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Co-expression of Fos immunoreactivity in protein kinase (PKC γ)-positive neurones: quantitative analysis of a brain region involved in learning

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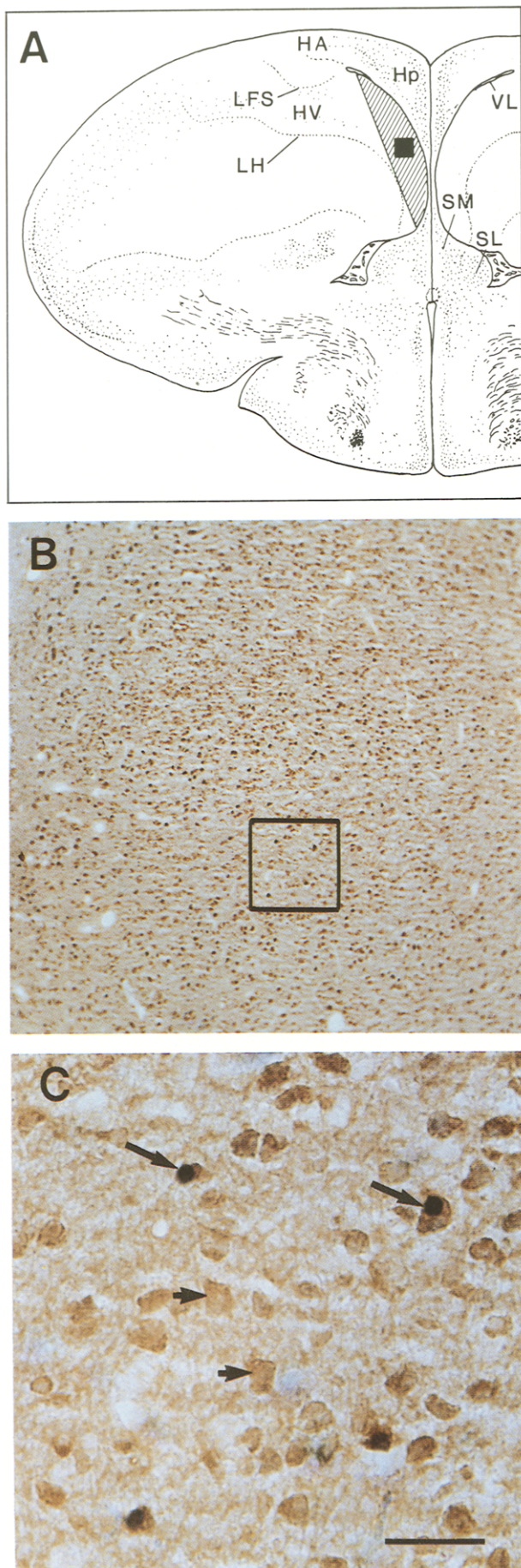
Key words: Protein kinase C; Protein kinase C γ ; *c-fos*; Fos protein; Chick; Intermediate and medial hyperstriatum ventrale; Imprinting; Learning

The expression of the gamma protein kinase C isoenzyme (PKC γ) and of the *c-fos* immediate early gene protein product Fos in the intermediate and medial hyperstriatum ventrale (IMHV) of day-old chicks was determined immunocytochemically. Previous research has shown that (a) there is a learning-related increase in the expression of Fos in the IMHV of the chick after imprinting; (b) PKC γ is expressed in neurones in most regions of the chick forebrain, including the IMHV. In the present study it was found that in imprinted chicks, 96.5% of neurones in the IMHV that expressed Fos also stained positively for PKC γ . These results raise the possibility of a functional connection between PKC γ activation and *c-fos* expression in neurones in general, and in particular in neurones in the IMHV that are involved in learning.

