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Temporal and Spatial Patterns of *c-fos* mRNA Induced by Intravenous Interleukin-1: A Cascade of Non-Neuronal Cellular Activation at the Blood-Brain Barrier

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ABSTRACT

Brain cells responsive to a peripheral immune challenge, identified by in situ hybridization of *c-fos* mRNA following intravenous administration of the proinflammatory cytokine interleukin-1β (IL-1) or sterile saline, were investigated at 0.5, 1, and 3 hours postinjection in rats. Doses of IL-1 ranged from 0.05 to 10 μg/kg; induction of *c-fos* mRNA occurred at ≥ 0.5 μg/kg. The majority of IL-1-induced *c-fos* mRNA-positive cells were non-neuronal cells located in barrier regions of the brain. The cells became radiolabeled in two separate but related spatiotemporal patterns. The first pattern, occurring at 0.5 hour, was characterized by *c-fos* mRNA labeling of cells of the outer meninges (mainly arachnoid), blood vessels (arteries, veins, and capillaries), and choroid plexus. This activation pattern disappeared at 1 hour. At 3 hours, a second activation pattern appeared in cells located just inside the now quiescent barrier cells. In addition, the circumventricular organs each showed characteristic spatiotemporal labeling patterns resulting from successive activation of specific cell types, with a general spread of activation directed away from the circumventricular organs over time. At 3 hours post IL-1, *c-fos* and glial fibrillary acidic protein (GFAP) mRNAs showed colocalization in the arcuate nucleus/median eminence/glia limitans region. We propose that the first wave of activation is elicited by blood-borne immune signals, but the second wave is caused by molecules generated within the first set of activated cells. The transduced signal appears to propagate to neighboring receptive cells by extracellular diffusion. In this manner, blood-brain barrier cells can transduce peripheral IL-1 signals in widespread areas of the brain, although the circumventricular organs may be the most effective loci for signal transduction. J. Comp. Neurol. 400:175–196, 1998. © 1998 Wiley-Liss, Inc.†

Indexing terms: cytokine; endothelia; glia; choroid plexus; circumventricular organ; inflammation

It is well known that the brain participates in the body's response to infection and injury. Immune challenges trigger the production of proinflammatory cytokines, notably interleukin-1β (IL-1), which act locally or are released into the bloodstream for transport to target cells and organs where they coordinate and amplify the immune response (Dinarello, 1994). The brain is one of those target organs, and in response to immune activation by IL-1 and several other cytokines (e.g., tumor necrosis factor, interleukin-6), the brain serves to activate the pituitary-adrenal axis and to produce fever, sleep, loss of appetite, and "sickness" behavior (Rothwell, 1991). Unlike other target organs, however, the brain is protected from access by immune signaling molecules because cytokines do not readily cross the blood-brain barrier (BBB). This fact poses one of the greatest enigmas in neuroimmunology.

Several mechanisms for immune-to-brain information transfer have been proposed (Watkins et al., 1995; Elmquist et al., 1997b; Licinio and Wong, 1997). Specifically: (1) cytokines such as IL-1 could directly enter the brain, (2) they could circumvent the BBB by generating a neural signal in peripheral autonomic sensory ganglia, or (3) they could stimulate the production of another bioactive molecule that more easily crosses the BBB. In the first category, cytokines may cross the BBB either via a specific

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transport mechanism or by passive diffusion. A saturable transporter has been identified for some of the cytokines, significantly IL-1 (Banks et al., 1991). Levels of IL-1 in cerebrospinal fluid (CSF) after peripheral administration were below the limits of detection in one assay which could detect plasma IL-1 at a concentration 100 times lower than the dose of injected IL-1 (Coceani et al., 1988) but were relatively much greater in cerebral cortex in another assay (Banks and Kastin, 1997). Therefore, whether a physiologically significant amount of IL-1 entering from circulation into the brain is a controversial issue, and to the extent that it does occur, leakage into the CSF from blood vessels in the meninges and choroid plexus is a likely mechanism (Broadwell and Brightman, 1976). Alternatively, local cytokine diffusion into brain parenchyma could occur at the circumventricular organs (CVOs), which have leaky BBBs. This is an attractive hypothesis because it allows for restricted elevations in cytokine concentrations whereby specific local responses could be generated depending on the roles and functions of the particular CVOs and adjacent territories. Thus, the vascular organ of the lamina terminalis (OVLT), by virtue of its proximity to preoptic nuclei controlling body temperature, could allow cytokine molecules to stimulate thermoregulatory neurons and cause fever; the median eminence (ME), with its endowment of corticotropin-releasing hormone (CRH)-containing nerve fibers, could be a site where IL-1 influences the activity of the hypothalamic-pituitary-adrenal (HPA) axis by affecting levels of CRH, the principal axis secretagogue; and the area postrema (AP), by its location adjacent to the nucleus of the solitary tract (NTS), could be site where IL-1 affects visceral sensory pathways and autonomic control centers.

In the second category, cytokines might influence brain activity by generating a neural message in the periphery, beyond the boundaries of the BBB. Thus, nerve endings or cells in autonomic ganglia and paraganglia may come in direct contact with circulating or locally generated cytokines. A nerve signal produced in these locations would travel to the brain via the vagus nerve. The NTS would be the first way station in an ascending pathway reaching the key central nervous system (CNS) control centers (Watkins et al., 1995).

