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Induction of Fos in glia-like cells after focal brain injury but not during wallerian degeneration

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Focal brain injury or perforant-path transections respectively led to an increase in the number of glial-fibrillary acidic protein (GFAP)-immunopositive astrocytes around the focal wound or in the terminal fields of the perforant path in the dentate molecular layer. This GFAP accumulation occurred 48-72 h after focal brain injury or perforant-path transection (wallerian degeneration). Focal brain injury also led to an accumulation of c-fos protein (Fos) in glial cells, ependyma and cells in the pia mater of the brain within 6 h of injury and this effect dissipated within 72 h. However, perforant-path lesions were not associated with accumulation of Fos in glial cells in the dentate molecular layer suggesting that c-fos induction in glial cells after injury is not necessary for GFAP accumulation. Induction of Fos in glia, ependyma and pia after focal brain injury may be associated with proliferation of these cells after injury.

INTRODUCTION

The transcription modulator c-fos is induced in adult mammalian neurons after seizures, sensory stimulation and traumatic brain injury[7,10,14,18,21]. C-fos protein (Fos) is also induced in glial-like cells after brain injury[10]. The functional consequences of Fos induction in glial cells is unknown, although, a role in reactive gliosis has been postulated[10]. Reactive gliosis and glial scar formation after brain injury may have important consequences for neural repair by providing growth factors for axonal regeneration or by blocking axonal regrowth by activating the physiological stop pathway[4]. The process of reactive gliosis is characterized by a dramatic increase in both the number of intermediate filaments and the quantity of glial fibrillary acidic protein (GFAP) within astrocytes of the glial scar[2].

Therefore, if Fos induction in glia is involved in reactive gliosis then Fos ought to be induced in glia by stimuli that lead to reactive gliosis. This hypothesis was tested using two different treatments that both lead to reactive gliosis and GFAP accumulation: (1) traumatic brain injury[2]; and (2) anterograde axonal degeneration (wallerian degeneration) produced by cutting perforant-path axons[12]. At different times after injury or axon transection, rats were perfused and processed for immunocytochemical detection of Fos and GFAP. We also used a double-antigen labelling procedure[15] to determine which type of glial cell expressed Fos after injury.

MATERIALS AND METHODS

To produce traumatic brain injury male Wistar rats were anaesthetized with pentobarbital and positioned in a stereotaxic apparatus. A burr hole was drilled above the cortex at coordinates AP –3.5, L 2.5 (Bregma = zero, mouth bar set at I/A zero). A 25-gauge needle was inserted 3 mm down from brain surface, down into the underlying cortex, corpus callosum and hippocampus. The needle was left in place for 1-min then withdrawn and reinserted for a further 1 min and then withdrawn. The skin overlying the exposed cranium was then sutured shut. At various times after injury (10 min, 6, 24 or 72 h) rats (n = 2 per time point) were anaesthetized and perfused transcardially for immunocytochemical processing of brains as described below. Two rats also had needles inserted more laterally into the fornix-fimbria and internal capsule (coordinates AP –3.5, L 3.0) to look for induction of Fos in glia in these regions. These rats were perfused 6 h after focal injury.

To produce perforant-path lesions male Wistar rats were anaesthetized with pentobarbital and positioned in a stereotaxic apparatus. A thin razor blade knife (2 mm wide) was inserted 6 mm below the skull surface along a line made by drilling burr holes laterally from lambda as previously described[6]. At various times after perforant-path lesion (10 min, 6, 12, 24, 48 or 72 h) rats (n = 2 per time point) were anaesthetized and perfused transcardially for immunocytochemical processing of brains as described below.

After lesions rats were anaesthetized with sodium pentobarbital and perfused transcardially first with saline and then with 4% paraformaldehyde in 0.1 M phosphate-buffer, pH 7.4 at room temperature. Brains were removed and immersed in the above fixative overnight. Sections (100 μm) were then cut coronally on a vibratome; during sectioning the brain was immersed in 0.01 M

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phosphate-buffered saline (PBS). Alternate sections were then processed for the immunocytochemical detection of Fos or GFAP.

Before processing sections for immunocytochemistry, they were incubated in 1% hydrogen peroxide in 100% methanol for 10 min to remove endogenous peroxidase staining from erythrocytes. Following this treatment brain sections were washed for 10 min in PBS, then incubated in 10% normal goat serum (for blocking, Cappel) for 20 min. Sections were then incubated for 48 h in one of two different primary antibodies: (1) to a synthetic 27 amino acid fragment (called the M-peptide) of Fos protein raised in rabbits and purified on peptide-conjugated affinity gels at a dilution of 1/1000[5]; (2) to a monoclonal antibody (Amersham) to GFAP at a dilution of 1/50 in 1% normal goat serum made up in 0.01 M PBS, at room temperature. For purposes of comparison some sections (only from focal injured rats) were incubated with a commercially available sheep antibody to Fos directed against the N'-terminal region of Fos (CRB, OA-11-821, dilution 1:1000 in normal swine serum), although for the time course and double-label studies (see below) only the rabbit anti-Fos serum was used to detect Fos. Next, sections were washed in PBS and incubated with biotinylated goat anti-rabbit serum (Vector) for the rabbit Fos antibody for 1 h, with biotinylated donkey anti-sheep (Sigma) for the sheep anti-Fos antibody overnight, or with secondary biotinylated anti-mouse (Vector) for the GFAP antibody for 1 h, using a modification of the avidin–biotin–horseradish peroxidase technique as described by Hsu et al.[13]. After washing in PBS, sections were incubated with the ABC solution (Vector) for 3 h, rinsed in PBS and placed in the chromogen diaminobenzidine (DAB, Sigma) containing hydrogen peroxide.

To control for specificity of staining some sections were incubated with Fos antiserum pre-adsorbed and others without Fos or GFAP antibody, which were replaced by normal rabbit serum. All incubations were performed at room temperature except where otherwise stated.

For double-antibody labelling reactions brain sections taken 6 h after focal brain injury were first stained for Fos as described using DAB as the chromogen and giving a brown nuclear reaction product. Then sections were washed in PBS and incubated for 48 h at 4 °C with the monoclonal antibody against GFAP (1:50). This reaction was visualized with the same avidin–biotin–horseradish peroxidase method except that benzidine dihydrochloride (BDHC, Sigma) was used as the chromogen giving a blue reaction product after addition of nitroprusside and hydrogen peroxide[15]. We also tried double-antigen labelling studies using antisera to Fos (DAB) and a rabbit antibody to the oligodendrocyte marker glycerol-phosphate dehydrogenase (GPDH), which was detected with BDHC.

RESULTS

In control non-injured rat brains Fos protein-like immunoreactivity (FOS-IR) as detected with the rabbit anti-Fos was localized to the nucleus of neurons scattered in the neocortex and limbic system. FOS-IR was not detected in non-neural brain cells in controls. However, control brains incubated with the Sheep anti-Fos serum showed little, if any, FOS-IR in any cell type.

Induction of FOS-IR in non-neural brain cells after focal brain injury and perforant-path lesions

Six h and 24 h (but not 10 min) after focal brain injury we observed an induction of FOS-IR in the nucleus of neurons (Fig. 1) and glial-like cells in white matter regions (corpus callosum, internal capsule, fornix-fimbria) around the wound (Figs. 2 and 3). The two anti-Fos sera revealed an identical pattern of immunostaining in non-neural cells (Figs. 2 and 3). Staining in non-neural brain cells was greatly reduced back to background 72 h after brain injury (Fig. 4), although in neurons (Fig. 1) there was some induction at this time point. However, when we perfused two rats 6 days after focal brain injury levels of FOS-IR in cortical neurons had returned to baseline. Fos-positive glial cells also appeared in grey matter regions around the edge of the wound following neocortical and hippocampal injury (Fig. 5).

