Expression of Fos and Jun Proteins Following Passive Avoidance Training in the Day-Old Chick

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Abstract

It has been shown previously that the immediate-early genes, c-fos and c-jun mRNA are induced in the 1-day-old chick forebrain after one-trial passive avoidance training in which chicks learn to avoid pecking at a bitter-tasting bead. Here, we have studied the expression of their proteins using antibodies to Fos and Jun. Western blotting disclosed two immunoreactive bands for the anti-Fos antibody (47 and 54 kD) and two immunoreactive bands for the anti-Jun antibody (39 and 54 kD). Two hours post-training there was an increase in the number of Fos-positive stained nuclei in right intermediate medial hyperstriatum ventrale (IMHV) (P < 0.01), left IMHV (P < 0.05), right lobus parolfactorius (LPO) (P < 0.025) and left LPO (P < 0.05) of birds trained on the bitter bead compared with controls that had pecked a water-coated bead. Staining for Jun protein was significantly greater in the right LPO of trained chicks (P < 0.01). Other forebrain regions showed no increase over quiet control levels. The findings are discussed in the context of the cascade of events involved in passive avoidance memory consolidation in the day-old chick.

Introduction

Passive avoidance training in the day-old chick is an attractive model in which to study the cascade of molecular events that occur during long-term memory consolidation, because it takes only a single brief trial to form the memory thus allowing one to distinguish the concomitants of the training experience from those of consolidation. In the passive avoidance task, chicks are presented with a small bright bead coated in the bitter tasting methylantranilate (M). The birds peck spontaneously at the bead, evince a disgust response, and avoid similar but dry beads subsequently for at least 24 hr. Control birds, which peck at a water-coated bead on trial (W), continue to peck dry beads on test. Comparing M- with W-trained birds has revealed a molecular cascade of events in two brain regions, the intermediate medial hyperstriatum ventrale (IMHV) and lobus parolfactorius (LPO), in the hours following training, which beginning with synaptic transients, culminates in synaptic remodelling, achieved via the insertion of de novo synthesized cell adhesion molecules into pre- and postsynaptic membranes (for recent review, see Rose and Stewart 1999).

A general feature of long-term memory formation in all species and tasks in which it has been studied is the requirement for protein synthesis within 1–3 hr of the training experience. Anisomycin or cycloheximide, antibiotic protein synthesis inhibitors, injected around the time of training, block protein synthesis for an hour or so subsequently and are amnestic; memory is retained for a period of a few hours and then lost (Davis and Squire 1984). This early phase of memory-related protein synthesis includes the expression of immediate-early gene products (IEGP; Freeman and Rose 1995) that in turn activate or repress the transcription of late genes important in the synaptic restructuring thought to underlie long-term memory, although the specificity of this response has been questioned (Tischmeyer et al. 1990; Nikolaev et al. 1991; Demmer et al. 1993; Dragunow 1996).
the chick, induction of the IEGs c-fos and c-jun has been demonstrated after passive avoidance training (Anokhin and Rose 1990) and in a visual discrimination (pebble floor) task (Anokhin et al. 1991). In the latter, c-jun expression seems to be specifically related to acquiring the discrimination. Fos protein expression increases following exposure to an imprinting stimulus (McCabe and Horn 1994). Blockade of Fos synthesis by pretraining intracranial injections of antisense oligodeoxynucleotides for c-fos results in amnesia for the passive avoidance task (Mileusnic et al. 1996). Injection of anisomycin at or around the time of training is also amnestic in the chick (Freeman et al. 1995). However, we have found that although anisomycin injected just downstream of this early time window of sensitivity, 2–3 hr post-training in the chick, is not amnestic, in conformity with the earlier studies on the effects of protein synthesis blockade, injection 4 hr post-training is once again amnestic; chicks injected with anisomycin at this time show no recall when tested at 24 hr. We have interpreted this as interference with a “second wave” of protein synthesis, presumably of structural proteins including the cell adhesion molecules, switched on via the activation of IEGs in the first wave.