The third category of hypotheses for entry mechanisms invokes a transduction process occurring at the BBB. Cytokines acting on barrier cells could stimulate the production and/or release of small, lipophilic molecules that diffuse across the BBB. Indeed, an initial site of action of IL-1 at the brain's barrier cells is suggested by the distribution of type 1 IL-1 receptors (IL-1R1). The vast majority of such receptors are located on non-neural cells that reside at the blood-brain interface: vascular endothelia, meninges, and choroid plexus (Ban et al., 1991; Boraschi et al., 1991; Cunningham et al., 1992; Ericsson et al., 1995). Thus, receptor-bearing cells may respond to IL-1 stimulation by producing a molecule which diffuses easily across cell membranes and exerts potent biological effects on target cells. One candidate molecule is prostaglandin E_2 (PGE_2). In the brain, PGE_2 is known to be produced in response to systemic endotoxin and crude IL-1 stimulation (Sirko et al., 1989). Furthermore, PGE_2 is induced in cerebral microvessels by endotoxin and crude IL-1 (Bishai et al., 1987). Finally, blockade of PGE_2 by administration of the prostaglandin synthesis inhibitor indomethacin blocks many of the CNS-mediated effects of peripherally administered IL-1 (Murakami et al., 1990; Crestani et al., 1991; Dunn and Chuluyan, 1992).

The present study was designed to reveal the viability of the candidate pathways by mapping the patterns of responsive cells following a peripheral immune challenge. A useful means for surveying responsive areas is the use of in situ hybridization histochemistry (ISHH) to localize cells showing induction of mRNA for the immediate-early gene *c-fos*. The rapid and intense induction of *c-fos* mRNA expression has proved to be an effective tool in detecting increased intracellular activity, and it is considered to be an early marker of neuronal activation (Sheng and Greenberg, 1990; Morgan and Curran, 1991). Appearance of Fos protein is closely linked temporally with *c-fos* gene expression (Sharp et al., 1991). This method has been particularly useful in studies of brain pathways responsive to peripheral immune challenges (Rivest, 1995). Earlier, we used ISHH of *c-fos* mRNA to identify a number of neural structures and CVOs that show elevated message levels 1 hour after intraperitoneal (i.p.) administration of IL-1 (Brady et al., 1994). A late (3 hours post-IL-1 injection) pattern of response was also identified in the hypothalamic arcuate nucleus and in cells at and near the BBB.

The present choices of route of administration (i.v. rather than i.p.), inflammatory agent (IL-1 rather than the bacterial endotoxin lipopolysaccharide [LPS]), and detection method (ISHH of mRNA rather than immunohistochemistry of Fos protein) were made in an effort to best resolve the temporal progression of events and to maximize the sensitivity of the assay, as follows. Responses to i.v. cytokines are quicker than to i.p. cytokines (e.g., Bataillard and Sassard, 1994), and the bolus i.v. delivery form is discrete, allowing better temporal resolution of the ensuing events. Selection of IL-1 as the inflammatory mediator allows an evaluation of the actions of a single prototypic molecule (though IL-1 may rapidly induce other immune system molecules). The logical alternative, LPS, may have direct actions as well as initiating a sustained production of cytokines, including IL-1, resulting in a loss of temporal resolution (Tilders et al., 1994). Detection of *c-fos* mRNA rather than Fos protein is desired because transcribed mRNA has an earlier appearance and briefer life span than does the translated protein (Sheng and Greenberg, 1990). Finally, by using autoradiography of sections from fresh-frozen brains, detection of signal in cells along the brain's edge is not complicated by problems with edge-staining artifacts that can occur with perfusion-fixed brains and with immunohistochemistry.

The earliest time point at which *c-fos* mRNA can be reliably detected, 0.5 hour, was examined in order to address the question of how peripheral IL-1 first enters or signals the brain. CNS structures were also analyzed at later time points, 1 and 3 hours, to determine which brain areas may participate in delayed events and to compare the early and late response patterns. Finally, a range of doses was studied, from 0.05 μg/kg to 10.0 μg/kg, to determine whether patterns of *c-fos* mRNA activation at 0.5 hour could be dissociated on the basis of threshold of response.

An additional, ancillary study was performed to explore the generality of several of the parameters of the experimental design. In this experiment, intraperitoneal (i.p.) IL-1 was administered, and 1, 3, and 5 hour survival times were examined.

MATERIALS AND METHODS

Animals, procedures, and radioimmunoassays

All animal procedures were approved by the NIMH Animal Care and Use Committee. Male Sprague-Dawley rats (200–250 g; Taconic Farms, Germantown, NY) were maintained in the animal facility with food and water available ad libitum under a 12/12 hour light/dark cycle, with lights on at 06:00, and handled daily for several days. They were then anesthetized (chloral hydrate/pentobarbital mixture: "Chloropent") and implanted with sterile silastic catheters (Baxter Healthcare, McGaw Park, IL) in the external jugular vein. Indwelling catheters were flushed twice daily through their interscapular exit with 100 U/ml heparinized sterile saline for at least 2 days. Experiments were conducted on six separate occasions. Paradigms varied according to dose and survival time. On each occasion, between 09.00 and 11.00 hours, polyethylene tubing was attached to the free end of the catheters, and through it, rats were given i.v. injection of 0.9% sterile saline (n = 12) or 0.05 to 10.0 μg/kg freshly prepared human recombinant IL-1β (n = 60; IL-1β initial spec. act. 1.8×10^4 U/μg, obtained from the Biological Response Modifiers Program, National Cancer Institute, Frederick, MD) dissolved in sterile saline (0.1 μg/50 μl). Animals were killed by decapitation at 0.5 (n = 52), 1 (n = 7), or 3 hours (n = 13) after the treatment. Brains with attached pituitaries were removed, frozen by immersion in 2-methyl butane at −30°C, and stored at −70°C prior to sectioning. Trunk blood was collected on ice in tubes containing ethylenediaminetetraacetic acid and centrifuged, and the plasma was frozen at −70°C.