Ependymal cells lining the lateral ventricle on the damaged side and third ventricle showed FOS-IR (Figs. 3 and 6) with a time course similar to that for glia. Also, non-neural cells in the intercellular matrix between the base of the hippocampus and the rest of the brain (i.e. the pial surface) showed FOS-IR on the injured side (Fig. 7), again with a similar timecourse to the induction of FOS-IR in glia. Furthermore, non-neural cells in the pia-arachnoid covering the injured cerebral hemisphere showed FOS-IR (Fig. 7) with a similar time course. Induction of FOS-IR in these various cell types was mainly nuclear and was completely abolished in preadsorption control studies where the Fos antiserum was absorbed against excess antigen and in studies where normal rabbit serum was substituted for Fos antiserum.

The results of the double-labelling studies using antibodies to Fos protein and GFAP are presented in Fig. 8. These studies revealed some double-labelled cells with Fos-positive nuclei (stained with DAB, orange/brown) and GFAP-positive processes (stained with BDHC, grey/blue). Some glial cells appeared double-labelled (Fig. 8A,B), but in many cases Fos-positive nuclei appeared in close proximity to GFAP-positive cell processes (Fig. 8C–G) and in these cases it was difficult to determine whether these were actually double-labelled cells with Fos-positive nuclei and GFAP-positive processes or were two different cells in close proximity. Other Fos-immunoreactive glia were GFAP-negative.

In the double-labelling studies using antibodies to Fos and GPDH we could not detect double-labelled glial cells. The reason for this negative finding could be that although the anti-GPDH serum specifically detected oligodendrocytes in white matter regions contralateral to the injured site (Fig. 9), it did not detect oligodendrocytes around the wound site. It may be that injury non-specifically prevented the labelling of oligodendrocytes around the wound by the anti-GPDH serum in the same areas where Fos was induced.

Following perforant-path lesions there was a time-dependent increase in FOS-IR in neurons in the hippocampus (Fig. 10) and in glia, ependyma and pia around the lesion site as measured with the rabbit anti-Fos serum. However, there was no FOS-IR in glial cells (as

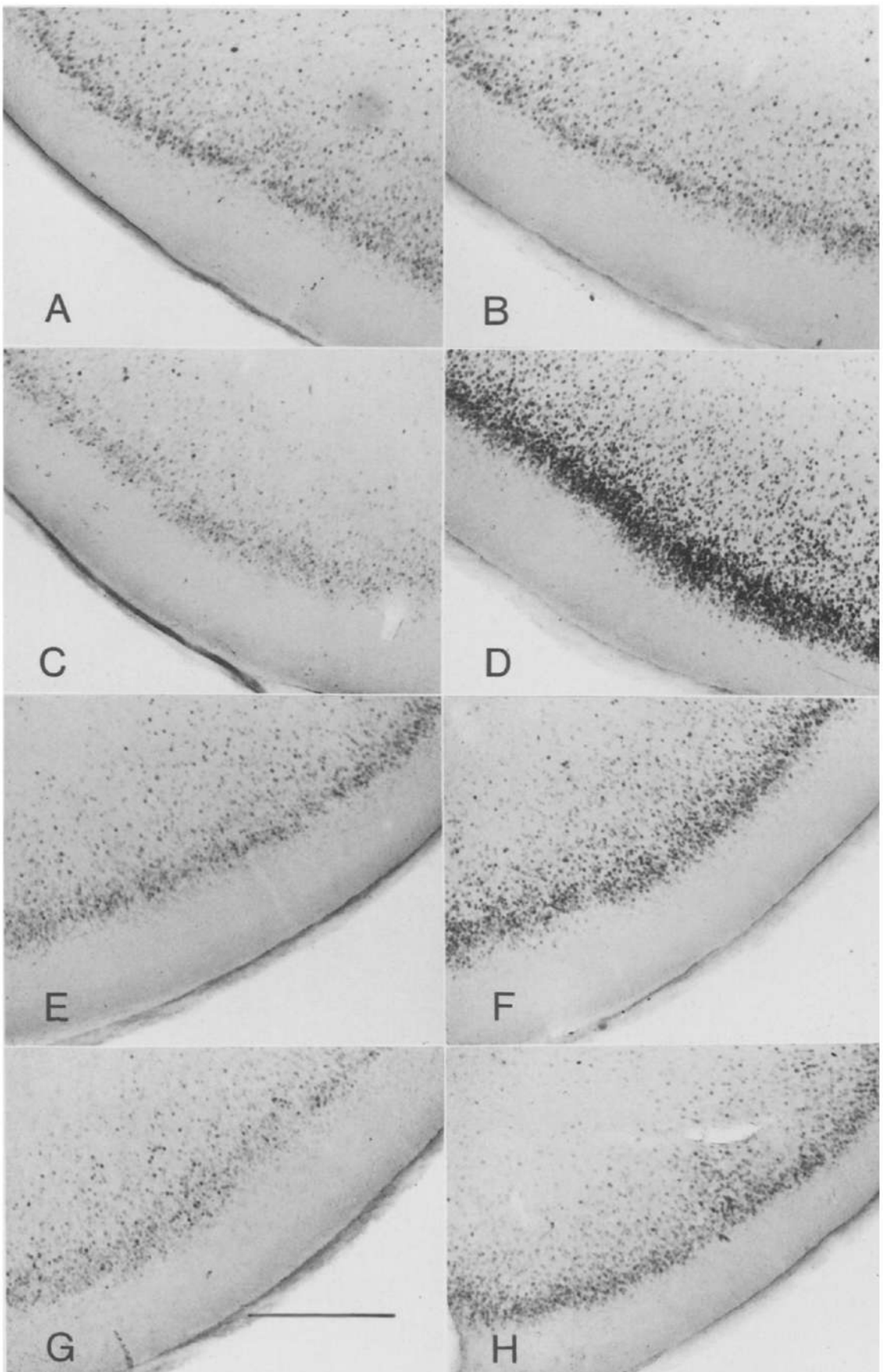

Fig. 1. Photomicrographs demonstrating the time course of the induction of FOS-IR in neurons in the piriform cortex after focal brain injury. A, C, E and G show piriform cortex contralateral to the damaged neocortex. B, D, F and H show piriform cortex ipsilateral to the damaged neocortex. A and B, 10 min after injury; C and D, 6 h after injury; E and F, 24 h after injury; and G and H, 72 h after injury. Sections were incubated with the rabbit anti-Fos serum. Bar = 400 μm.

