ELSEVIER

Behavioural Brain Research 114 (2000) 39–49

BEHAVIOURAL BRAIN RESEARCH

www.elsevier.com/locate/bbr

Research report

Expression of immediate early genes in the hippocampal formation of the black-capped chickadee (*Poecile atricapillus*) during a food-hoarding task

Tom V. Smulders *, Timothy J. DeVoogd

Department of Psychology, Uris Hall, Cornell University, Ithaca, NY 14853, USA

Received 15 December 1999; received in revised form 8 March 2000; accepted 8 March 2000

Abstract

Black-capped chickadees store food in many different locations in their home range and are able to accurately remember these locations. We measured the number of cells immunopositive for three different Immediate Early Gene products (Fra-1, c-Fos and ZENK) to map neuronal activity in the chickadee Hippocampal Formation (HF) during food storing and retrieval. Fra-1-like immunoreactivity is downregulated in the dorsal HF of both storing and retrieving chickadees compared to controls. In retrieving birds, the number of Fos-like immunoreactive neurons relates to the number of items remembered, while the number of ZENK-like immunoreactive neurons in the HF may be related to the accuracy of cache retrieval. These results imply that the brain might process complex information by recruiting more neurons into the network of active neurons. Thus, our results could help explain why food-hoarding birds have more HF neurons than non-hoarders, and why this number increases in autumn when large numbers of food items are cached.

Keywords: Food-storing birds; Spatial memory; Immediate early genes; Fra-1; Fos; Zenk; Memory retrieval; Immunocytochemistry

Scatter-hoarding birds cache food-items in many locations [57]. Their memory for these locations [7,8,14,59] involves the Hippocampal Formation (HF) [39,60,61,65], a homologue to the mammalian hippocampal and parahippocampal regions [4,17,19,33,36–38,55]. The avian HF is larger in food-hoarding birds than in non-hoarders [39,61] and lesioning this structure impairs the use of spatial memory in birds [23,40,60]. The HF of black-capped chickadees (*Poecile atricapillus*) is larger in the fall (during the peak in food hoarding) than at other times of the year, including winter [67]. This is due to a net addition of newly generated neurons in the fall [2], resulting in an increased cell number [63].

Why is the HF larger in the fall, but not in winter? During the fall, birds spend most of their time hoarding seeds, but only retrieving seeds sporadically. During winter, on the other hand, birds spend more time retrieving seeds, while they hoard much less frequently than during the fall [10,20–22,27,49]. It is possible that the HF is involved only in storing memories for cache locations, but not in retrieving them. This would explain the seasonal pattern in HF volume and neurogenesis [2,67].

In this study, we investigate whether the HF is involved in storing and/or retrieving the information about cache locations by measuring the expression of immediate early genes (IEGs). IEGs are genes whose expression is induced rapidly and whose gene products are typically transcription factors (reviewed in Ref. [26,54]). In some systems, this cascade has been linked to the permanent remodeling of the neuron and its synapses involved in encoding longer-term memory (reviewed in Ref. [18,54,69]). Very often, however, the

* Corresponding author. Present address: Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA. Tel.: +1-336-716-8691; fax: +1-336-716-8501.

E-mail address: tsmulder@wfubmc.edu (T.V. Smulders).

0166-4328/00/$ - see front matter
PII: S0166-4328(00)00189-3

molecular consequences of the expression of IEGs are not known, and they are used as markers of any areas that are particularly active during a certain behavior [26,51,56]. The expression of IEGs has recently been used successfully in songbirds to elucidate which areas of the brain are activated during song production and song perception [5,29,30,32,34,44–47] and in chicks in the context of imprinting [1,42]. In this study, we use antibodies to specific gene-products from two well-specified IEG families, the Fos family and the Krox family. Members of the two families are likely to have different functions and to be expressed differently in response to behavioral stimulation.

1. Materials and methods

1.1. Subjects

Eighteen black-capped chickadees were caught around Ithaca, NY in December 1995 under federal and NY State permits. They were fed a mix of ground beef, raisins, carrot baby food, hard-boiled eggs, wheat germ, and turkey pellets. They were housed in $45.7 \times 21.9 \times 25.4$ cm^3 ($w \times d \times h$) wire cages on a 9 h/15 h (l/d) light schedule.

1.2. Experimental set-up

The birds were carried individually, in their home cage, from the housing room to the experimental room. They entered the $4.5 \times 4 \times 2.5$ m^3 ($w \times d \times h$) room through a sliding door from their home cage. The room contained three dead trees, which had holes drilled into their trunks and branches at approximate intervals of 20 cm. Underneath each hole was a wooden perch. The holes could be closed off with a knotted piece of string that was attached above the hole. The three trees contained a total of 51 holes.

The observer sat behind a one-way window and recorded the behavior using The Observer© Version 2.0 (Noldus) on an IBM XT computer.

1.3. Behavior

The birds were acclimated to the experimental room by letting each one fly around in it for 20 min each day for 6 days. The birds were food deprived 21 h before being run in the experiment. They were assigned to groups of three, on the basis of similarities in outer rectrix appearance and tarsometatarsus length (indicators respectively of age and possibly of sex [62]). One bird of each trio was randomly assigned to each of the following three behavioral treatments:

1.3.1. Storing

The birds entered into the experimental room, where husked sunflower seeds were available. They were left in the room until they had stored a minimum of five seeds or 75 min had elapsed. If a bird did not store at all during that period, it was rerun on a later date. Birds that stored fewer than five, but more than one seed, were used in the experiment. Birds were usually not interrupted during a hoarding bout, but taken out of the room after the bout was over. This resulted in more than five seeds being hoarded by several birds.

1.3.2. Retrieving

Birds were allowed to store seeds as in the first group. They then had access to ad-lib food for ≈ 2 h before being food-deprived again. Twenty-four hours after the storage phase, birds were returned to the experimental room and allowed to retrieve their caches. At this time, all the holes were covered and pieces of sunflower seed were available in the holes in which the bird had stored the previous day. No other food was available in the room. The birds were left in the room until at least 60% of the seeds had been retrieved or 90 min had elapsed.

1.3.3. Control

The birds were put in the experimental room just like the birds in the Storing condition, but had only unstorable sunflower seed flour available to them. Each was left in the room for the same amount of time as the Storing bird with which it had been matched up.

After being taken out of the room, the birds in each of the three conditions were given one whole sunflower seed in their home cage (to give all of them exposure to handling and eating whole seeds) and then left in the dark for 75 min, after which they were perfused. Because the birds determined the onset of hoarding and retrieving themselves, and because different birds will take different amounts of time to store similar amounts of seeds, we decided to perfuse them all at equal times after the end of the exposure. We chose 75 min, since the c-Fos protein typically is present at maximal levels between 1 and 2 h after the stimulus that induces it [48].