In an ancillary study, animals (175–225 g) were given i.p. injections of 2.5 μg IL-1 per rat (dissolved in 250 μl sterile saline) or 250 μl sterile saline alone and examined at 1 (n = 3/group), 3 (n = 3/group), or 5 (n = 6/group) hours after injection. These animals were processed as above. Single labeling of *c-fos* and double labeling of *c-fos* and glial fibrillary acidic protein (GFAP) mRNAs was performed on tissues from this group. GFAP mRNA, a marker for astrocytes, was selected because the results of the i.v. study had suggested an astrocytic phenotype of the labeled cells in certain locations (see Results). Our choice of marking message rather than antigen was based on our experience that immunohistochemistry is difficult to perform in unfixed tissue.

In situ hybridization histochemistry

Cryostat-cut 15-μm-thick coronal sections were thaw-mounted onto gelatin-coated slides, dried and stored at −40°C until processing. The in situ hybridization histochemistry (ISHH) procedure was performed as described elsewhere (Whitfield et al., 1990; Miller et al., 1993). The ribonucleotide probe directed against bases 1256–2116 of the rat *c-fos* cDNA inserted into the pSP65 plasmid (gift of Dr. T. Curran, Roche Institute of Molecular Biology, Nutley, NJ) was transcribed using the Riboprobe kit (Promega, Madison, WI) with SP6 RNA polymerase and [^{35}S]UTP (sp. act. > 1,000 Ci/mmol; New England Nuclear, Boston, MA) after linearization with SacI restriction enzyme (Promega). The ribonucleotide probe directed against bases 473–1826 of the rat IL-1R1 cDNA inserted into the pBluescript SK plasmid (gift of Dr. R. Hart, Rutgers University, Newark, NJ), was transcribed with T3 RNA polymerase after linearization with EcoRI. The ribonucleotide probe directed against bases 647–1320 of the rat GFAP gene inserted between the Kpn1/Sac1 sites of the Bluescript KS+ plasmid (gift of Dr. D. Feinstein, Cornell University School of Medicine, New York, NY) was transcribed after linearization with SacI with T7 RNA polymerase and a digoxygenin RNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Slides were treated with 500,000 cpm of radioactive probe per tissue section (plus approximately 200 ng/section digoxigenin probe for double-label hybridization), incubated overnight at 52°C, then washed (in 2× standard saline citrate (SSC) for 1 hour at 50°C, briefly rinsed in 0.2× SSC at 55°C followed by washes in 0.2× SSC at 55°C and 60°C for 1 hour each) and dried. For the digoxygenin-labeled sections, prior to autoradiography, the sections were treated in alkaline phosphatase-conjugated anti-digoxigenin serum (Boehringer Mannheim), reacted with a chromagen (Miller et al., 1993), stopped, and air-dried.

Data analysis and presentation

Slides and ^{14}C standards containing known amounts of radioactivity (American Radiochemicals, St. Louis, MO) were placed in x-ray cassettes, apposed to film (Biomax MR, Kodak, Rochester, NY) for 3 days, and machine-developed (X-OMAT, Kodak). Slides were subsequently coated with nuclear track emulsion (NTB2, Kodak for radioactive sections; LM1, Amersham for double-label sections with digoxigenin), exposed for 3–4 weeks, and then developed for 2 minutes (D-19, Kodak; Herkenham, 1988). Single-label sections were Nissl- counterstained. Microphotography in brightfield and darkfield illumination was done with a Leitz DMR microscope and Vario-Orthomat camera (Leica, Deerfield, IL) using Tech Pan film (4415, Kodak). The printed photographs were mounted and subsequently digitized on a flatbed scanner using Adobe Photoshop software (Adobe Systems, Mountain View, CA). In later phases of the project, photomicrography was done digitally using a cooled CCD camera (Sensys 1600, Photometrics, Tucson, AZ). The captured images were contrast-enhanced in Adobe Photoshop using Unsharp Mask and Adjust Levels features. Structures were identified with the aid of the atlas of Paxinos and Watson (1986).

Adrenocorticotropic hormone (ACTH) and corticosterone in plasma were measured by radioimmunoassay (RIA; ICN Biochemicals kit, Cleveland, OH). The intra-assay and interassay coefficients of variance were < 10%. Statistical significance between control and experimental group means was determined by analysis of variance and the Student Newman Keuls post-hoc test, using SuperANOVA software (Abacus, Berkeley, CA). A P value of 0.05 was chosen as the level of statistical significance.