Filial imprinting is the process through which the young domestic chick (*Gallus gallus domesticus*) learns to recognise characteristics of an object to which it is exposed. When subsequently given a choice between this object and other objects, the chick selectively approaches the familiar one (see ref. 3 for a recent review). A series of studies has demonstrated that a restricted region of the chick forebrain, the left intermediate and medial hyperstriatum ventrale (IMHV) is crucially involved in the learning process of imprinting and is a likely site of information storage^{8,10}. McCabe and Horn¹⁶ demonstrated that imprinting leads to elevated immunoreactivity for the Fos protein in the IMHV. Fos is the product of the immediate early gene *c-fos*, the expression of which has been used as a marker for neuronal activation^{7,19}. There was a significant positive correlation between preference scores (a measure of the strength of learning) and Fos expression in cell nuclei in the IMHV¹⁶, but not in a number of control regions in the chick forebrain (McCabe and Horn, in preparation). Increased expression of the

c-fos gene in the IMHV has also been reported after passive avoidance learning in the chick¹.

Protein kinase C (PKC) is a key enzyme for signal transduction, and can be activated by receptor-stimulated turnover of phosphoinositides^{2,17,18}. PKC is implicated in the regulation of neuronal function^{17,18} and in the synaptic plasticity that underlies hippocampal long-term potentiation (LTP) in the rat^{11,13}. Van der Zee et al.²³ demonstrated an increase in immunoreactivity of the PKC γ isoenzyme in the mouse hippocampus after spatial learning. This study employed the monoclonal antibody 36G9 which recognises the gamma subspecies of PKC^{5,23}. Recently, the distribution of PKC α , β and γ isoenzymes in the chick forebrain was examined, with emphasis on the IMHV^{4,24}. In the IMHV, PKC γ was present in a subset of neuronal cell bodies, whilst PKC α , β immunoreactivity appeared to be present in a restricted band of afferent fibres. In the present study, by means of double-labelling, we investigated whether the expression of Fos in the IMHV of imprinted chicks is in neurones containing PKC γ .



Domestic chicks (Ross I) were hatched and maintained in darkness until they were trained and tested. At approximately 24 h after hatching, the chicks were trained by exposure to an imprinting object for 2×1 h, as described elsewhere⁸. One hour after the end of training, the chicks received a preference test involving sequential exposure to the training object and a novel object. A measure of the chick's preference for the training object was calculated as a score. This preference score, expressed as a percentage, is given by the expression: approach activity towards the training object divided by total approach activity during the test^{3,8}. In order to maximise the expression of Fos-like immunoreactivity in the IMHV¹², six chicks with a high preference score for the training object were selected (mean preference score: 98.3 ± 1.2 , S.E.M.).

Between 45 and 60 min after the end of the preference test, the chicks were anaesthetised deeply with pentobarbitone sodium (20–25 mg i.p./chick) and received transcardial perfusion of approximately 50 ml of Krebs solution followed by 250–300 ml of the fixative containing 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB: pH 7.4). The brains were removed and cryoprotected by immersion overnight at 4°C in phosphate buffer containing 20% sucrose. Transverse sections (10 μ m) at the level of the IMHV region^{8–10} were cut on a cryostat.

The sections were washed in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and incubated overnight at 4°C with mouse monoclonal antibody to a peptide fragment from chicken Fos (1:1,000; Oncogene Science). They were then rinsed and processed with an avidin–biotin complex horseradish peroxidase kit (Vector). The bound enzyme complex was visualised using the chromogen protocol according to Shu et al.²¹. This method employs a buffered glucose oxidase, nickel and diaminobenzidine (DAB) solution and gives a dense blue black reaction product. The sections were then treated with avidin/biotin blocking solution (Zymed kit). For the PKC γ staining, the same sections were

Fig. 1. Representative photomicrographs showing staining in the left IMHV. A: simplified diagram of a coronal section of the chick brain at the level of the IMHV. The extent in the coronal plane of the left IMHV¹⁰, as removed in previous biochemical studies^{9,14,15,20} is indicated by the hatched area. B: low-power photomicrograph of a coronal section stained for Fos and PKC γ , represented by the filled square within the IMHV in diagram A. The area within the square in B is shown at a higher power in C. PKC γ -positive cells are stained brown and Fos-expressing nuclei are dark blue. Some of the double-stained cells are indicated by arrows. Arrowheads indicate some of the cells that were stained for PKC γ only. Bar = 200 μ m for B and 30 μ m for C. The diagram in A was adapted, with permission, from ref. 12.