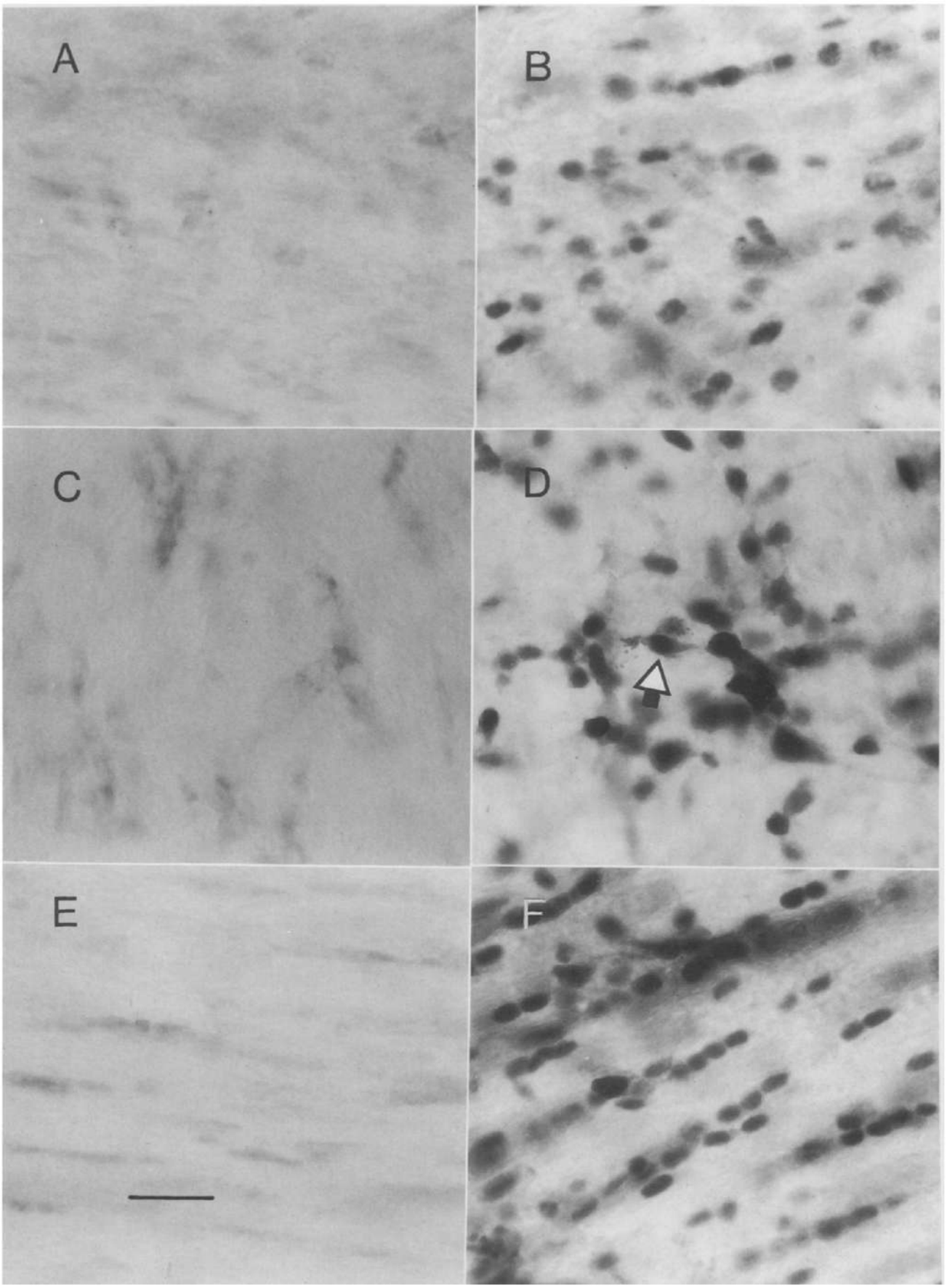

Fig. 2. Photomicrographs showing FOS-IR in the corpus callosum (A and B), internal capsule (C and D), and fornix-fimbria (E and F) ipsilateral (B, D and F) and contralateral (A, C and E) to a focal brain wound. Bar = 20 μm. Note the absence of FOS-IR in glia contralateral to the wound and the massive induction of FOS-IR in glial cells ipsilateral to the wound. Note also the bead-like arrangement of glia in F, a pattern consistent with staining of oligodendrocytes (or perhaps endothelial cells?). The rat was perfused 6 h after focal brain injury and sections were incubated with the rabbit anti-Fos serum.

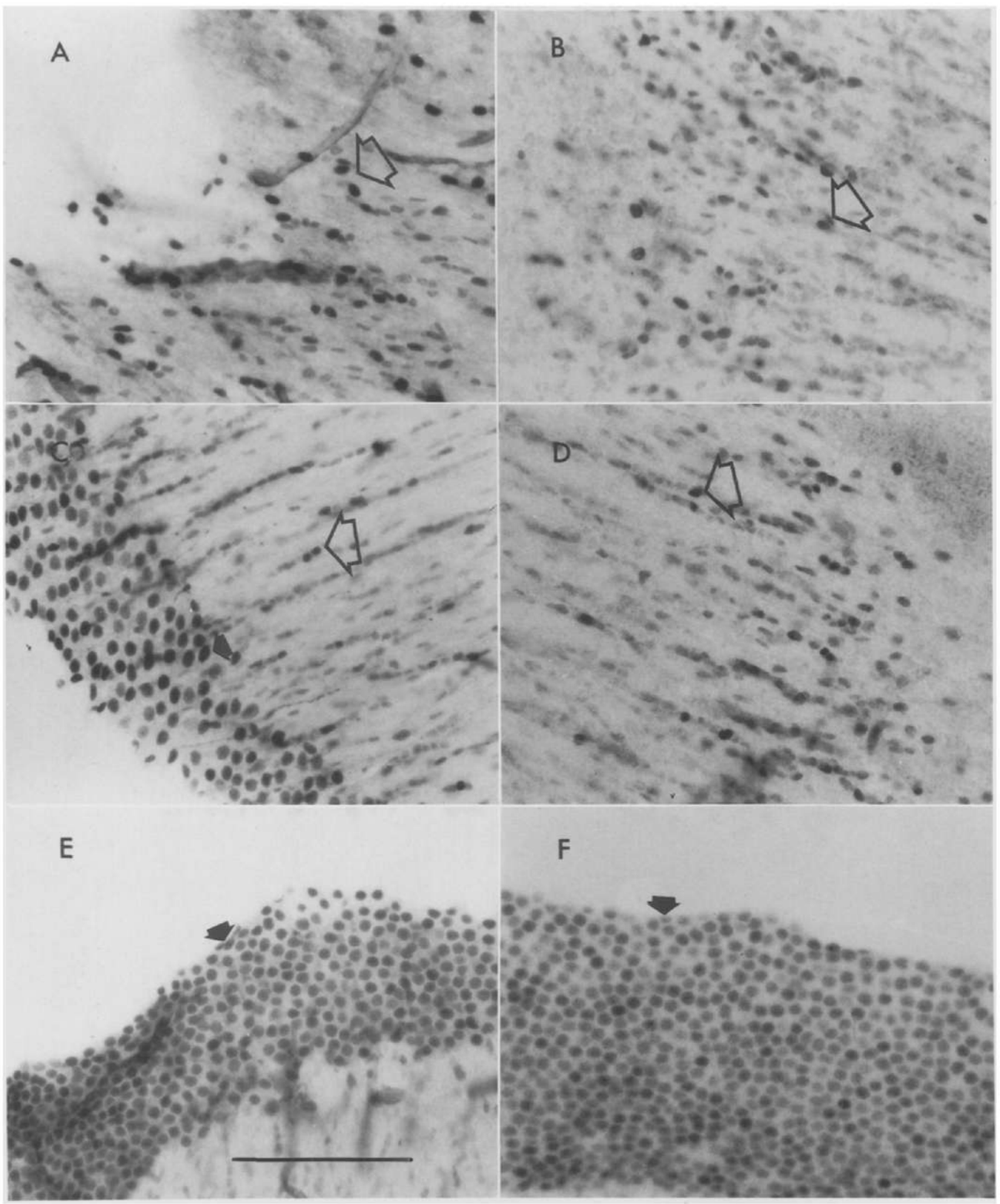

Fig. 3. Photomicrographs showing FOS-IR in the corpus callosum (A, B), fornix-fimbria (C, D) and third ventricle (E, F) ipsilateral to a focal injury. A, C and E were immunostained using the CRB anti-Fos serum, and B, D and F were immunostained using the rabbit-anti-Fos serum. Hollow arrows show Fos-positive glia and dark arrows show Fos-positive ependyma. Note that the pattern of Fos staining is identical for both antisera. Bar = 100 μm.