RESULTS

In both i.v. and i.p. saline-injected animals, low levels of *c-fos* mRNA expression were observed in widespread areas of the brain. Structures showing low to moderate levels of neuronal labeling at 0.5 hour were the cerebral cortex, pons, and cerebellum. Scattered positive cells were also found throughout the rest of the brain (data not shown). The levels of "background" expression decreased over time; thus, the control material at the 3 hour time point showed very low levels of *c-fos* mRNA. The description of IL-1-induced expression is based on observations of labeling

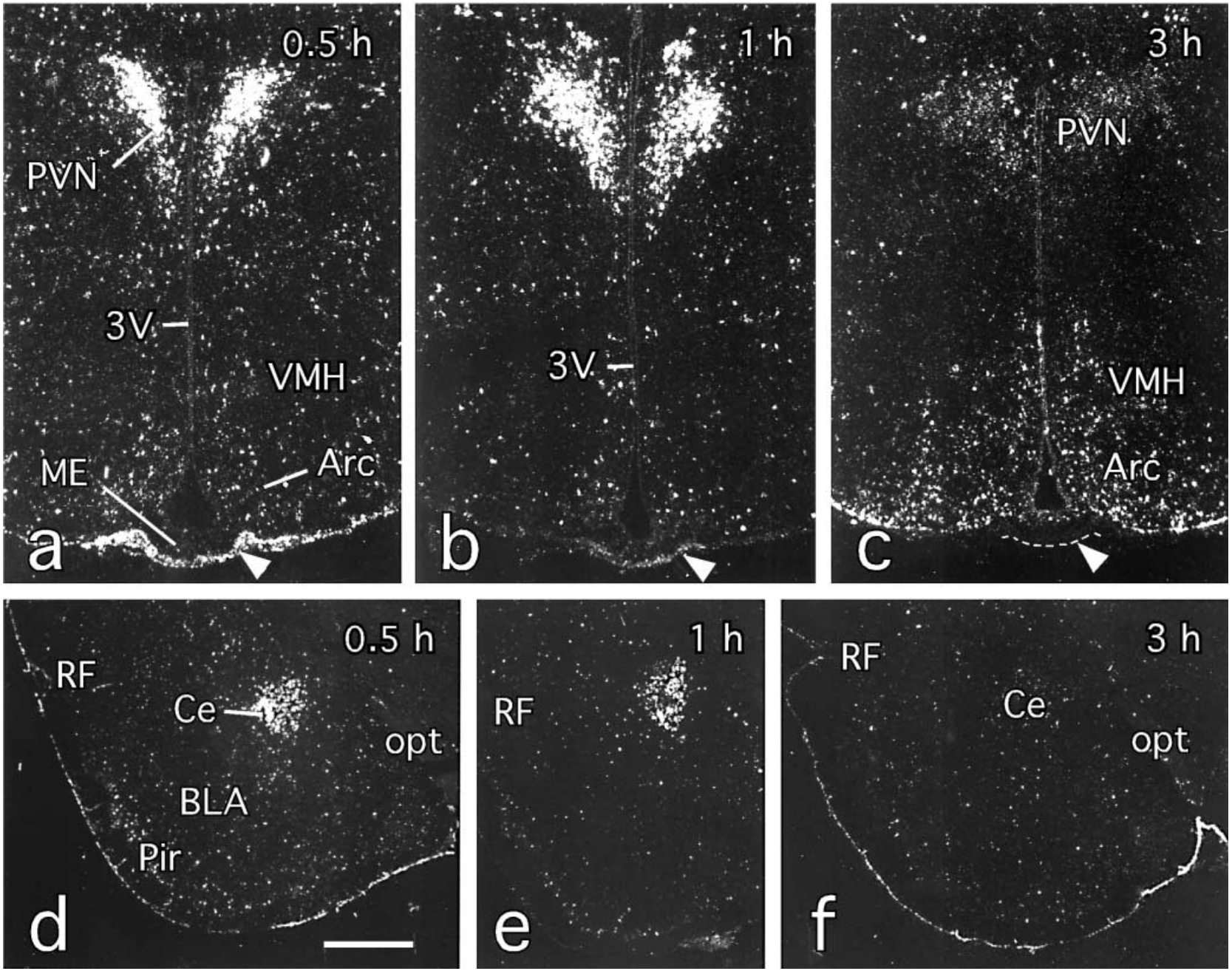
0.5 h
PVN
3V
VMH
ME
Arc
a
1 h
3V
b
3 h
PVN
VMH
Arc
c
0.5 h
RF
Ce
opt
BLA
Pir
d
1 h
RF
e
3 h
RF
Ce
opt
f

Figure 1

that clearly exceeded the levels seen in the control material for each time point. Notably, patterns of non-neuronal labeling were never observed in any of the control material.

Time course studies

Labeling at 0.5 hour. In the first experiment, the dose of i.v. IL-1 was either 2 (n = 2) or 10 µg/kg (n = 2), and the survival time was 0.5 hour. Similar patterns and intensities of *c-fos* mRNA labeling were seen at the two doses and in all animals. Subsequently in two experiments, a single dose (4 µg/kg) was administered, and the animals were killed 0.5 (n = 4), 1 (n = 7), or 3 hours later (n = 7).