The measurement of IEGPs is a useful markers of metabolic activity in brain and spinal cord (Sagar et al. 1988). This has the advantage of being more sensitive than indirectly measuring the metabolic rate of neurones through the build-up of the anti-metabolite, 2-deoxyglucose, as it gives an indication of nuclear rather than dendritic activity (Dragunow and Faull 1989). Here, we describe the effects of training chicks on the passive avoidance task on the expression of Fos and Jun proteins in the left and right IMHV and LPO, detected immunocytochemically.

Materials and Methods

REAGENTS

The anti-Fos polyclonal antibody (gift from Prof. P.J. Sharp; AFRC, Roslin, Edinburgh) was raised against a 22-amino-acid synthetic peptide corresponding to the carboxyl terminus of chicken c-Fos. Anti-Jun, PC07, a monoclonal antibody, was from Oncogene Sciences. Anti-rabbit secondary antibody was from Sigma. The ECL protein detection kit was from Amersham International. All other chemicals were reagent grade.

ANIMALS AND TRAINING PROCEDURES

Ross 1 Chunky chicks (Gallus domesticus) of both sexes were hatched in a communal incubator on a 12-hr light/12-hr dark cycle at 38°–40°C. On the evening following hatching, chicks were placed in pairs into pens, each illuminated with a 25-W red light. The birds were then left to equilibrate overnight with food and water, allowing Fos and Jun proteins to fall to basal levels.

The following morning, chicks were divided into groups. One third of the animals (quiet controls; Q) were left undisturbed, in their pens, throughout the experiment. The remaining chicks were pretrained and trained as described by Lössner and Rose (1983). Pretraining consisted of three 10-sec presentations of a small (2.5-mm-diam.) white bead. Chicks that pecked at least twice out of the three pretraining trials (at least 80%) were then trained by a 10-sec presentation of a chrome bead (4 mm diam.) dipped in either MeA (M) or water (W). Chicks were tested for recall by presentation of a similar but dry chrome bead 2 hr post-training. The M and W chicks that met the criterion of avoiding the test bead if M trained (at least 80%) or pecking if W trained (at least 95%) together with Q chicks (n = 9 in each case) were taken for immunocytochemical analysis.

IMMUNOCYTOCHEMISTRY

Q, M, and W chicks were anesthetized with sagatol (phenobarbitone) and perfused through the heart with saline followed by Zambonin’s fixative [15% picric acid, 4% paraformaldehyde, 0.1 M phosphate buffered saline (PBS)]. The brains were then removed and left overnight in Zambonin’s fixative before washing in PBS and rapidly frozen in an isopentane/solid CO₂ bath. They were then mounted on a chuck for cryostating. Slices, 20 µm thick, were cut from areas of the brain that contained the LPO and IMHV and mounted on chrome–alum–gelatine coated slides. Figure 1 shows representative sections from these regions. Sections were collected from each area every 200 µm, resulting in three sections for each brain region being analyzed, allowing a mean density of positive stained nuclei per region per chick to be calculated. The slides were stored at −70°C for up to 1 month prior to immunocytochemistry.

The mounted tissue sections were left to warm up to room temperature and then washed for three 10-min sessions in PBS to remove excess Zambon-
in’s fixative. Quenching of endogenous peroxidase activity was achieved by incubating sections in 0.1 M PBS containing 1% hydrogen peroxide for 30 min. After washing with PBS, nonspecific binding was blocked by incubating sections for 30 min in PBS containing 10% normal goat serum (NGS). The slides were then left overnight at room temperature in a solution of anti-Fos (or anti-Jun) antibody [1:9000; (vol/vol) in 1% NGS/PBS]. The following day, after stringent washes with PBS, the slices were incubated overnight at 4°C with a peroxidase-labeled anti-rabbit secondary antibody at 1:100 (vol/vol) in 1% NGS/PBS. All secondary antibody was removed by washing twice for 20 min in PBS and 20 min in 50 mM Tris-buffered saline (TBS). The peroxidase-labeled secondary antibody was probed for by a short 5-min incubation with 0.03% H₂O₂/TBS followed by an additional incubation with the same solution containing 0.01% diaminobenzidine (DAB). The reaction was terminated with copious quantities of TBS. Coverslips were placed over fixed sections, and blue/black staining of DAB was visualized using a light microscope. Histological identification of regions was performed by staining the adjacent section with cresyl violet and the aid of a chick brain atlas (Youngren and Phillips 1978).