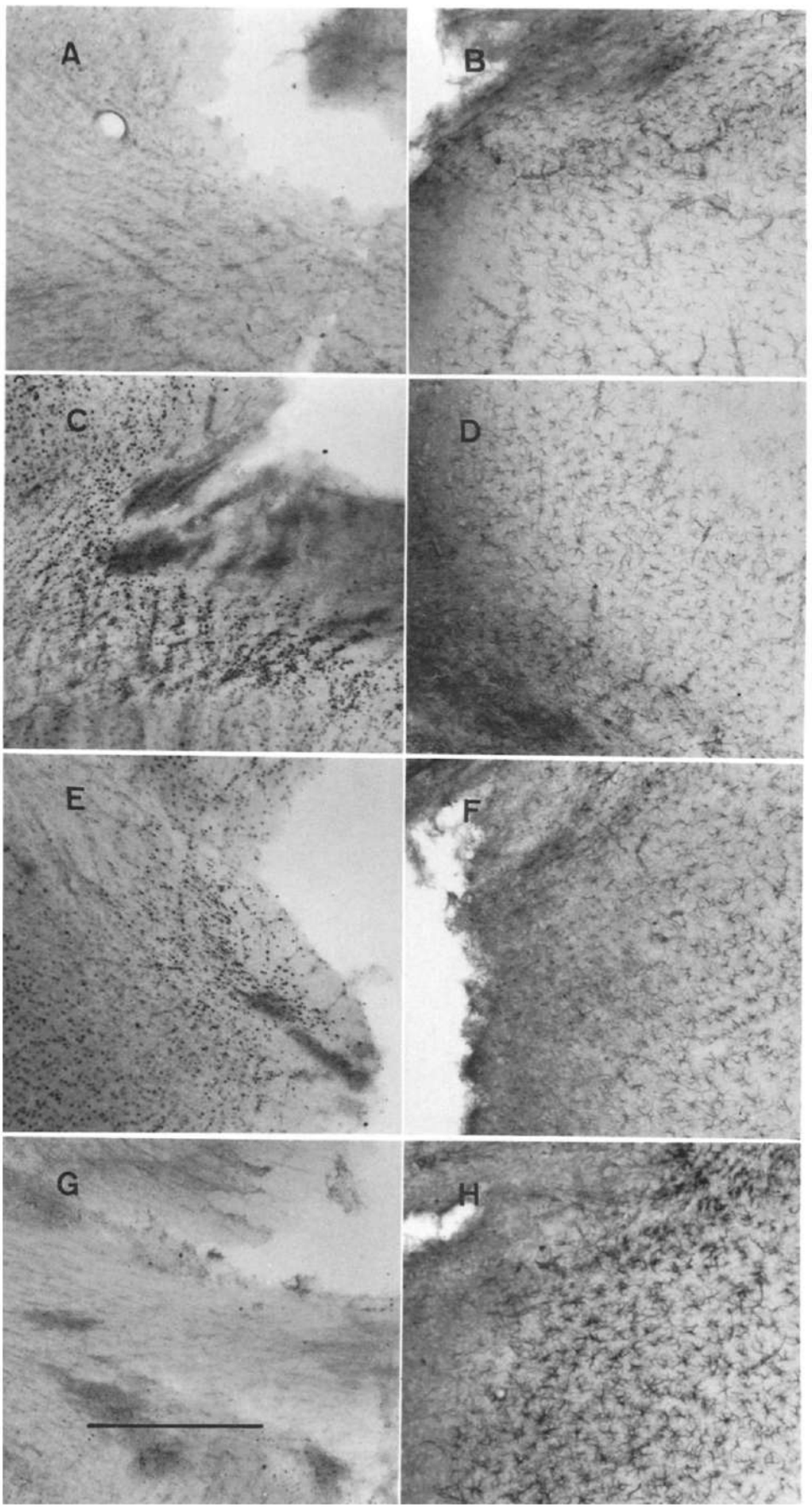

Fig. 4. Photomicrographs showing time-dependent accumulations of FOS-IR (using rabbit anti-Fos, A, C, E, G) and GFAP-immunoreactivity (B, D, F, H) 10 min (A and B), 6 h (C and D), 24 h (E and F), and 72 h (G and H) after focal brain injury in coronal sections taken from around the focal wound of rat brain. Bar = 400 μm. Note the appearance of FOS-IR in glia at 6 h and disappearance by 72 h and the increase in GFAP only at 72 h after injury.

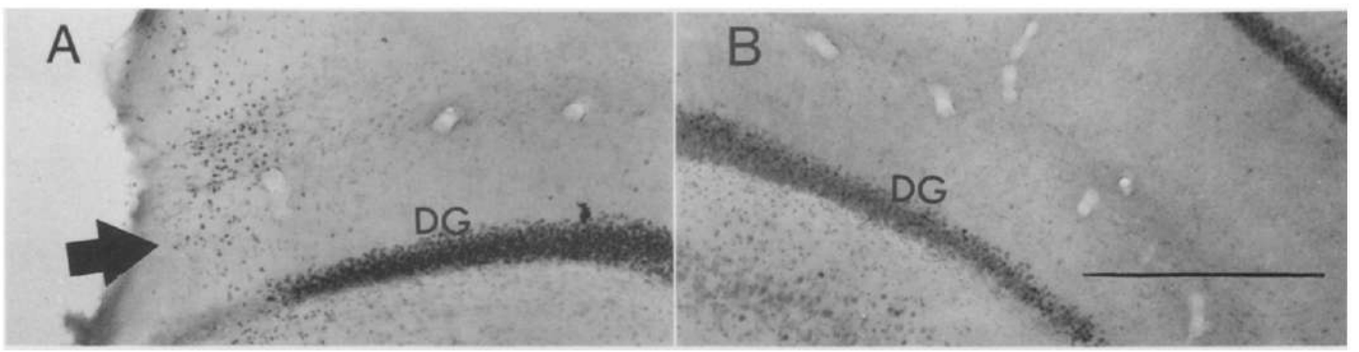

Fig. 5. Photomicrographs showing hippocampus ipsilateral (A) and contralateral (B) to hippocampal focal brain injury. Bar = 200 μm. Note the appearance of FOS-IR in glial-like cells in regions ipsilateral to the focal injury (arrow); the presence of FOS-IR can also be seen in neurons (DG, dentate granule cells) on both the ipsilateral and contralateral side with an increase on the side ipsilateral to the damage. The rat was perfused 6 h after focal brain injury and sections stained with the rabbit anti-Fos serum.

measured with rabbit anti-Fos) in the dentate molecular layer (Fig. 10) at any time point used.

Time-dependent changes in GFAP-immunocytochemistry in astrocytes after focal brain injury and perforant-path lesions

Seventy-two hours, but not 10 min, 6 h or 24 h after focal brain injury there was a large increase in the