1.4. Perfusion and tissue preparation

The birds were injected with an overdose of Chloropent (Fort Dodge Labs). They were then perfused transcardially with 0.9% NaCl ($+0.1\%$ $NaNO_2$) in 0.1M NaPB ($pH = 7.3$), followed by 4% paraformaldehyde ($+0.1\%$ $NaNO_2$) in 0.1 M NaPB. The brain was post-fixed in the paraformaldehyde for at least 1 h (up to 18 h for some bad perfusions ($< \frac{1}{4}$ of all birds)) and then stored in sodium phosphate-buffered saline. Brains were cryoprotected by immersing them in 20% sucrose in NaPB until they sank to the

bottom of the jar. They were then cut frozen on a sliding microtome into 40 μm sections, which were collected in 0.02 M KPBS (pH = 7.3) with 0.0025% Sodium Azide. These sections were then stored at 4°C until they were stained. Every 6th section was stained with the Fos antibody, and another set of every 6th section was stained with the ZENK antibody (see Section 1.4.1). The brains of each matched set of three birds (one of each behavioral treatment) were run through the procedure together, to control for any variability among the different runs of the protocol.

1.4.1. Fos immunocytochemistry

Sections were washed in KPBS with Bovine Serum Albumin and then incubated in 3% normal horse serum for 2 h. Sections were then moved to primary antibody (Fos AB-1 mouse monoclonal, Oncogene Science, diluted 1/2000) in KPBS + 0.1% triton-X for 66 h at 4°C. This primary antibody has been used in birds before [42] and is known to cross react with other gene products in the Fos family, such as Fra-1 and Fra-2 [31]. After three washes, the tissue was then transferred to secondary antibody (horse anti-mouse, Vector Laboratories, 1/500) in KPBS + 0.1% triton-X for 3 h at room temperature. After another three washes the tissue was transferred to ABC solution (Vector Elite kit, 1/200) for 2 h at room temperature. After the last three washes, the tissue was incubated in a mix of 2.5% Nickel Ammonium Sulfate, 0.2% glucose, 0.04% Ammonium Chloride, 0.025% DAB in 0.1M NaAc buffer (pH = 6.0), with 40 units of glucose oxidase for 30–45 min. The tissue was mounted on gelatin-covered slides, cleared with Histoclear® and coverslipped with Eukitt®.

1.4.2. ZENK immunocytochemistry

The ZENK protein is a member of the Krox family. Its name is an acronym of the gene's other four names: Zif-286, EGR-1, NGFI-A and Krox-24 [50]. The protocol was similar to the one for Fos with the following exceptions. We used the polyclonal rabbit anti-EGR-1 (Santa Cruz) at a 1/10 000 dilution and a goat anti-rabbit secondary (Vector Laboratories). The primary antibody has been well characterized for use in birds [47]. The final staining step was done in a solution containing 0.05% DAB, 0.025% CoCl and 0.02% NiCl in 0.1M NaPB, to which 0.0015% H_2O_2 was added. This staining method provides stain in 1–2 min. Again the tissue was mounted on gelatin-covered slides, cleared with Histoclear® and coverslipped with Eukitt®.

1.5. Volume analysis and cell counts

In addition to the expected nuclear stain in the tissue stained with the anti-Fos antibody, we also found many cells with cytoplasmic label in the HF. We believe that this stain represents a cross-reactivity of the antibody with other members of the Fos family. The primary antibody we used has been shown to cross-react with Fra-1 and Fra-2 [31]. In rats, these proteins are expressed in the cytoplasm of neurons, rather than in the nucleus, and their expression in adults is specific to the hippocampus [3,52]. In our tissue as well, cytoplasmic stain is limited to the HF, with the possible exception of a few individual neurons in other brain areas. Therefore, we will refer to the cytoplasmically stained neurons as Fra-1-like immunoreactivity and treat them as a separate category of neurons in our analyses. The cells that showed nuclear stain with the anti-Fos antibody will be referred to as Fos-like, and those stained with the anti-ZENK primary (all of which showed nuclear stain) ZENK-like.

The volume of the Hippocampal Formation (HF) was estimated by measuring the surface area of the HF on each section stained for ZENK, multiplying this by 0.24 mm (the distance between every 6th 40 μm section) and adding up the resulting volumes. Surface areas were measured using NIH Image 1.61 on an Apple Macintosh IIci computer, connected to a high-resolution black and white video camera (COHU Solid State Camera). Using our estimate of the total volume, we divided the HF into a rostral and a caudal part, each representing $\approx 50\%$ of the total HF volume. In addition, we also drew an imaginary horizontal line tangent to the top of the ventricle, dividing the HF into a dorsal and a ventral part (Figs. 2 and 3). Cells stained for either immediate early gene were counted separately in the resulting four subdivisions (dorsal-rostral, ventral-rostral, dorsal-caudal and ventral-caudal).

Stained cells were counted semi-automatically. Using a 10X objective, images of regions of interest were digitized with NIH Image 1.61. In pilot work, we determined that a staining intensity threshold set at 70% of the difference between background staining and maximal nuclear staining ($0.7*(M-B)+B$; M = maximum stain in that section, B = mean background stain density in the area under investigation) would reliably discriminate between labeled and unlabeled cells. Labeled cells were counted this way throughout the entire HF (every cell in every section) for all three categories. All counts were done blind to treatment. Final counts for each subdivision were divided by the volume in which they had been counted to obtain an estimate of the density of Fos-like, Fra-1-like and ZENK-like immunoreactivity in each subdivision. All analyses were performed on this density measure. Our staining procedure did not allow us to use stereological methods. Our densities should therefore be considered as relative measures that are comparable within our study, but not as absolute numbers.

1.6. Statistical analysis

In order to quantify the retrieval performance of the birds in the Retrieving group, we calculated a Retrieval Quotient (RQ). This quotient is calculated as follows:

$$RQ = \frac{r(n) - c(n)}{n - c(n)}$$

with n = the total number of seeds stored by that bird on the previous day, $r(n)$ = the number of seeds actually retrieved after n looks and $c(n)$ = the number of seeds expected to be recovered by pure chance after n looks. To calculate $c(n)$, we simply divided the total number of seeds stored by the total number of possible storage sites, and multiplied this number by n looks (Fig. 1). For perfect performance, $RQ = 1$, for chance performance, $RQ = 0$.