**ANALYSIS OF DATA**

Sections containing IMHV also contained hippocampus (Hp); those containing LPO also contained hyperstriatum accessorium (HA), and immunopositive nuclei in all four regions were counted. (Fig. 1). For each region and tissue section, three areas were selected at random and the number of immunoreactive (stained) nuclei counted. Three tissue sections containing each region per bird
were processed giving a total of nine estimates of IEGP-positive nuclei per region for each bird from which the mean number (density) of immunoreactive nuclei was calculated for each region. Each such mean was then combined with that from the same region of all birds within the treatment group, giving an $n$ of 9, from which statistical comparisons were made between M and Q or M and W birds using the Mann-Whitney two-tailed $U$ test.

WESTERN BLOTTING

Sample preparation, electrophoresis, and immunoblotting were as described by Freeman and Rose (1995). Briefly, 50 µg of crude nuclear protein from IMHV or LPO was separated by 5%–15% SDS-PAGE. Blots were poststained with Coomassie blue to determine even loading and transfer. The contents of each gel were then transferred to nitrocellulose as described by Burnette (1981). Membranes were blocked overnight using a modification of BLOTTO [Johnson et al. 1984; 150 mM NaCl, 100 mM phosphate, 10% (wt/vol) marvel nonfat milk powder]. The nitrocellulose membrane was incubated for 2 hr with anti-Fos antibody at 1:1000 dilution in BLOTTO. After stringent washing in 0.1 M PBS/0.05% Triton, the membrane was incubated with HRP conjugated to a goat anti-rabbit IgG (whole molecule) at 1:500 dilution in BLOTTO. Immunoreactive bands were visualized using the ECL system according to the manufacturer’s protocol. The membrane was then stripped of all antibodies (according to the protocol) and reprobed under the same conditions using the anti-Jun antibody at 1:1000 dilution in BLOTTO for 2 hr. A representative autoradiograph is shown in Figure 2.

Results

Preliminary experiments (data not shown) revealed an increase in titer detected by Western blotting of both Jun and Fos proteins in all four brain regions in both M and W birds compared with Q controls 1–3 hr post-training; we therefore chose a post-training time of 2 hr for the immunocytochemistry. At this time after M training, there was an increase in the number of Fos-positive stained nuclei in right IMHV ($P < 0.01$), left IMHV ($P < 0.05$), right LPO ($P < 0.025$) and left LPO ($P < 0.05$) compared with these regions in W birds. The density of Fos-positive staining in the W trained birds was not significantly different from that in untrained Q birds (Fig. 3). Immunopositive staining for Jun protein was only significantly greater in M compared with W chicks in the right LPO ($P < 0.01$). In both IMHVs, M training resulted in a significant increase in the number of Jun-labeled neurones above Q levels but not above that in W chicks. There was no increase in the density of Jun-positive nuclei in the left LPO after either training experience. The number of nuclei immunopositive for Fos and Jun were counted in the HA and Hp for the slice containing the LPO and IMHV, respectively. Neither the HA or Hp showed an increase due to either training experience compared with Q levels.