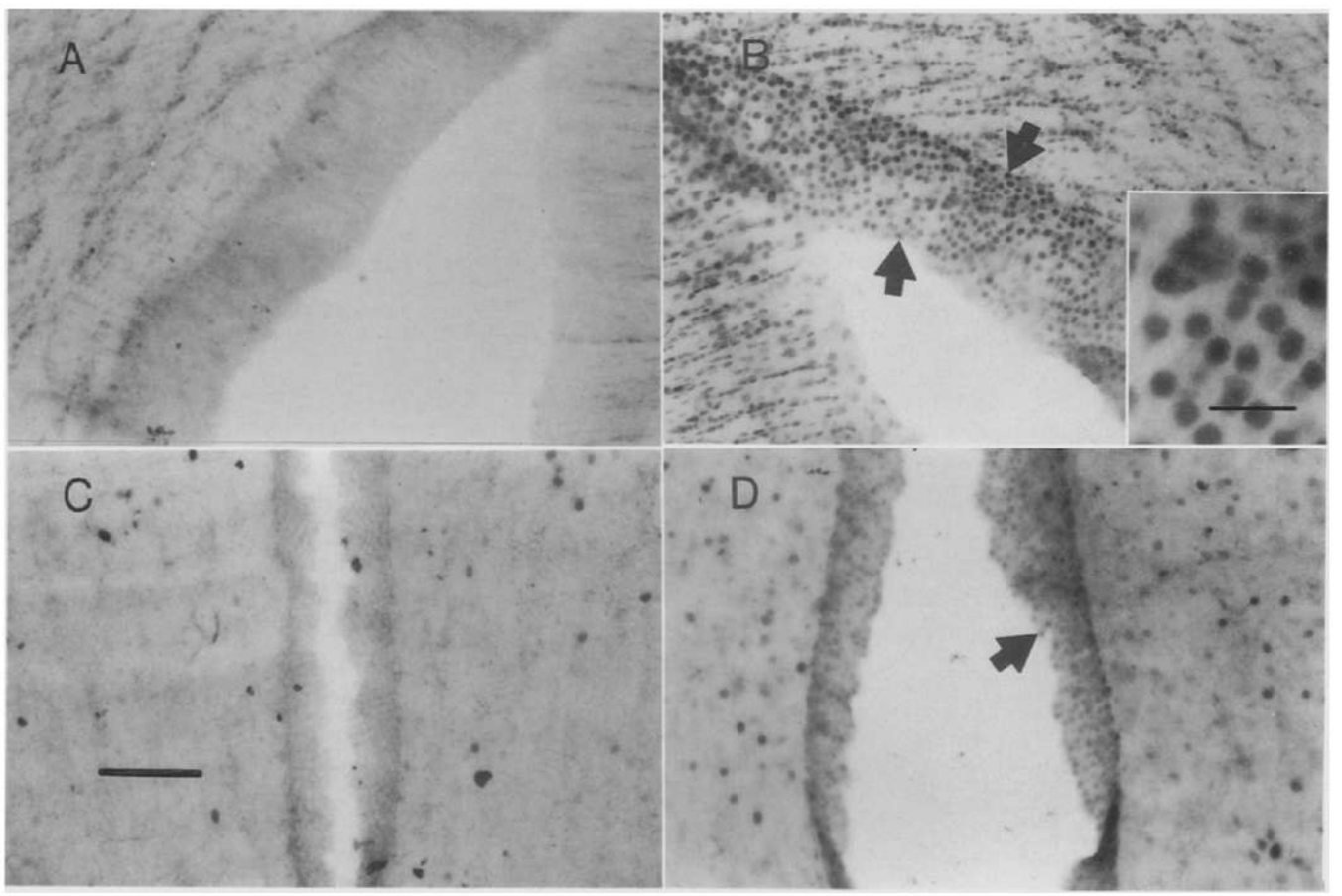

Fig. 6. Photomicrographs showing FOS-IR in the lateral ventricle (A and B) ipsilateral (B) and contralateral (A) to a focal brain wound. Inset in B shows a high power photomicrograph of FOS-IR in the nuclei of ependyma lining the lateral ventricle on the damaged side (inset bar = 20 μm). Note the large accumulation of FOS-IR in ependymal cells lining the lateral ventricle on the damaged side (arrows) and the total lack of FOS-IR in ependyma on the intact side of the brain. C and D show third ventricles from a control rat which had not received a focal brain injury (C) and from a brain injured rat (D). Note the accumulation of FOS-IR in ependyma in the third ventricle from the damaged rat brain (arrow) and the absence of FOS-IR in the ependyma of the third ventricle of a control brain. Bar = 100 μm. Rats were perfused 6 h after focal brain injury, and sections incubated with the rabbit anti-Fos serum.

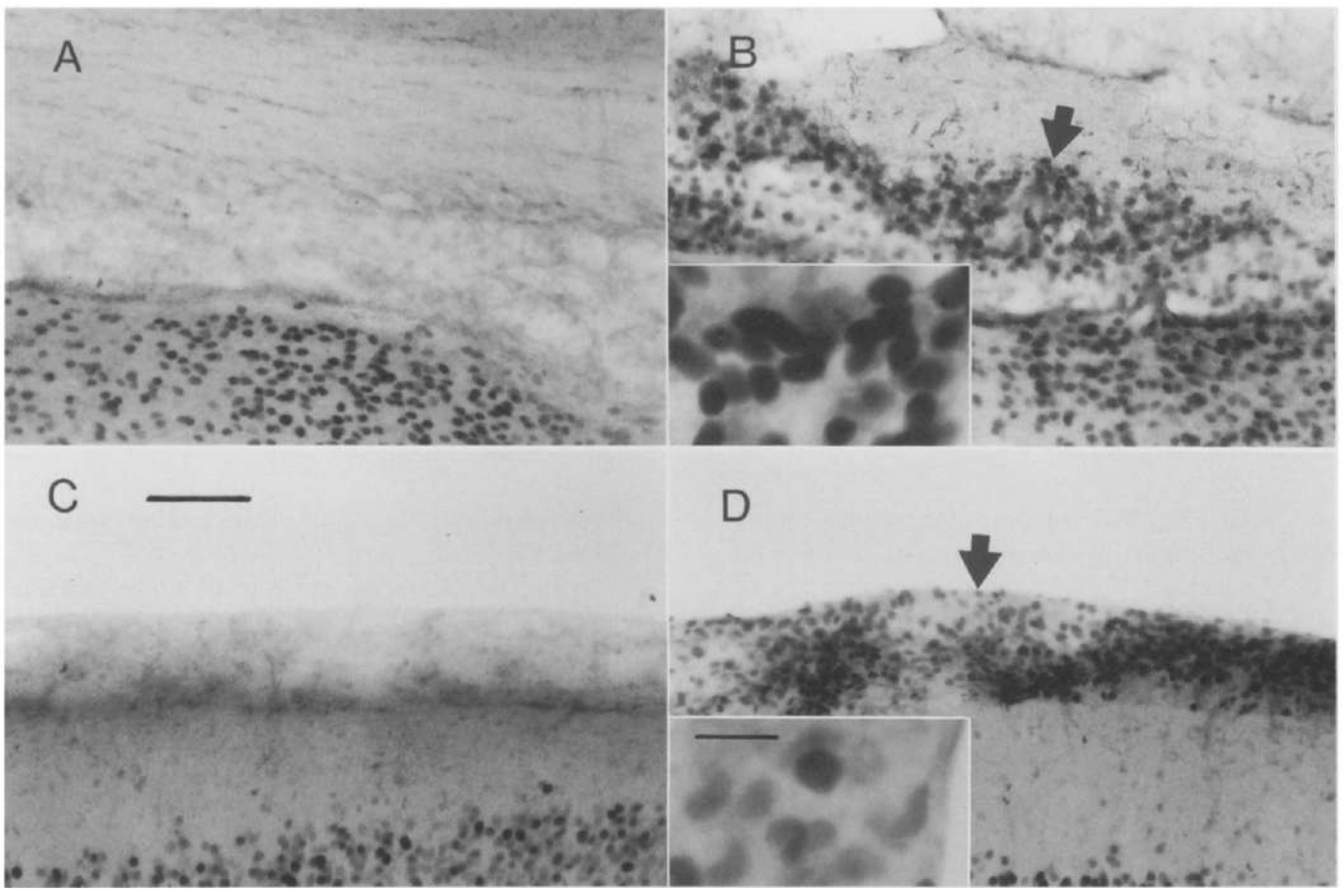

Fig. 7. Photomicrographs showing the intercellular matrix (pial surface) between the base of the hippocampus and the rest of the brain (thalamus) ipsilateral (B) and contralateral (A) to a focal brain wound. Note the appearance of cells showing FOS-IR ipsilateral to the focal injury (arrow). Inset shows high power photomicrograph of section in B. C and D show the pia-arachnoid covering the neocortex contralateral (C) and ipsilateral (D) to a focal brain wound. Note the accumulation of FOS-IR in cells in the pia-arachnoid ipsilateral to the injury (arrow) and the absence of FOS-IR in the contralateral pia-arachnoid. Bar = 100 μm. Inset shows high power photomicrograph of D (inset bar for B and D = 20 μm). Rats were perfused 6 h after focal brain injury and sections were incubated with the rabbit anti-Fos serum.

number of GFAP-immunopositive astrocytes around the wound in the neocortex and corpus callosum (Fig. 4). Similarly, following perforant-path lesions there was a time-dependent increase (at 48–72 h) in the number of GFAP-positive astrocytes in the outer ⅔ of the dentate molecular layer (Fig. 11). No increase was found at 10 min, 6, 12 or 24 h after perforant-path lesion (Fig. 11).