Data were analyzed using Systat© 5.2.1 on an Apple Macintosh Centris 610 computer. Analyses were performed using a repeated measures General Linear Model. This analysis is the general statistical model that includes both ANOVA and Multiple Regression as special cases. It allows the simultaneous testing of within- as well as between-subject variables, both categorical and continuous, as independent variables. In our analyses, we used three categorical within-subject variables (dorsal versus ventral subdivision of HF, rostral versus caudal subdivision of HF and Immediate Early Gene identity), while the between-subject variables were either categorical (treatment assignment) or continuous (time in experiment, number of seeds stored, …). The analysis also tests for possible interactions between all the within-subject variables and the between-subject variable(s). The dependent measure in every case was the density of stained neurons. In the analyses in which we investigated effects of behavioral variables on the expression of the IEGs, we log-transformed the IEG densities, in order to detect log-linear relationships (as suggested by preliminary plotting of the data). Findings are considered significant when $P < 0.05$.

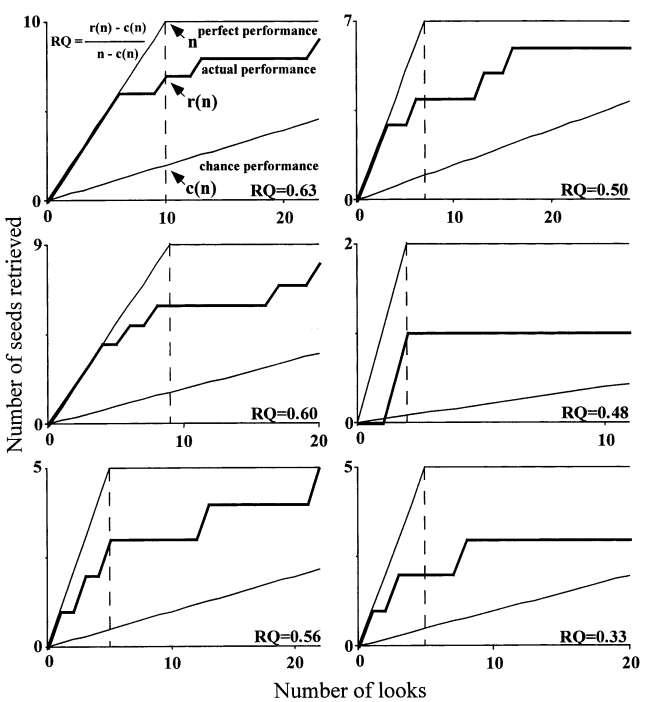

Fig. 1. Behavioral performance of the six birds in the Retrieval group. Calculation of the Retrieval Quotient (RQ) is explained in the first panel. n = the number of seeds stored during the previous day, $r(n)$ = the number of seeds retrieved after n looks, $c(n)$ = number of seeds expected to be retrieved by pure chance after n looks.

2. Results

2.1. Behavior

Birds in the Storing group stored a median of six seeds (range: 3–21). The birds in the Retrieval group stored a median of six seeds (range: 2–10) and retrieved a median of five (range 1–9) of those. The mean retrieval performance was well above chance levels of discovery ($RQ = 0.52 \pm 0.1$ (mean $\pm$ SD), ranging from 0.33–0.63; Fig. 1).

2.2. Distribution of the different IEGs

The three different IEGs had similar densities of labeled cells across the entire HF ($F(2,34) = 0.679$, NS; $3 \times 2 \times 2$ repeated measures ANOVA with IEG identity (IEG), dorsal versus ventral subdivision (DV) and rostral versus caudal (RC) subdivision as within-subject variables). All IEGs showed a higher density of labeled cells in the dorsal HF than in the ventral HF ($F(1,17) = 35.956$, $P < 0.0001$; same analysis). The more detailed distribution of labeled cells across the four different sub-parts of the HF was different for different IEGs, however (same analysis, significant interactions: IEG by DV: $F(2,34) = 9.072$, $P = 0.0007$; IEG by RC: $F(2,34) = 14.388$, $P < 0.0001$; IEG by DV by RC: $F(2,34) = 11.523$, $P = 0.0002$). Because of these differences, we decided to analyze the detailed distribution of each IEG separately.

ZENK-like immunoreactive cells (Fig. 2A,B) could be found all across the forebrain. In the HF, the stained cells were mainly found along the dorsal and medial edges, as well as on the lateral edge (Fig. 2C). Densities were generally lower away from the edges of the HF. More ZENK-like immunoreactive cells are found in the dorsal than in the ventral part ($F(1,17) = 25.903$, $P = 0.0001$; 2×2 repeated measures ANOVA with DV and RC subdivisions as within-subject factors), and more are found in the rostral than in the caudal half of the HF ($F(1,17) = 12.077$, $P = 0.0029$). The dorsal-rostral