The 0.5 hour time point was characterized in all animals by greatly elevated *c-fos* mRNA expression in discrete neural structures, all CVOs examined, and a collection of non-neuronal cells associated with the BBB. Saline-injected animals (n = 3) showed no induction of message in these locations. Labeled cells were identified as neurons if they satisfied the following criteria: they showed indistinct outer membranes, were very pale-staining or had no visible staining at all (these Nissl-staining characteristics are the consequence of the RNase treatment), were greater than 8–10 µm in diameter, and were organized in locations and patterns that reflected the underlying neuronal architecture. In cases of silver grain accumulations without any visible underlying stain, the spread of silver grains was scrutinized; the criterion for neuronal labeling was a diffuse distribution of grains with an overall diameter of approximately 20 µm. Labeling concentrated over smaller cells (clear borders and more darkly stained, presumably glial or endothelial), in contrast, had both a smaller diameter of distribution and a sharper decline in grain density from the central source of radioactivity. Neural structures that were consistently (in all cases) and strongly labeled at 0.5 hour post-IL-1 injection were the hypothalamic paraventricular nucleus (PVN; Fig. 1a), central nucleus of the amygdala (Ce; Fig. 1d), the caudal portions of the NTS (Fig. 2a,c), and the dorsolateral bed nucleus of the stria terminalis (BST; Fig. 3a). In the PVN, the elevated message levels were largely confined to the parvicellular portion, though at higher doses in some animals, the magnocellular portion was also labeled. In the NTS, the label was confined mainly to the medial and commissural subnuclei (Kalia and Sullivan, 1982). In all of these locations, the criteria for neuronal labeling were met.

All of the examined CVOs showed distinct patterns of *c-fos* mRNA labeling at 0.5 hour. Labeling in the OVLT was concentrated over the outermost cells of the meninges at the ventral and especially rostral margins of the OVLT, and also over the midline strip of darkly staining small cells that appeared to be continuous at the rostral end with the labeled meningeal cells (Fig. 4a). Labeled cells lateral to this midline stripe were of two distinct types: vascular cells, identified as such because they were dark, small, round, or elongated, and typically arranged in small circular clusters (Fig. 4b), and occasional neurons, identified as such by their larger size and weak staining characteristics (not shown). Labeling was present in medial preoptic area neurons adjacent to the OVLT, but neuronal labeling was seen also in control material. In the subfornical organ (SFO), cell labeling was confined mostly to scattered small, dark-staining cells, some of which could be identified as vascular due to their clustering (Fig. 5a,c). Labeling was more abundant on the lateral margins of the SFO where a rich vascular plexus innervates the adjacent choroid plexus. Small blood vessels dorsal to the SFO, in the ventral hippocampal commissure, were also labeled (Fig. 5e). In the ME, cells forming the thick outer meningeal wall were labeled, as were cells forming small penetrating blood vessels (Fig. 6a). The ME proper was not labeled. In the AP, dispersed cells were labeled (Fig. 2a), and pale staining as well as darker staining cells were *c-fos* mRNA-positive. We could not determine which cells were neuronal and which were non-neuronal.

Widespread labeling of meninges, blood vessels, choroid plexus, and other unidentified non-neuronal cells was prominent at 0.5 hour in all cases. Hybridization signals were localized over the outermost cells of the arachnoid membrane, most prominently along the ventral surface of the hypothalamus and amygdala (Fig. 7a), but everywhere else as well (Fig. 8a). Less intense but conspicuous pial *c-fos* mRNA labeling was found in all other subarachnoid cisterns and recesses, the outer surface of the brain (Fig. 8d), and the longitudinal cerebral fissure. Intensely labeled cells of the meninges and blood vessels appeared in folded meninges at the border between the medulla and cerebellum (Fig. 9a).

Blood vessels in the subarachnoid spaces and within the brain were labeled at 0.5 hour (Figs. 7a, 8d, 9a, 10). Labeled vessel types included surface veins and arteries, smaller venules (Fig. 10a) and arterioles (Fig. 10b), and capillaries. Not all blood vessels were labeled, however, so that an unlabeled vessel might be found in close proximity to a labeled vessel. In fact, most vessels were unlabeled. Prominently labeled vessels were those sectioned longitudinally and localized to the lateral hypothalamus, cerebral cortex, and ventral medulla. Vessels cut in cross- section were apparent in many edge sites along the surface epithelium. Examples are the vessels at the dorsal edge of the thalamus bordering the hippocampus and in the folds of the cerebellum. In larger vessels where cells types could be discerned, labeling in the lumen of the vessels was in the endothelium and occasionally the muscular coat, identified by the very elongated shape of its cells (Fig. 10b).

The choroid plexus in all locations examined was lightly labeled at 0.5 hour (Fig. 11a). Only a minority of choroid plexus cells were labeled (Fig. 11b). Grain densities over choroid plexus cells were approximately one-fifth the densities counted over intensely labeled cells of the PVN (data not shown). Labeled cells appeared to be both vascular endothelial and choroidal epithelial types (Fig. 11b).

Fig. 1. Darkfield photomicrographs of *c-fos* mRNA-positive cells in coronal brain sections taken at the level of the paraventricular nucleus (PVN) of the hypothalamus (**a–c**) and central nucleus of the amygdala (Ce; **d–f**). The levels in a–c and d–f correspond to -2.12 and -2.56 mm from bregma, respectively (Paxinos and Watson, 1986). Time points after i.v. IL-1 injection are 0.5 hour (a, d), 1 hour (b, e), and 3 hours (c,f). White spots are *c-fos* mRNA-positive cells in the brain (concentrated in the PVN and Ce at 0.5 and 1 hour and in the vicinity of the arcuate nucleus [Arc] at 3 hours) and along the outer margins of the brain, as described in the text. Arrowheads in a–c point to the outer edge of the median eminence (ME), which is outlined for emphasis in c. 3V, third ventricle, BLA, basolateral nucleus of amygdala; opt, optic tract; Pir, piriform cortex; RF, rhinal fissure; VMH, ventromedial nucleus of hypothalamus. Scale bars = 0.5 mm for a–c,1 mm for d–f.