DISCUSSION

Focal brain injury in rats produced a time-dependent increase in FOS-IR in glial-like cells in white and grey matter regions around the wound margin, ependymal cells lining the lateral ventricle on the damaged side and the third ventricle, pial cells in the intercellular matrix between the base of the hippocampus and the rest of the brain on the damaged side, and in cells in the pia-arachnoid covering the damaged neocortex. This induction of Fos was not present at 10 min, was maximal at 6 h, and had returned to baseline 72 h after injury. The induction of FOS-IR in these various cell types occurred adjacent to the wound suggesting that the wound itself, rather than some secondary event, caused the increase in FOS-IR. The induction of Fos-like protein(s) in non-

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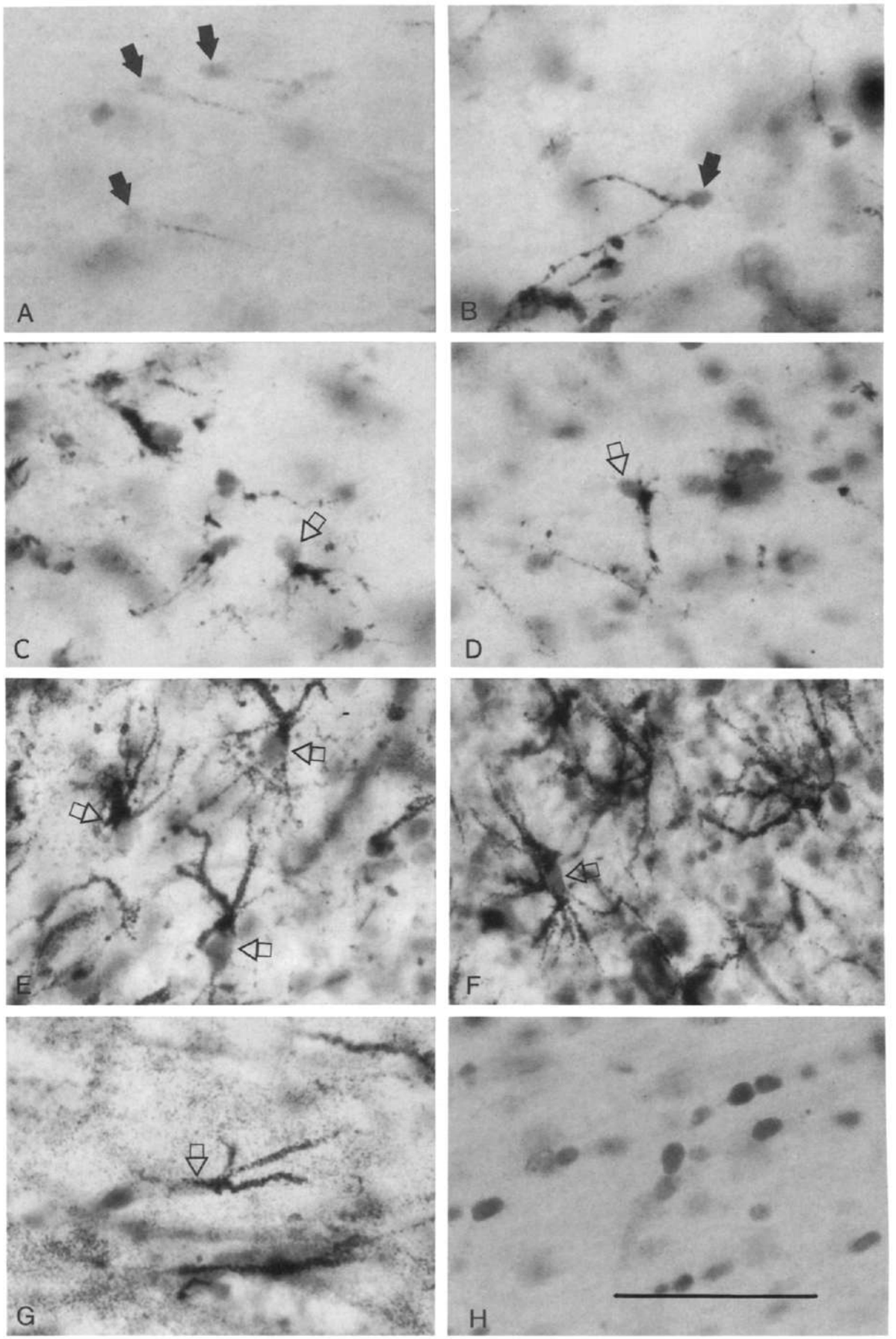
Fig. 8. Photomicrographs from a number of experiments showing the extent of double-labelled cells in the rat corpus callosum adjacent to a focal brain injury. The nuclear reaction product shows Fos-positive glial cell nuclei using the chromogen DAB and the rabbit antiserum. The process reaction product shows GFAP-positive astrocytic cell processes using the chromogen BDHC. Note that some cells are clearly double-labelled with Fos-positive nuclei and GFAP-positive processes (closed arrows, A, B), whereas others show Fos-positive nuclei in close apposition to GFAP-positive processes (open arrows, C–G). In these cases it is difficult to ascertain whether these are actually double-labelled cells or separate cells that are in close apposition. Other Fos-positive glial cells were not double-labelled (H) and many of these formed long bead-like arrays typical of oligodendrocytes or perhaps swollen endothelial cells. Bar = 50 μm.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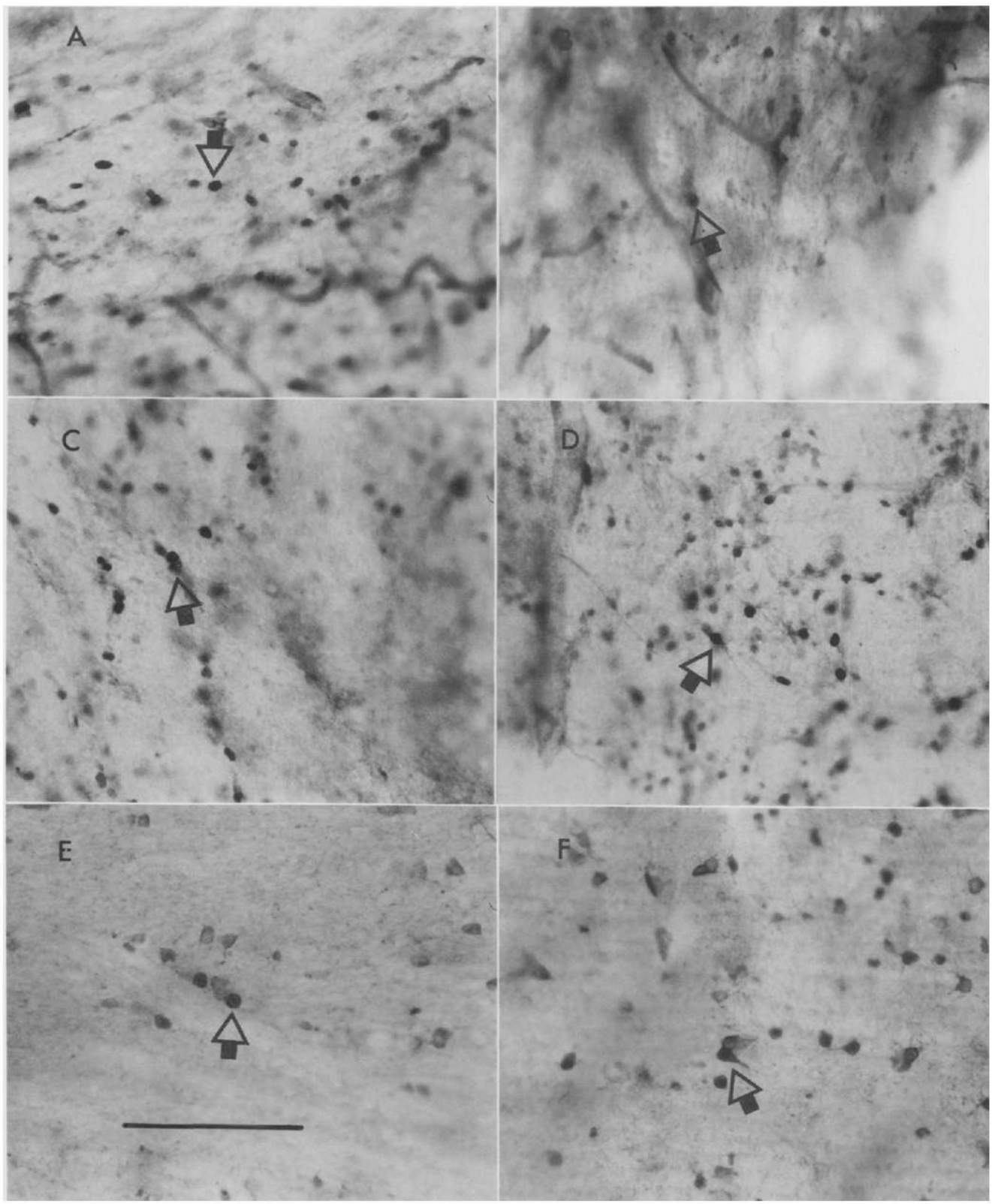

Fig. 9. Photomicrographs showing glial cells as indicated by arrows in the corpus callosum (A, C, E) and internal capsule (B, D, F) detected with the CRB anti-Fos serum (A, B), the rabbit anti-Fos serum (C, D) or the rabbit anti-GPDH serum (E, F). Note that in the corpus callosum and internal capsule the Fos-positive glia detected with both Fos antisera (A–D) show the same morphology. Bar = 100 μm.