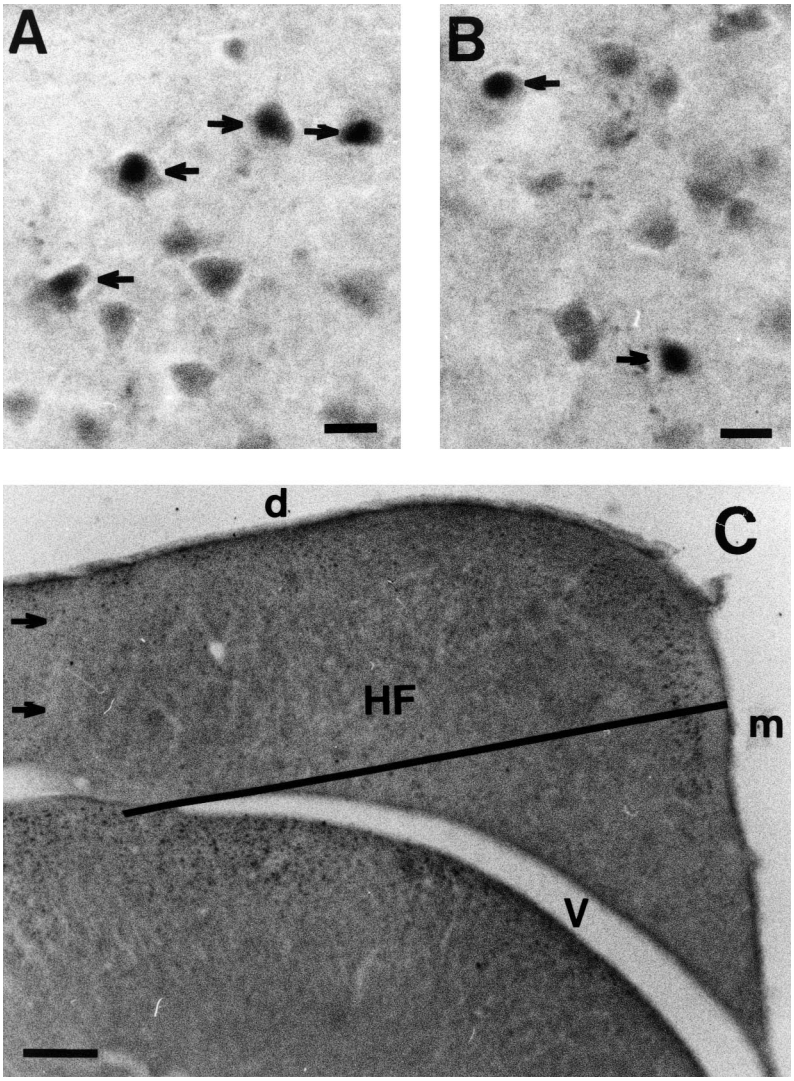

Fig. 2. ZENK-like immunoreactive nuclei in HF (A,B) and distribution of ZENK-like immunoreactive cells in the left HF (C). Notice the large number of ZENK-like immunoreactive cells near the medial (m) and dorsal (d) edges. (V: ventricle). Arrows indicate the lateral boundary of the HF and the horizontal line represents the division between dorsal and ventral HF. (scale bar = 10 μm (A,B) and 200 μm (C)).

part of the HF in particular has the highest density of ZENK-like immunoreactivity, more than expected from just the combination of the two main effects (interaction: DV by RC: $F(1,17) = 15.137$, $P = 0.0012$).

Fos-like immunoreactive cells (Fig. 3A) were distributed mostly towards the lateral boundary of the HF, as well as a few along the ventricle (Fig. 3C). There were more Fos-like cells in the dorsal than in the ventral HF ($F(1,17) = 21.038$, $P = 0.0003$; 2×2 repeated measures ANOVA with DV and RC subdivisions as within-subject factors), but there were no significant differences in density between rostral and caudal HF, nor was there an interaction between DV and RC.

Fra-1-like immunoreactive cells (Fig. 3B) tended to be distributed near the dorsal and medial boundaries of the HF (Fig. 3C). Again, there were more Fra-1-like cells in the dorsal than in the ventral part of the HF ($F(1,17) = 15.303$, $P = 0.0011$; 2×2 repeated measures ANOVA with DV and RC subdivisions as within-subject factors), but there were no differences between rostral and caudal, and no interactions between DV and RC.

2.3. Patterns across treatments

There was a significant effect of treatment on the density of Fra-1-like immunoreactive cells in the HF ($F(2,15) = 3.738$, $P = 0.0482$). This effect was carried completely by the dorsal HF ($2 \times 2 \times 3$ ANOVA with two within-subjects repeated measures (DV and RC) and one between-subjects variable (behavioral treatment), interaction between treatment and DV: $F(2,15) = 4.994$, $P = 0.0218$; Fig. 4). There were more Fra-1-like immunoreactive cells in the dorsal HF of control birds than in the dorsal HF of either the storing

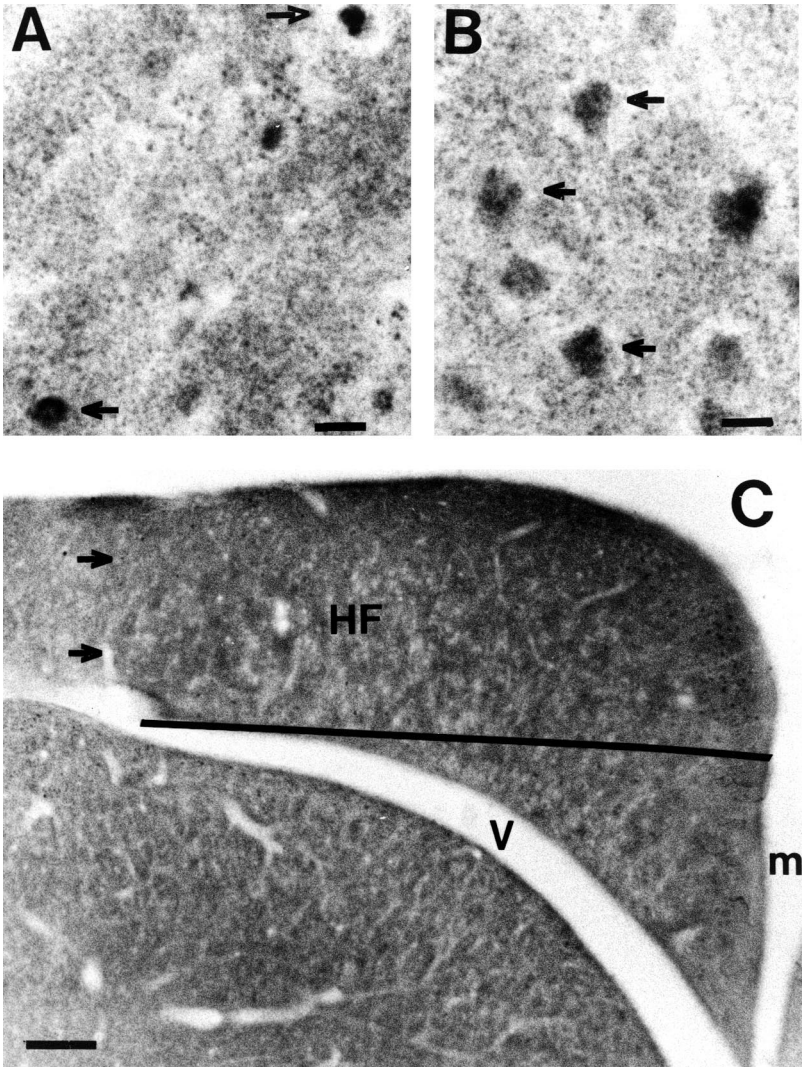

Fig. 3. Close-ups of Fos-like immunoreactive nuclei near the lateral edge of HF (A) and Fra-1-like immunoreactive cell bodies near the medial edge of HF (B). A few examples are indicated by arrows. Distribution of Fos-like and Fra-1-like immunoreactive cells in the left HF (C). Notice the large number of Fra-1-like stained cells near the medial (m) edge. (V: ventricle) Arrows indicate the lateral boundary of the HF and the horizontal line represents the division between dorsal and ventral HF. (scale bar = 10 μm (A,B) and 200 μm (C)).

or retrieving groups ($F(2,15) = 8.544$, $P = 0.0033$), but no differences in the ventral HF ($F(2,15) = 0.937$, NS). There were no significant differences in the density of either Fos-like or ZENK-like immunoreactive cells in the HF among the three treatment groups, nor was there any interaction between treatment and subdivision within the HF. There are no differences in total HF volume among treatments.