Discussion

As Figure 1 shows, and we have reported previously (Freeman and Rose 1995), the polyclonal raised against c-Fos and the monoclonal against c-Jun each recognized two bands. The 47-kD band recognized by the anti-Fos antibody is assumed to correspond to c-Fos, and the 39-kD band recognized by the anti-Jun antibody to c-Jun. The second band at 54 kD was common to both antibodies and, we believe, represents one of the family of activation protein-1 (AP-1) binding proteins. These assumptions were based on the fact that both the antibodies were raised against either Fos or Jun proteins and throughly characterized. Previously, we have shown that both of the bands immunoreactive to Fos or Jun demonstrated a memory-related increase; inhibition of the NMDA-receptor not only abolished the induction of Fos and Jun, it also prevented memory formation (Freeman and Rose, 1995).
demonstrated that c-fos and c-jun mRNA was induced after training in the chick forebrain. However, other studies using different ages of animals and species suggest that other members of the Fos and Jun families are more abundant in forebrain tissue (Mellström et al. 1991). Training on either the methylantranilate or the water-coated bead induced the expression of Fos-p47, Jun-p39, and Fos/Jun-p54 in both LPOs and IMHVs 1–3 hr post-training as determined by increases in optical density revealed by Western blotting (data not shown).

Using the induction of Fos and Jun as a measure of brain region activity, there was a M-training-related recruitment of neurones in the IMHV and LPO, but not the Hp or HA, at 2 hr post-training (Fig. 3). Fos staining was significantly higher in M-trained birds than either W-trained or Q controls in all brain regions. In contrast, Jun expression was increased both by M and W training in both left and right IMHV; there were no changes in left LPO, but an increase in M- over W-trained birds in right LPO.

These results raise three issues: the different pattern of Jun versus Fos expression, the fact that some Jun expression is observed in W-trained birds, and the relationship between the roles of IMHV and LPO. These differences cannot be explained by differences in the basal levels of Fos or Jun expression (high basal levels of Jun masking any further expression), because in the Quiet controls the density of Jun and Fos labeled nuclei were comparable. M training increased the density of Fos and Jun labeled nuclei to approximately the same degree in the left IMHV (Fig. 3A). However, this is not the case for the right IMHV or either LPO where, although no statistical comparisons were made, there appears to be more Fos labeled nuclei than Jun (Fig. 3B-D). Differences between the right and left IMHV and the right and left LPO in physiology and morphology as a consequence of learning are well established in the chick (Patterson et al. 1990; Nicol et al. 1995; Rose and Stewart 1999), and difference in Jun and Fos staining could be a reflection of this. That is, we may be looking at the involvement of at least two different populations of neurones each expressing different species of transcription factor that contain Fos and/or Jun proteins, where M or W training may cause differential expression and hence the activation of different neuronal pathways that do not share the exact same molecular or biochemical cascades. This could certainly be true for the IMHV where Anokhin et al. (1991) found that c-jun expression seemed to be specifically related to acquiring the discrimination of an appettive learning task, thus explaining why W training causes an increase in immunolabeling of Jun producing neurones in the IMHV, an area associated with visual discrimination tasks involving food (Andrew 1991).

There are two distinct waves of protein synthesis in the chick after passive avoidance training: the first wave lasting up to 90 min (Patterson et al. 1986) and the second between 4 hr and 5 hr post-training (Freeman et al. 1995). There is a period, 2–3 hr after training, that is not dependent on protein synthesis. This raises the question as to why Fos and Jun protein levels should be elevated at this 2-hr time point when there was no susceptibility to protein synthesis inhibition. A simple explanation could be that we are observing a persistent elevation of these proteins that were produced during the first phase of protein synthesis. The c-fos studies seem to suggest that this is likely because their mRNAs are elevated 30 min after training (Anokhin et al. 1991). However, it does bear noting that levels due to training for Fos proteins were elevated in the left IMHV (Fig. 3) but not its mRNA, which appears to be depressed (Anokhin et al. 1991). Suggesting that an initial suppression of c-fos expression in the left IMHV is required followed by its induction. These results show that both the IMHV and LPO are involved in the first wave of protein synthesis. Fos and Jun form transcription factors that control the expression of other genes (Angle and Karin 1991). Whether these transcription factors are regulating the expression of late genes during the first wave is unknown. The c-fos antisense study seems to indicate that Fos levels had not yet reached their peak; amnesia is only evident with pretraining injections and testing 3 hr after training (Mileusnic et al. 1996). However, because they failed to test at a time point in-between 30 min and 3 hr, it is difficult to determine more accurately the exact time of memory decay due to antisense c-fos. Interestingly, they found that only pretraining injections of antisense c-fos caused amnesia, which lead them to the assumption that Fos expression was only important in the induction of the second wave of protein synthesis. The time in-between the two waves of protein synthesis could reflect inhibition of late gene expression by these transcription factors.
Down-regulation of gene expression appears to be an important factor in passive avoidance memory formation; *c-fos* in the left IMHV is a prime example (Anokhin et al. 1991). Whether Fos or Jun causes the expression of late genes during this second phase of protein synthesis is also not known, but they do seem to be important in the induction of it for both IMHV and LPO.