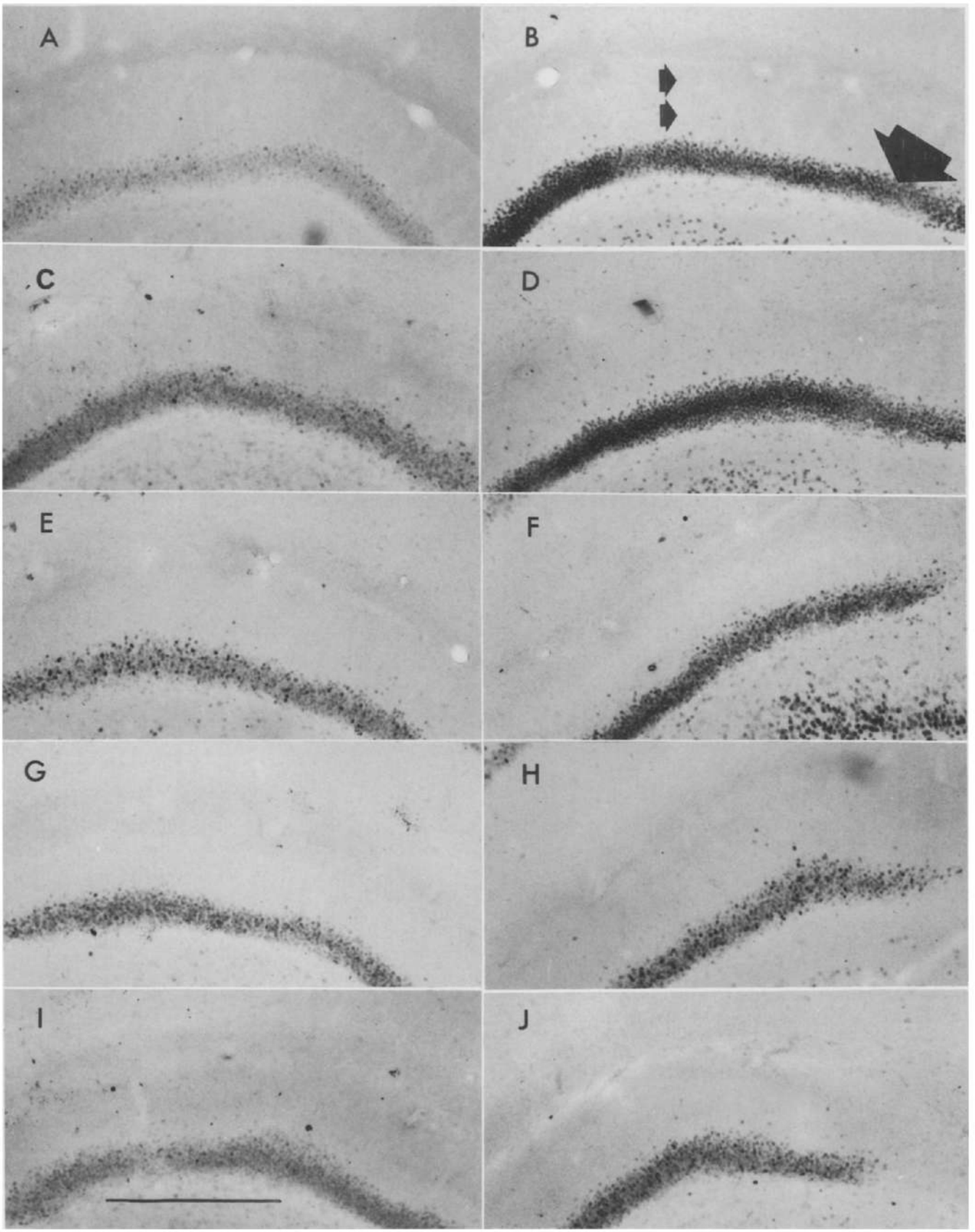

Fig. 10. Photomicrographs showing FOS-IR in dentate granule cells (upper blade, large arrow) contralateral (A, C, E, G and I) and ipsilateral (B, D, F, H and J) to perforant-path transection. Rats were perfused at different times after transection and sections were incubated with rabbit antisera: A, B, 6 h; C, D, 12 h; E, F, 24 h; G, H, 48 h; I, J, 72 h. Note the absence of FOS-IR in glial cells in the outer ⅔ of the dentate molecular layer (the region between the small arrows) at all time points. Bar = 200 μm.