subsequently pre-incubated for 15 min in 0.1% H₂O₂ in PBS, rinsed in PBS and immersed in 5% normal sheep serum (NSS) in PBS for 30 min to reduce non-specific binding. The sections were then incubated with the primary antibody (36G9, a monoclonal mouse anti-PKC γ IgG), diluted 1:200, and left in 1% NSS in PBS overnight at 4°C in a wet chamber. Sections were then rinsed in PBS and again preincubated with 5% NSS for 30 min before the secondary incubation step in biotinylated sheep anti-mouse IgG (1:200; Amersham). Finally, after treatment with Streptavidin-HRP (1:200; Zymed), the sections were subjected to a DAB reaction. The resultant brown reaction deposits indicating PKC γ were easily distinguishable from the dense blue-black Fos-positive nuclei. At two coronal levels in the IMHV (see Fig. 1A), the degree of co-localisation of Fos and PKC γ was determined for each chick. Preabsorption tests showed that both monoclonal antibodies were specific for their respective antigens. As a further control, one of the two primary antibodies, or both, were omitted from the staining procedure. When this was done, non-specific binding of the secondary antibodies was absent.

Fig. 1 shows part of a coronal section at the level of the IMHV, stained for Fos and PKC γ . Fos immunoreactivity was observed in nuclei of IMHV somata, either covering the entire nucleus, or fragmented and close to the nuclear membrane. In both cases, Fos-positive cells were immunoreactive for PKC γ . PKC γ immunoreactivity was found in the cytoplasm of cell somata and associated dendrites, and some of the cells also displayed nuclear staining. Of the mean number of Fos-positive cells counted (186.9 ± 32.6 , S.E.M., for 2 sections from left and right IMHV), $179.2 (\pm 30.0)$, i.e. 96.5% (± 1.0) were also stained for PKC γ . The degree of co-expression may have been underestimated, as some of the Fos-positive cells lacked clear cytoplasm, probably due to the plane of sectioning. The high incidence of co-localisation of PKC γ and Fos appeared to be a general phenomenon that was also seen e.g. in the hippocampus and the hyperstriatum accessorium. Fos-positive cells are present in the IMHV of dark-reared chicks, though such cells occur less frequently than in trained chicks¹⁶. In a pilot study in which we examined sections from 3 dark-reared chicks, PKC γ and Fos were also found to be co-localised. The vast majority of PKC γ -stained cells are neurones²⁴. Thus it is likely that the expression of Fos after imprinting is in neuronal nuclei. The present results show that nearly all of the neurones in the IMHV that express Fos after imprinting also contain PKC γ . Using the same antibody as in the present study, Van der Zee et al.²³ found a substantial increase in expression of

PKC γ after applying the phorbol ester PDBu to fixed mouse brain sections, and following the application of carbachol to rat brain slices²⁵ (see ref. 25 for a discussion of the differences between the characteristics of 36G9-binding for in situ localized PKC γ and for purified PKC γ). This, together with their findings on the effects of spatial learning makes it probable that 36G9 recognises the *activated* form of PKC γ . Thus, the learning-related increase in Fos expression in the IMHV that occurs after imprinting¹⁶ is likely to be in neurones with activated PKC.

It has been suggested that PKC-dependent phosphorylation is required for the activation of immediate early genes such as *c-fos*⁶. Furthermore, it is possible that PKC γ is responsible for the phosphorylation of certain phosphoproteins that are involved in the plastic neural changes that accompany the learning process of imprinting. For instance, Sheu et al.^{15,20} found a significant positive correlation between preference scores (i.e. a measure of the strength of learning) and phosphorylation of a component of a protein kinase C substrate²² (MARCKS; $M_r = 67$ kDa in chick brain) in the left IMHV. Experiments are underway to investigate the relationship between imprinting and changes in PKC isoenzyme immunoreactivity.

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