2.4. Patterns within treatments

Because there were obvious individual differences among birds in the number of cells expressing the three different staining patterns, we investigated if these individual differences correlated with any relevant behavioral variables in the three treatment groups. In all these analyses, we used the dorsal versus ventral (DV)

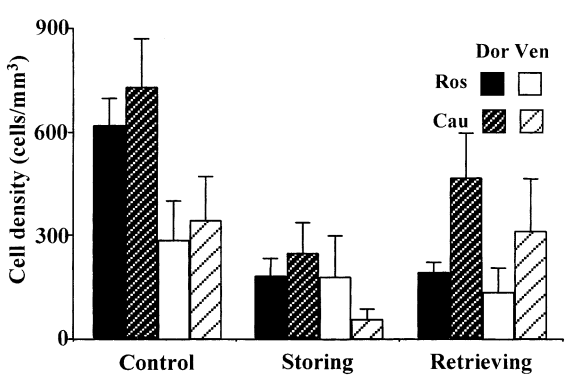

Fig. 4. Densities of Fra-1-like stained cells in the four subdivisions of the HF for the three treatment groups. Densities in the dorsal HF are significantly higher in the control group than in either of the other two groups. Dark bars are dorsal regions, and striped bars are caudal regions. Error bars represent the standard error of the mean.

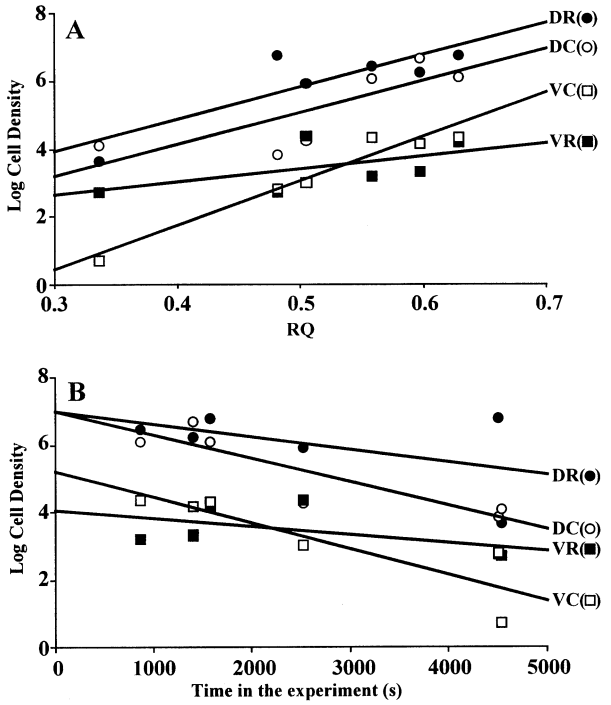

Fig. 5. Positive relationship of the density of ZENK-like immunoreactive cells, split up among the four HF subdivisions, with the Retrieval Quotient (RQ) (A), and negative relationship with the amount of time spent in the experimental room (B). See Section 2 for details on the statistical analysis. (D = dorsal, V = ventral, R = rostral, C = caudal).

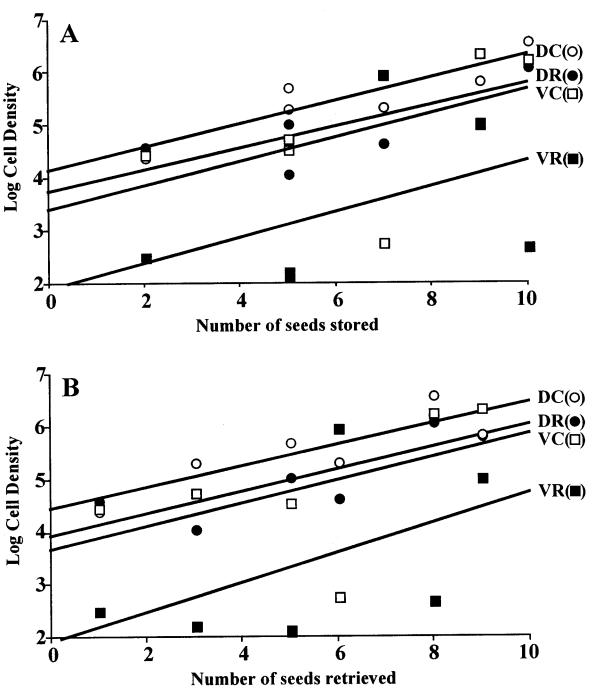

Fig. 6. Positive relationship of the density of Fos-like immunoreactive cells with the number of seeds stored the previous day (A) and the number of seeds actually retrieved (B). See Section 2 for details on the statistical analysis. (D = dorsal, V = ventral, R = rostral, C = caudal).

and the rostral versus caudal (RC) subdivisions of the HF as within-subject variables, while using the behavioral variable under investigation as a between-subject variable. A significant interaction between the between-subjects variable and one of the within-subject variables would indicate that the effect of the between-subjects variable is different for different subdivisions of the HF.

In the Control group, we only had one behavioral variable to test: total time in the experiment. There was a significant effect of this variable on the Fra-1-like immunoreactivity ($F(1,4) = 14.219$, $P = 0.0196$). However, one bird stayed in the experiment 4–10 times longer than any of the other birds, and without this outlier the effect was not significant ($F(1,3) = 3.585$, NS). In the Storing group, we tested four variables: time in the experiment, time since the start of hoarding, total number of seeds cached, and hoarding intensity (measured as the median inter-store interval). No significant effects were found on any of the three IEG expression patterns.

In the Retrieving group, we tested six variables: total time in the experiment, number of seeds stored the previous day, number of seeds (re)stored during the retrieval session, number of seeds retrieved, number of sites investigated and the Retrieval Quotient (RQ). ZENK-like immunoreactivity was significantly influenced by the total time in the experiment ($F(1,4) = 13.996$, $P = 0.0201$) and by RQ ($F(1,4) = 247.4234$, $P = 0.0001$) (Fig. 5). There were no significant interactions between these variables and subdivision within the HF. Because of the design of the experiment, animals that did not perform well at recovery were left in the experiment longer, such that time in the experiment and RQ were negatively correlated ($r = -0.833$, $P = 0.0393$). Because most birds started retrieving soon after entering the experimental room, total time in the room also correlates with the time from when the bird retrieves its first seed to the end of the session ($r = 0.992$, $P = 0.0001$). This latter variable also has a significant effect on ZENK-like immunoreactivity ($F(1,4) = 24.654$, $P = 0.0077$). None of the other variables had a significant effect on ZENK-like expression.

There was a significant effect of the number of seeds stored the previous day ($F(1,4) = 20.746$, $P = 0.0104$) and the number of seeds retrieved ($F(1,4) = 38.857$, $P = 0.0034$) on the Fos-like immunoreactivity in the HF (Fig. 6). Again, there were no significant interactions with location within the HF. Number of seeds retrieved naturally correlates positively with the number of seeds stored the previous day ($r = 0.955$, $P = 0.0030$). No other variables correlated significantly with Fos-like expression, including the number of locations visited, which is a measure of foraging activity. None of the variables examined correlated significantly with Fra-1-like immunoreactivity.