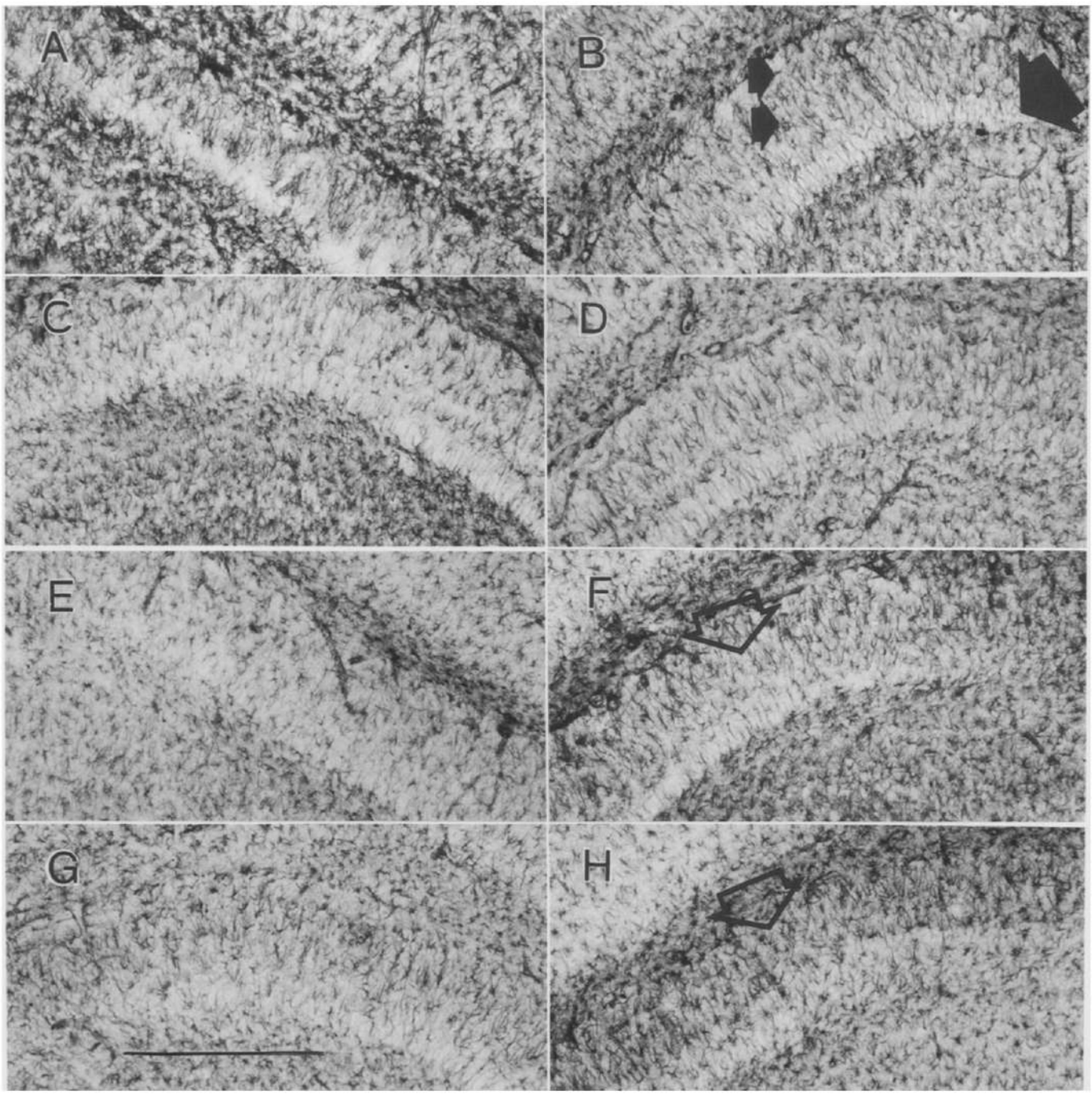

Fig. 11. Photomicrographs showing GFAP-positive astrocytes in the dentate molecular layer (between small dark arrows) contralateral (A, C, E, G) and ipsilateral (B, D, F, H) to perforant-path transection. Rats were perfused at different times after transection: A, B, 12 h; C, D, 24 h; E, F, 48 h; G, H, 72 h. Large dark arrow shows the dentate granule cell layer which is devoid of immunostaining. Hollow arrows show accumulation of GFAP-positive astrocytes 48 and 72 h after perforant-path transection in the outer ⅔ of the dentate molecular layer. Bar = 200 μm.

neural brain cells after focal injury is striking because these cells do not show basal or seizure-induced Fos-like protein(s)[8]. Thus, whereas Fos-related protein(s) exists basally in neurons, expression in non-neural brain cells is not observed basally. This observed induction of Fos in ependyma, pia, and glia after traumatic brain injury is consistent with our previous results showing Fos induction in these same cell types after traumatic brain injury to mouse brain[8].

Neurons throughout the neocortex and hippocampus on the damaged side especially in the dentate granule layer and piriform cortex also showed a large accumulation of FOS-IR 6 h after focal brain injury. This increase was not present at 10 min, was maximal at 6 h, was

reduced at 24 h, was greatly reduced at 72 h, and was absent at 6 days after injury. Thus, the increase in FOS-IR in neurons after focal brain injury was longer lasting than the increase in non-neural cells (which was gone by 72 h), and is also longer lasting than the increase in FOS-IR in neurons after seizures[7]. The reason for this longer time course is unclear, but may be related to the severity of the inducing stimulus and to the excitable nature of neurons versus glial and ependymal cells.

The observed Fos-immunostaining in brain cells was largely nuclear. Immunostaining was completely abolished when the Fos antiserum was preabsorbed with excess peptide or replaced with preimmune normal rabbit serum. The results of these specificity studies, together with the largely nuclear localization of the FOS-IR, where Fos is known to accumulate[20], and the rapid, transient time course of the increase of FOS-IR suggests that Fos or a closely related nuclear molecule was detected in these studies. One of the antisera used, raised against Fos amino acids 127–152, recognizes Fos as well as a set of Fos-related antigens (FRAs) with similar biochemical characteristics[11]. Thus, the FOS-IR observed in this study using this antibody may represent a composite of a class of Fos DNA-binding proteins. However, the CRB sheep anti-fos serum which is directed against the N′-terminal region of the Fos molecule revealed an identical pattern of immunostaining after injury in glia, ependyma and pia to that seen with the rabbit anti-Fos serum. This result, together with the specificity controls indicates that Fos and possibly FRAs are being detected in neural and non-neural cells after focal brain injury. However, in control brains the rabbit anti-Fos serum detected immunoreactivity whereas the sheep anti-Fos serum did not. The most likely reason for this result is that whereas the rabbit anti-Fos detects Fos and FRAs the sheep antiserum appears specific for Fos. Therefore, basal immunostaining in neurons is most likely FRAs which are constitutively expressed in nerve cells in the neocortex and limbic system, whereas Fos is not constitutively expressed. However, because both antisera detected immunostaining in glia and ependyma after brain injury it seems very likely that Fos (and possibly FRAs) were induced in these cells.

Our double-antigen labelling studies showed that some glial-like cells appeared to be double-labelled with FOS-IR in their nuclei and GFAP immunoreactivity in their processes (Fig. 8A,B). These cells were most likely astrocytes. Other Fos-positive glia appeared in close proximity to GFAP-positive astrocytes (Fig. 8C–G). Some of these may actually have been double-labelled but it was not possible to state this with certainty without electron microscopic studies. Other Fos-positive glial cells were not double-labelled (Fig. 8H). This lack of double-labelling may reflect the sensitivity of our double-labelling procedure. Also, although fibrous (type 2) astrocytes are GFAP-positive, protoplasmic (type 1) astrocytes are low in GFAP, except after brain injury when type 1 astrocytes proliferate and express GFAP (become reactive astrocytes). Thus, Fos-positive GFAP-negative glial cells may become GFAP-positive many days after the injury, when their Fos levels have returned to baseline. It is also possible that these Fos-positive glial-like cells were not astrocytes but other types of cells (e.g. microglia, fibroblasts, oligodendrocytes, endothelial cells).

Although we detected some Fos/GFAP double-labelled cells, we could not detect any double-labelled Fos/GPDH glial cells. However, this negative result is most likely explained by the observation that the GPDH antibody did not stain oligodendrocytes around the wound margin, which was the only region where Fos-positive glia appeared. However, based upon the morphology of the majority of Fos-positive glial cells in the corpus callosum and in the fornix-fimbria in particular (i.e. bead-like arrays of cells) it seems that some Fos-positive glia that appeared after injury were oligodendrocytes (see Figs. 2, 3 and 8H). However, the possibility exists that these long strings of cells were not oligodendrocytes but swollen endothelial cells.

Focal brain injury also led to an increase in GFAP-positive astrocytes around the wound. This increase was present at 48 and 72 (but not 6 or 24) h after injury. This result confirms previous observations[2]. This delayed induction of GFAP may account for the lack of double-labelling of Fos-positive glial cells with GFAP as discussed above. Thus, the increase in Fos in glia around the wound (6–24 h after injury) preceded GFAP accumulation (at 48–72 h), suggesting that Fos induction may lead to GFAP induction. However, the results of the perforant-path lesion experiments cast doubt on this hypothesis.

Perforant-path lesions lead to a destruction of the entorhinal inputs to the outer ⅔ of the dentate molecular layer in the hippocampus[12]. This is followed by a dramatic increase in the number of GFAP-positive astrocytes in the outer ⅔ of the dentate molecular layer[12]. The results of the present study confirm this observation (see Fig. 11). The time course of accumulation of GFAP at 48–72 h after perforant-path transection is similar to the time course of GFAP accumulation after focal brain injury. However, in contrast to focal brain injury, perforant-path transection did not result in Fos induction in glia in the outer ⅔ of the dentate molecular layer. Thus, GFAP induction after brain injury does not require previous Fos induction in glial cells.

The functional consequences of Fos induction in

glia-like cells, ependyma and pia adjacent to the wound after focal brain injury are unclear. However, the induction of Fos in glia in vivo supports previous in vivo results[8–10] and complements in vitro studies showing that Fos is induced in rat[17] and human[3] astrocytoma cells and in secondary astrocytic cultures[1] by growth factors and other mitogens and in non-neural cells after injury to the fish optic nerve[22]. These in vivo and in vitro studies suggest that Fos induction in glia, ependyma and pia after focal brain injury may contribute to the cell proliferation which occurs after injury[16]. Recent evidence showing that Fos is induced in fibroblasts after wounding[23] and is involved in fibroblast division[19] would support this hypothesis.

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