increase by 64%. The observed mean increase of N-Me-D-Asp-sensitive binding was 59%. Although this result does not exclude an effect of imprinting on non$N$-Me-D-Asp-type sites, particularly since the calculation is necessarily approximate, there is clearly evidence for a major effect on N-Me-D-Asp-type sites. This suggestion is supported by the similarity in the absolute magnitude of the left IMHV changes in binding in experiments 1 and 2 (Figs. 1 and 2*A*).

There are several reasons why the changes reported in the present study cannot simply be attributed to side effects of the training procedure. (*i*) The studies that led to the localization of IMHV and to the demonstration of a crucial role of this region, especially of the left IMHV (30), in information storage had controlled for these and other side effects of training (see ref. 31 for review). (*ii*) In the present study a general effect of arousal would be expected to affect both right and left IMHV. This was not found either in experiment 2 or in experiment 3. An effect of arousal would also be expected to be expressed in behavior: the more aroused the chicks, the more vigorously would they be expected to approach the red box during training. However, the partial correlation coefficient between N-Me-D-Asp-sensitive binding and preference score was significant when the effect of approach activity during training was held constant. This latter finding also demonstrates that differences in locomotor activity during training cannot account for the correlation between binding and corrected preference score. (*iii*) Light exposure *per se* does not account for the findings, since the corrected mean left IMHV binding in chicks that had been exposed to the red box for 140 min, but had not developed a preference for it, was closely similar to the mean left IMHV binding of the dark-reared chicks in experiment 2. This similarity suggests that this level of binding (see Fig. 3) is the floor level of unimprinted chicks, whether dark-reared or visually experienced. Considerations *ii* and *iii* indicate that N-Me-D-Asp-sensitive binding in the left IMHV is not influenced by arousal, light exposure, or locomotor activity *per se*. Instead, the results of experiments 2 and 3 suggest that binding increases as the chicks learn about the imprinting object and so form a preference for it. This selective preference is evidence that the chicks recognize the object. It is possible (32) that an increased number of excitatory receptors, by increasing the effectiveness of

neurotransmission, would functionally weight the synapses in the left IMHV and hence form a neural basis for this recognition memory.

The consequences of the increased number of *N*-Me-D-Asp-type receptors are likely to be subtle. (*a*) The effectiveness of synaptic transmission using *N*-Me-D-Asp-receptor channels depends on the membrane potential of the postsynaptic cell (33). As a result the ligand for these receptors is effective in opening the channels only under certain conditions. One possibility is that the channels are gated by the depolarizing action of neurons the activity of which is controlled by the attentional or affective state of the animal or by input from neuronal assemblies representing other memories. (*b*) The activation of *N*-Me-D-Asp receptors leads to an influx of Ca^{2+} ions (34). These ions could activate a Ca^{2+}/calmodulin-dependent kinase (35, 36), which may lead to (31) and maintain changes in the structure of the PSD and influence its interactions with proteins in the cytoskeleton of the dendritic spine (37). (*c*) The conductance of *N*-Me-D-Asp channels is considerably greater than that of non-*N*-Me-D-Asp channels, raising the possibility that the *N*-Me-D-Asp channels may exert a correspondingly greater influence on the postsynaptic cell (33, 38).

N-Me-D-Asp receptors have been implicated in experience-dependent changes in the kitten visual cortex (39, 40), in the initiation of hippocampal long-term potentiation (20, 41), and in the acquisition of place-learning in rats (42). Our results provide evidence that these receptors are implicated in the storage of information during learning.

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