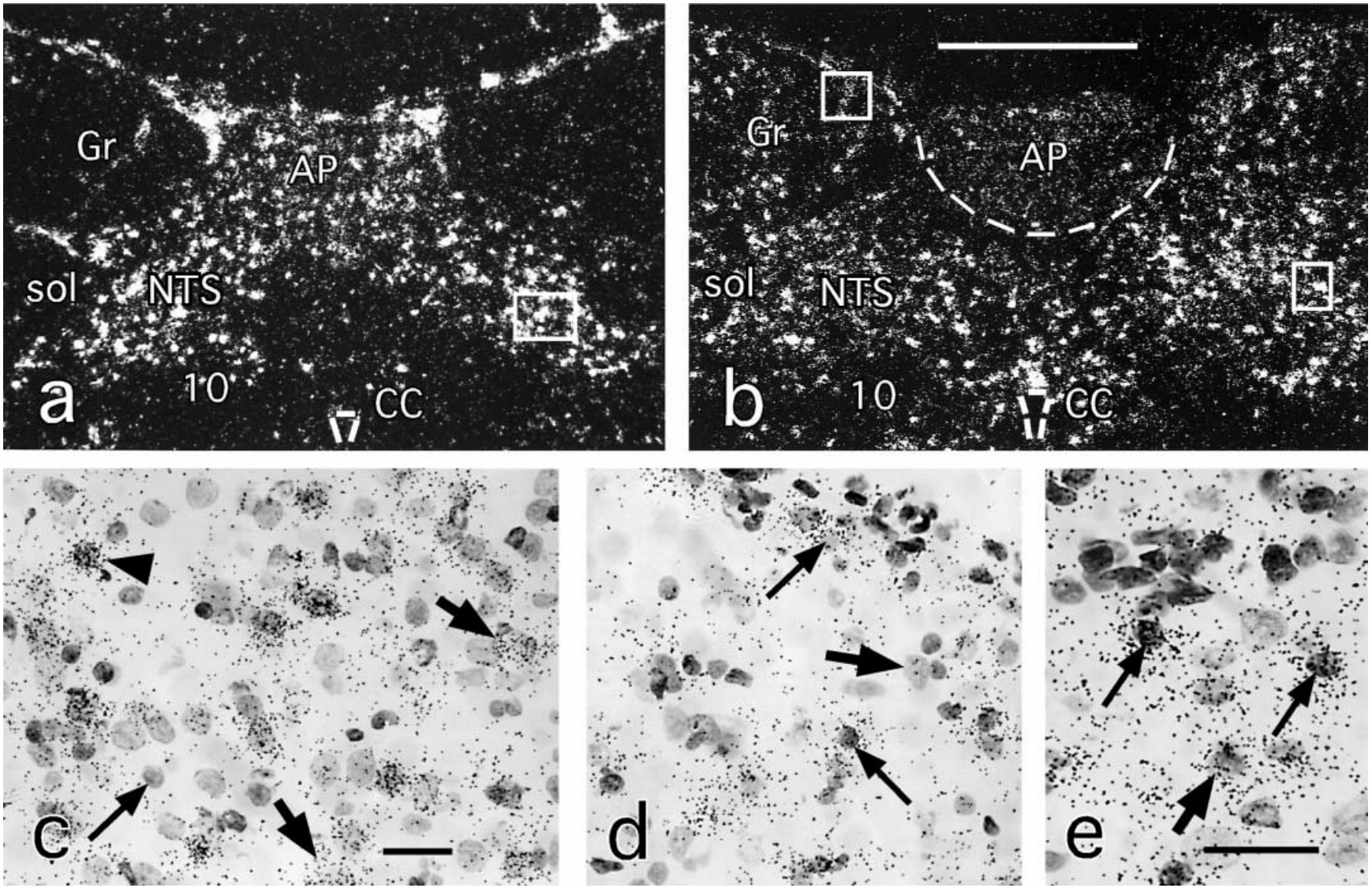

Fig. 2. Photomicrographs at the level of the area postrema (AP) and nucleus of the solitary tract (NTS), at -13.80 from bregma (Paxinos and Watson, 1986), showing *c-fos* mRNA-positive cells. At 0.5 hour (**a**,**c**), labeled cells in the NTS (c; location of scene is marked with an box in a) are neurons (large arrow), cells of indeterminate phenotype (arrowhead), and not smaller non-neuronal cells (small arrows), based on size and staining intensity. At 3 hours (**b,d,e**), cell labeling has spread beyond the NTS into adjacent nuclei such as the gracile nucleus (Gr); in the Gr (location indicated by box in b) labeled cells are mostly small non-neuronal cells (small arrows in d) and not larger neuronal cells (large arrow). In the NTS at 3 hours (e; location marked by box in b), labeled cells are non-neuronal (small arrows) and neuronal (large arrow in e). CC, central canal; sol, solitary tract; 10 dorsal motor nucleus of vagus. Dashed line in b marks the border between the AP and the NTS. Scale bars = 0.5 mm for a,b, 25 µm for c,d, 25 µm for e.