3. Discussion

3.1. Patterns of IEG expression across treatments

Fra-1-like immunoreactivity was down regulated in the dorsal HF of both storing and retrieving birds, compared to the control group. Since the function of Fra-1 in the cell is not yet clear, this result is not easily interpreted. This IEG product is present in the cytoplasm of hippocampal neurons, but not in the nucleus [52]. Thus, it is unlikely that it plays a role as a transcription regulator. It does, however, bind to members of the Jun family of IEGs. It is possible that Fra-1 sequesters Jun-like proteins in the cytoplasm. A downregulation of the cytoplasmic Fra-1 could then result in a release of other proteins, which in turn could influence transcription. Alternatively, the Fra-1-Jun complex might play an as yet unknown role in the cytoplasm itself [52], in which case downregulation in the perikaryon could be a consequence of relocation of the protein complex to the extremities of the neuronal processes. Regardless of the exact meaning of a downregulation of the protein, our results show that IEG expression is altered in the HF both during the storing and the retrieval phase of the hoarding behavior. We can therefore conclude that the HF is somehow involved in both phases of this natural memory process.

The downregulation of Fra-1-like immunoreactivity was limited to the dorsal HF. This is of interest in regard to identifying functional subdivisions in the avian HF. Our dorsal subdivision in which Fra-1-like expression is observed corresponds roughly to the Dorsomedial part of the HF as described by Szèkely [68]. According to her tract-tracing studies, this region projects mainly to other parts of the HF. Not enough is known to date about this region to make any comparisons to the mammalian hippocampus meaningful. There is no reason to assume that dorsal HF in birds has any relationship to dorsal hippocampus in mammals.

3.2. Patterns in the retrieving treatment

In the Retrieving birds, the number of ZENK-like immunoreactive cells correlated negatively with the time the bird spent in the environment and positively with their retrieval performance. Because of the co-linearity between these two variables, these results can be interpreted in two different ways. First, perhaps many cells express ZENK-like immunoreactivity initially, and then over time fewer and fewer cells retain levels high enough to be counted. This could happen if the half-life of the ZENK protein is short compared to other IEGs. The ZENK protein in birds returns to baseline levels between 2 and 4 h after induction [47], while ZENK-expression as a consequence of a behavioral task in mice returned to baseline after ≈ 2 h [6]. This time course is short enough to explain the observed expression patterns in our results.

Alternatively, the pattern in ZENK-like immunoreactivity is due to differences in retrieval performance. A high score on the Retrieval Quotient (RQ) indicates that a bird retrieved most of its seeds close to the beginning of its search/retrieval bout. The fact that our birds were severely food-restricted before the experiment suggests that differences in motivation are unlikely to underlie the observed differences in performance. Therefore, we consider RQ to be an index of memory accuracy. Birds with more ZENK-like immunoreactive cells in their HF remembered the locations of their caches more accurately. Because the two variables (accuracy and time spent in the environment) are interrelated, it is impossible to say which one is the primary correlate of the individual differences in ZENK-like expression. If time since the start of retrieval is the primary correlate, then that implies that there was an initial general upregulation of ZENK protein at the onset of retrieval. If retrieval accuracy was the primary correlate, then animals that retrieve more accurately experience ZENK upregulation in larger numbers of neurons to begin with. Either way, retrieval of caches is accompanied by an upregulation of the ZENK protein in HF neurons.

The number of Fos-like immunoreactive cells correlates significantly with the number of seeds stored the previous day, and the number retrieved during the session. Since these two numbers are closely correlated, it is again difficult to say which is the primary correlate of the observed effect. The pattern in Fos-like immunoreactive cells is not due to differences in foraging activity, nor in the number of seeds eaten: one of the hoarding birds ate many more seeds than any other bird, but Fos-like expression in its HF was about median. Interestingly, the best behavioral correlate with the number of Fos-like immunoreactive cells is the absolute number of seeds remembered, rather than the accuracy with which they are retrieved. This suggests that remembering the locations of more items is accompanied by Fos-expression in (and presumably activation of) more HF cells.

It is somewhat surprising that we can detect such subtle within-group variability in the retrieval group, while we cannot find differences between the number of either Fos-like or ZENK-like immunoreactive cells in the different treatment groups. This suggests to us that the HF is also being activated in the other two conditions (control and storing), but that we have not been able to identify the relevant behavioral variables that trigger the expression of either IEG. A control group of animals that were just kept in their normal housing conditions without disturbance would be useful in testing this hypothesis.

3.3. Implications for the role of the Hippocampal Formation in food hoarding

These findings could have important implications for the evolution of the HF in food-hoarding birds. If more hippocampal neurons are activated when a bird remembers more caches (Fos-like results) and remembers them more accurately (one possible interpretation of the ZENK-like results), then birds that have to process the locations of a few thousand items with increased accuracy could possibly need more neurons (a larger neural network) than animals that do not have this requirement. Food-hoarding birds, which have more neurons in their HF than do non-hoarding species [13,24,25,39,61], have also been shown to have a more accurate spatial memory performance than non-hoarders [43]. Memory capacity is more difficult to test, and has not been compared between the two groups of birds.

Could our results also shed light on why we find more neurons in the HF during the autumn peak of hoarding than at other times of the year [63]? Both the present study and Clayton and Krebs [15] present results implying that the HF is involved during the encoding of memories for cache sites, but we found no quantitative relationship between the number of items stored and the number of neurons activated. We also showed that hippocampal neurons are activated during the retrieval of spatial memories, and more of them express IEGs when (more accurately) remembering more cache locations. However, most cache retrieval takes place after the hoarding peak, and neuronal number in the HF has returned to baseline by this time [63]. It is therefore unlikely that the animals in winter actually recall the many thousands of caches made in the fall. Parids have been shown to remember cached items only up to 4-6 weeks after caching [10,28] and both theoretical [12,64] and empirical [11,41,53] work suggests that they use different strategies for retrieving hoards over these longer time spans.

Then why are there more neurons in the HF in October? We hypothesize that this increase in cell number is related to a different need for accurate spatial memory during the hoarding peak itself: the efficient management and distribution of caches. In order to lose as few caches as possible to thieves, the chickadees should distribute their caches as uniformly as possible and at a specific, optimal density [58]. This can only be done by redistributing previously stored items [9,16,35] and/or by remembering where previous caches are located, in order to guide the placement of new ones [64,66]. Both processes require a very accurate memory for the locations of many caches. The results of the present study suggest that this memory may require the presence and activation of a large number of HF neurons, which are there during the fall, but not during winter [63].