Following training on the task, a molecular and cellular cascade occurs in the left IMHV. The cas-

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**Figure 3:** Density of Fos- and Jun-positive nuclei in the left IMHV (A), right IMHV (B), left LPO (C), right LPO (D), left HA (E), right HA (F), left HP (G), and right HP (H). Error bars represent s.e.m. n = 9. Statistical comparisons were made between M and W or M and Q. (***) P < 0.01; (**) P < 0.025; (*) P < 0.05.
cade, reviewed by Rose and Stewart (1999), begins with synaptic transients including enhanced glutamate release (Daisley et al. 1998), up-regulation of NMDA receptor activity, and increased calcium flux into synaptoneurosomes (E.J. Salinska, D. Chaudhury, R.C. Bourne, and S.P.R. Rose, in prep.). It is this cascade that we assume results in the intracellular signaling through which IEGs are switched on. The left IMHV remains a site of physiological and biochemical activity for at least several hours post-training. Thus, electrophysiological studies have shown an increase in high-frequency neuronal bursting in the left IMHV 3 hr post-training (Gigg et al. 1993). Furthermore, pretraining lesions of the left IMHV have implicated this area in the acquisition of long-term memory (Patterson et al. 1990). However, in a series of double dissociation studies Patterson and Rose (1992) were able to conclude that following training, there was a sequential involvement of right IMHV and both left and right LPO. Thus, bilateral lesions of the LPO post- but not pretraining are also amnestic, whereas post-training lesions of the left IMHV are not (Gilbert et al. 1991). As soon as 1 hr after training there are transient morphological changes in the right IMHV; synapse volume decreases, although there is a significant increase in synaptic density; however, by 24 hr these changes have disappeared (Doubell and Stewart 1993). Training-induced high-frequency neuronal firing also switches from left to right IMHV (Gigg et al. 1993). A reason for the transient changes could be connected to the role of the right IMHV as a temporary or buffer store for memory, as occurs in filial imprinting of chicks (Horn 1991). In any event, early 2-deoxyglucose studies (Kossut and Rose 1984) and, more recently, both the lesion (Gilbert et al. 1991) and the electrophysiological data (Gigg et al. 1994) suggest that within a relatively short time after the training experience, the LPO, which is connected disynaptically to the IMHV by way of the archistriatum (Csillag et al. 1994, 1997), becomes engaged. Many of the more lasting changes in synaptic morphology observed following training are localized bilaterally to the LPO (Stewart 1991; Rose and Stewart 1999) and presumably must require the full mobilization of protein synthetic machinery.

From lesion studies it appears that the right IMHV is required for recall and that the mechanism for this is not evident until 4 hr after training (Gilbert et al. 1991), and whether this involves de novo protein synthesis is a possibility given that Fos and Jun levels are elevated in this area. As for the LPO, because most events appear to occur much later after training, it is highly likely that too is a site manufacturing proteins during the second of synthesis. Because memory for passive avoidance training appears to involve different spatial and temporal components, it is probable that the array of late genes whose expression is controlled by certain transcription factors such as those containing Fos and Jun proteins is different for individual areas and/or neurones, and the training-induced increase in IEG expression is fully in accord with this data.

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