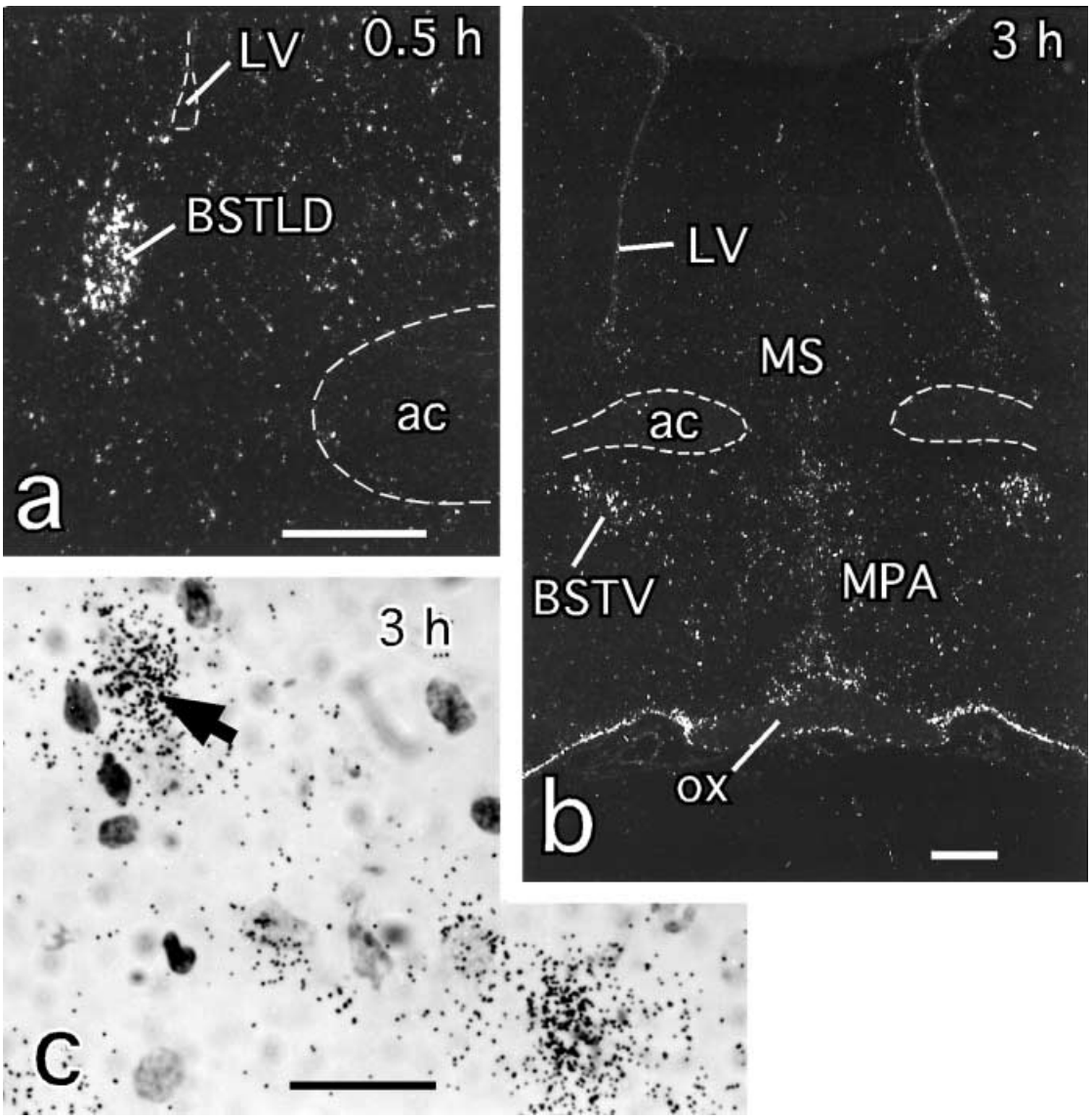

Fig. 3. Photomicrographs of *c-fos* mRNA labeling at the level of the bed nucleus of the stria terminalis (BST) at 0.5 hour (**a**) and 3 hours (**b,c**) after i.v. interleukin-1β (IL-1) injection. The angle of section is skewed relative to the atlas of Paxinos and Watson (1986): in b, the BST is at the atlas level of -0.26 mm, and the optic chiasm (ox) is at the level of -0.80 mm; in a, the BST is slightly more caudal. At 0.5 hour, labeling appears in the dorsolateral BST (BSTLD). At 3 hours, labeling appears below the anterior commissure, in the ventral portion of the BST (BSTV), and the marked cells are neurons (large arrow in c). Dashed lines mark the anterior commissure (ac) and the location of the lateral ventricle (LV). MPA, medial preoptic area; MS, medial septum. Scale bars = 0.5 mm for a,b, 25 μm for c.

Endothelial cells were more rounded and formed circular patterns within the choroid plexus; choroidal epithelial cells were slightly more squared and formed strings of cells moving away from the vascular elements. Anchor points containing the larger vessels were typically the most densely labeled.

Labeling at 1 hour. The pattern of *c-fos* mRNA labeling at 1 hour after i.v. IL-1 injection was dramatically different than it had been at 0.5 hour, except in neurons. At 1 hour, *c-fos* mRNA signals in the neural structures, NTS, Ce, dorsolateral BST, and PVN, were very nearly identical to those seen at 0.5 hour (Fig. 1b,e).