4. Conclusion

The three IEGs under study each show a different pattern of expression in the HF of birds performing a food-hoarding task. Fra-1-like immunoreactivity in the dorsal HF is downregulated when the birds are storing as well as when they are retrieving. Fos-like immunoreactivity is upregulated when recalling more items. ZENK-like immunoreactivity is also upregulated during the retrieval process. The possible recruitment of more neurons into the active circuitry as more locations are remembered (more accurately) could provide insights into how the brain processes complex information. Thus it may explain why food-hoarding birds, which have a need for processing more spatial information more accurately, have more hippocampal neurons than non-hoarders, and why this neuronal number varies seasonally.

Acknowledgements

The authors would like to thank Brian McCabe and David Clayton for sharing immunocytochemistry protocols with us. We also thank Sarah W. Newman for her invaluable advice on Fos immunocytochemistry, and Joyce Reed for helping out with bird care whenever needed. Sarah W. Newman, Tora K. Smulders-Srinivasan and four anonymous reviewers supplied invaluable comments on earlier drafts of this manuscript. This work was part of TVS's doctoral dissertation, and was supported by MH56093 to TJD.

References

[1] Anokhin KV, Mileusnic R, Shamakina IY, Rose SPR. Effects of early experience on c-fos gene expression in the chick forebrain. Brain Res 1991;544:101–7.
[2] Barnea A, Nottebohm F. Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees. Proc Natl Acad Sci USA 1994;91:11217–21.
[3] Beer J, Mielke K, Zipp M, Zimmermann M, Herdegen T. Expression of c-jun, junB, c-fos, fra-1 and fra-2 mRNA in the rat brain following seizure activity and axotomy. Brain Res 1998;794:255–66.
[4] Bingman VP, Bagnoli P, Ioale P, Casini G. Behavioral and anatomical studies of the avian hippocampus. In: Chan-Palay V, Köhler C, editors. The Hippocampus. New Vistas, vol. 52. New York: Alan R. Liss, 1989:379–94.
[5] Bolhuis JJ, Zijlstra GG, den Boer-Visser AM, Van Der Zee EA. Localized neuronal activation in the zebra finch brain is related to the strength of song learning. Proc Natl Acad Sci USA 2000;97:2282–5.
[6] Brennan PA, Hancock D, Keverne EB. The expression of the immediate-early genes c-fos, egr-1 and c-jun in the accessory olfactory bulb during the formation of an olfactory memory in mice. Neuroscience 1992;49:277–84.
[7] Brodbeck DR. Memory for spatial and local cues: A comparison of a storing and a nonstoring species. Anim Learn Behav 1994;22:119–33.

[8] Brodbeck DR, Burack OR, Shettleworth SJ. One-trial associative memory in black-capped chickadees. J Exp Psych: Anim Behav Proc 1992;18:12–21.
[9] Brodin A. Cache dispersion affects retrieval time in hoarding Willow Tits. Ornis Scand 1992;23:7–12.
[10] Brodin A. The disappearance of caches that have been stored by naturally foraging willow tits. Anim Behav 1994;47:730–2.
[11] Brodin A. Separation of caches between individual willow tits hoarding under natural conditions. Anim Behav 1994;47:1031–5.
[12] Brodin A, Clark CW. Long-term hoarding in the *Paridae*: a dynamic model. Behav Ecol 1997;8:178–85.
[13] Clayton N, Krebs J. The brain and behavioural development of food-storing in birds: marsh tits as a model. Montreal: 3rd International Neuroethology Conference, 1992.
[14] Clayton NS, Krebs JR. Memory for spatial and object-specific cues in food-storing and non-storing birds. J Comp Physiol A 1994;174:371–9.
[15] Clayton NS, Krebs JR. Lateralization in memory and the avian hippocampus in food-storing birds. In: Alleva E, Fasolo A, Lipp H-P, Nadel L, Ricceri L, editors. Behavioural Brain Research in Naturalistic and Semi-Naturalistic Settings, vol. 82. Dordrecht: Kluwer Academic, 1995:139–57.
[16] Cowie RJ, Krebs JR, Sherry DF. Food storing by marsh tits. Anim Behav 1981;29:1252–9.
[17] Craigie EH. The hippocampal and parahippocampal cortex of the emu (*Dromiceius*). J Comp Neurol 1935;61:563–91.
[18] Dragunow M. A role for immediate-early transcription factors in learning and memory. Behav Genet 1996;26:293–9.
[19] Erichsen JT, Bingman VP, Krebs JR. The distribution of neuropeptides in the dorsomedial telencephalon of the pigeon (*Columba livia*): a basis for regional subdivisions. J Comp Neurol 1991;314:478–92.
[20] Haftorn S. Contribution to the food biology of tits especially about storing of surplus food. Part I. The crested tit (*Parus c. cristatus* L.). K Norske Vidensk Selsk Skr 1954;1953:1–123.
[21] Haftorn S. Contribution to the food biology of tits especially about storing of surplus food. Part III: The willow tit (*Parus atricapillus*). K Norske Vidensk Selsk Skr 1956;1956:1–79.
[22] Haftorn S. Contribution to the food biology of tits especially about storing of surplus food. Part IV: a comparative analysis of *Parus atricapillus* L., *P. cristatus* and *P. ater* L. K Norske Vidensk Selsk Skr 1956;1956:1–54.
[23] Hampton RR, Shettleworth SJ. Hippocampal lesions impair memory for location but not color in passerine birds. Behav Neurosci 1996;110:831–5.
[24] Healy SD, Clayton NS, Krebs JR. Development of hippocampal specialisation in two species of tit (*Parus* spp). Behav Brain Res 1994;61:23–8.
[25] Healy SD, Krebs KR. Development of hippocampal specialisation in a food-storing bird. Behav Brain Res 1993;53:127–31.
[26] Herrera DG, Robertson HA. Activation of *c-fos* in the brain. Prog Neurobiol 1996;50:83–107.
[27] Higuchi H. Stored nuts *Castanopsis cuspidata* as a food resource of nestling varied tits *Parus varius*. Tori 1977;26:9–12.
[28] Hitchcock CL, Sherry DF. Long-term memory for cache sites in the black-capped chickadee. Anim Behav 1990;40:701–12.
[29] Jarvis ED, Nottebohm F. Motor-driven gene expression. Proc Natl Acad Sci USA 1997;94:4097–102.
[30] Jarvis ED, Scharff C, Grossman MR, Ramos JA, Nottebohm F. For whom the bird sings — context-dependent gene expression. Neuron 1998;21:775–88.
[31] Jensen D. Defective induction of Jun and Fos-related proteins in phorbol ester-resistant EL4 mouse thymoma cells. Oncogene 1991;6:1219–25.
[32] Jin H, Clayton DF. Localized changes in immediate-early gene regulation during sensory and motor learning in zebra finches. Neuron 1997;19:1049–59.
[33] Källén B. Embryogenesis of brain nuclei in the chick telencephalon. Ergeb Anat Entwicklungsgesch 1962;36:62–82.
[34] Kimpo RR, Doupe AJ. FOS is induced by singing in distinct neuronal populations in a motor network. Neuron 1997;18:315–25.
[35] Kraft M. Bemerkenswertes Nahrungsdepot einer markierten Sumpfmeise *Parus palustris*. Anz orn Ges Bayern 1990;2:170–1.
[36] Krayniak PF, Siegel A. Efferent connections of the hippocampus and adjacent regions in the pigeon. Brain Behav Evol 1978;15:372–88.
[37] Krayniak PF, Siegel A. Efferent connections of the septal area in the pigeon. Brain Behav Evol 1978;15:389–404.
[38] Krebs JR, Erichsen JT, Bingman VP. The distribution of neurotransmitters and neurotransmitter-related enzymes in the dorsomedial telencephalon of the pigeon (*Columba livia*). J Comp Neurol 1991;314:467–77.
[39] Krebs JR, Sherry DF, Healy SD, Perry VH, Vaccarino AL. Hippocampal specialization of food-storing birds. Proc Natl Acad Sci USA 1989;86:1388–92.
[40] Krushinskaya NL. Some complex forms of feeding behaviour of nut-cracker *Nucifraga caryocatactes*, after removal of old cortex. Zh Evol Biokhim Fisiol 1966;11:563–8.
[41] Lens L, Adriaensen F, Dhondt AA. Age-related hoarding strategies in the crested tit Parus cristatus: should the cost of subordination be re-assessed? J Anim Ecol 1994;63:749–55.
[42] McCabe BJ, Horn G. Learning-related changes in Fos-like immunoreactivity in the chick forebrain after imprinting. Proc Natl Acad Sci USA 1994;91:11417–21.
[43] McGregor A, Healy SD. Spatial accuracy in food-storing and nonstoring birds. Anim Behav 1999;58:727–34.
[44] Mello C, Nottebohm F, Clayton D. Repeated exposure to one song leads to a rapid and persistent decline in an immediate early gene's response to that song in zebra finch telencephalon. J Neurosci 1995;15:6919–25.
[45] Mello CV, Clayton DF. Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. J Neurosci 1994;14:6652–66.
[46] Mello CV, Clayton DF. Differential induction of the ZENK gene in the avian forebrain and song control circuit after metrazole-induced depolarization. J Neurobiol 1995;26:145–61.
[47] Mello CV, Ribeiro S. ZENK protein regulation by song in the brain of songbirds. J Comp Neurol 1998;393:426–38.
[48] Morgan JI, Curran T. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. Annu Rev Neurosci 1991;14:421–51.
[49] Nakamura H, Wako Y. Food storing behavior of willow tit *Parus montanus*. J Yamashina Inst Ornithol 1988;20:21–36.
[50] Nastiuk KL, Mello CV, George JM, Clayton DF. Immediate-early gene responses in the avian song control system: cloning and expression analysis of the canary *c-jun* c-DNA. Mol Brain Res 1994;27:299–309.
[51] Newman SW, Parfitt DB, Kollack-Walker S. Mating-induced c-fos expression patterns complement and supplement observations after lesions in the male Syrian hamster brain. Ann N Y Acad Sci 1997;807:239–59.
[52] Pozas E, Aguado F, Ferrer I. Fra-1 immunoreactivity in the rat brain during normal postnatal development and after injury in adulthood. Neurosci Res 1999;33:137–45.
[53] Pravosudov VV. Individual differences in foraging and storing behaviour in Siberian Tit *Parus cinctus* Bodd. and Willow Tit *Parus montanus* Bald. Sov J Ecol 1986;4:60–4.
[54] Robertson HA. Immediate-early genes, neuronal plasticity, and memory. Biochem Cell Biol 1992;70:729–37.
[55] Rose M. Über die cytoarchitektonische Gliederung des Vorderhirns der Vögel. J Psychol Neurol 1914;21:278–352.
[56] Rouiller EM, Wan XST, Moret V, Liang F. Mapping of c-*fos* expression elicited by pure tones stimulation in the auditory

pathways of the rat, with emphasis on the cochlear nucleus. Neurosci Lett 1992;144:19–24.
[57] Sherry DF. Food storing in the Paridae. Wilson Bull 1989;101:289–304.
[58] Sherry DF, Avery M, Stevens A. The spacing of stored food by marsh tits. Z Tierpsychol 1982;58:153–62.
[59] Sherry DF, Krebs JR, Cowie RJ. Memory for the location of stored food in marsh tits. Anim Behav 1981;29:1260–6.
[60] Sherry DF, Vaccarino AL. Hippocampus and memory for food caches in Black-Capped Chickadees. Behav Neurosci 1989;103:308–18.
[61] Sherry DF, Vaccarino AL, Buckenham K, Herz RS. The hippocampal complex of food-storing birds. Brain Behav Evol 1989;34:308–17.
[62] Smith S. The Black-Capped Chickadee: Behavioral Ecology and Natural History. Ithaca, NY: Cornell University Press, 1991:362.
[63] Smulders TV. Food Hoarding, Spatial Memory and the Avian Hippocampal Formation: the Neuroethology of a Nearctic Song Bird, the Black-Capped Chickadee (*Parus atricapillus*). Cornell University: Doctoral Dissertation, 1998.
[64] Smulders TV. A game theoretical model of the evolution of food hoarding: Applications to the Paridae. Am Nat 1998;151:356–66.
[65] Smulders TV, DeVoogd TJ. The avian hippocampal formation and the memory for hoarded food: Spatial learning out in the real world. In: Bolhuis JJ, editor. Brain Mechanisms of Perception, Learning and Memory: Essays for Gabriel Horn. Oxford: Oxford University Press, in press.
[66] Smulders TV, Dhondt AA. How much memory do tits need? Trends Ecol Evol 1997;12:417–8.
[67] Smulders TV, Sasson AD, Devoogd TJ. Seasonal variation in hippocampal volume in a food-storing bird, the black-capped chickadee. J Neurobiol 1995;27:15–25.
[68] Szekely AD. The avian hippocampal formation: subdivisions and connectivity. Behav Brain Res 1999;98:219–25.
[69] Tischmeyer W, Grimm R. Activation of immediate early genes and memory formation. Cell Mol Life Sci 1999;55:564–74.