In the CVOs at 1 hour, patterns and densities were altered in organ-specific fashions. Compared with the labeling seen at 0.5 hour, less label was observed in the OVLT, ME, and AP. Much of the loss of labeling in these CVOs was due to the disappearance of vascular labeling within the organs. At 1 hour after i.v. IL-1 injection, labeling was not apparent over the darkly staining meningeal cells of the OVLT nor on the cells comprising the midline stripe of the OVLT proper (Fig. 4c,d). Labeled cells satisfying the criteria for neurons were distributed in the body of the OVLT as well as in the territory surrounding the OVLT, especially in the ventrolateral corner of the medial preoptic area at caudal levels of the OVLT. A similar distribution of cells was seen in the saline-injected animals. The SFO showed a different pattern of labeling than it had at 0.5 hour; labeled cells occupied the center of

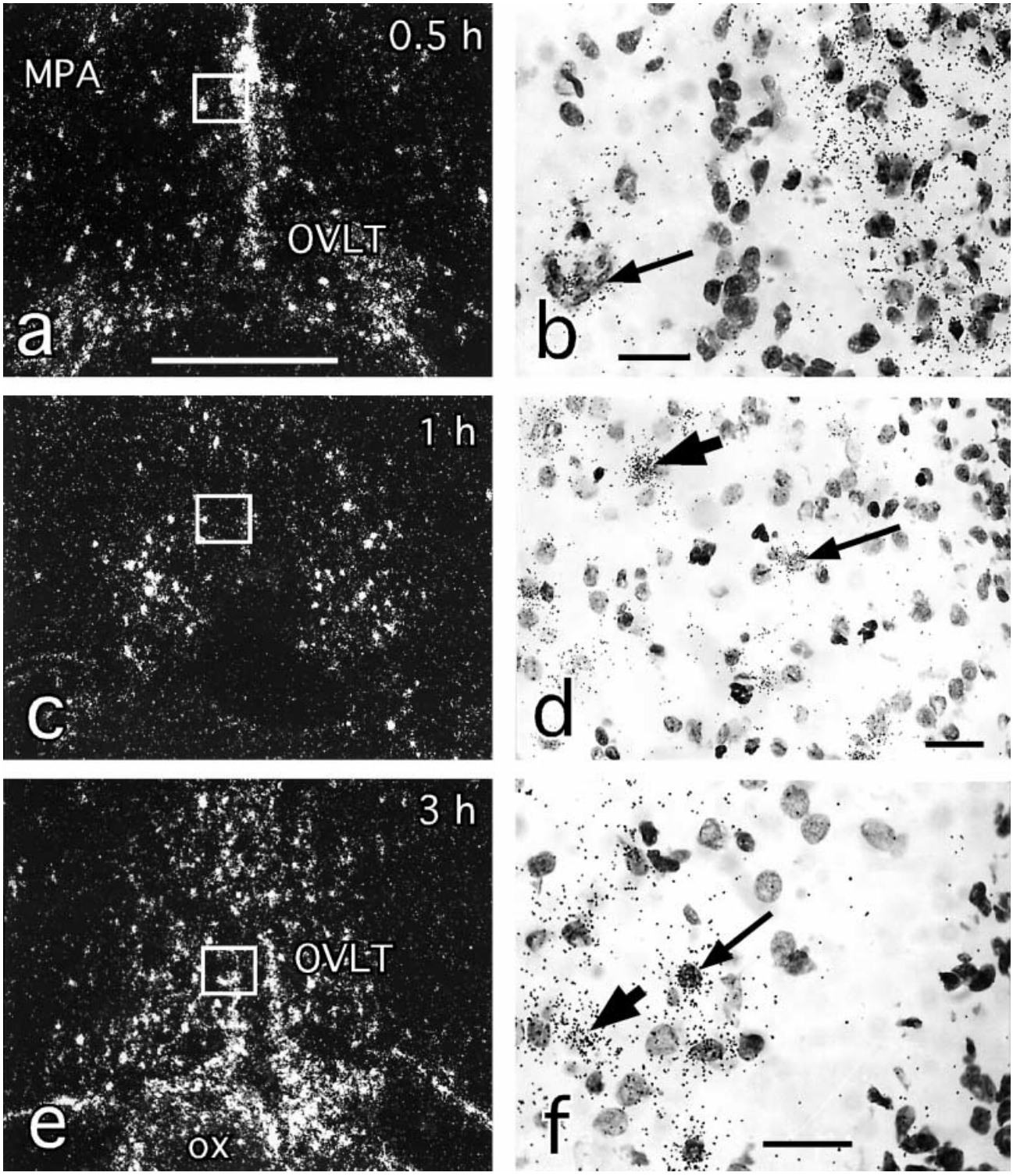

Fig. 4. Photomicrographs of *c-fos* mRNA labeling in the organum vasculosum of the lamina terminalis (OVLT) at 0.5 (**a,b**), 1 (**c,d**), and 3 (**e,f**) hours after i.v. interleukin-1β (IL-1) injection. The level is -0.20 mm from bregma (Paxinos and Watson, 1986). High-magnification scenes in b,d,f are taken from the same sections photographed at low magnification in a,c,e, respectively, and are marked by boxes. The midline is along the right margin in each. Cells labeled at 0.5 hour are the dark cells on the midline and small non-neuronal cells, including those comprising blood vessels, as seen at the left of b and marked by the arrow. At 1 hour, though absolute determination is impossible, both non-neuronal cells and neurons appear to be labeled (small arrow and large arrow, respectively, in d). The same is true at 3 hours (f). Note the complementary labeling patterns in a/b and e/d. MPA, medial preoptic area; ox, optic chiasm. Scale bars = 0.5 mm for a,c,e, 25 μm for b,d,f.

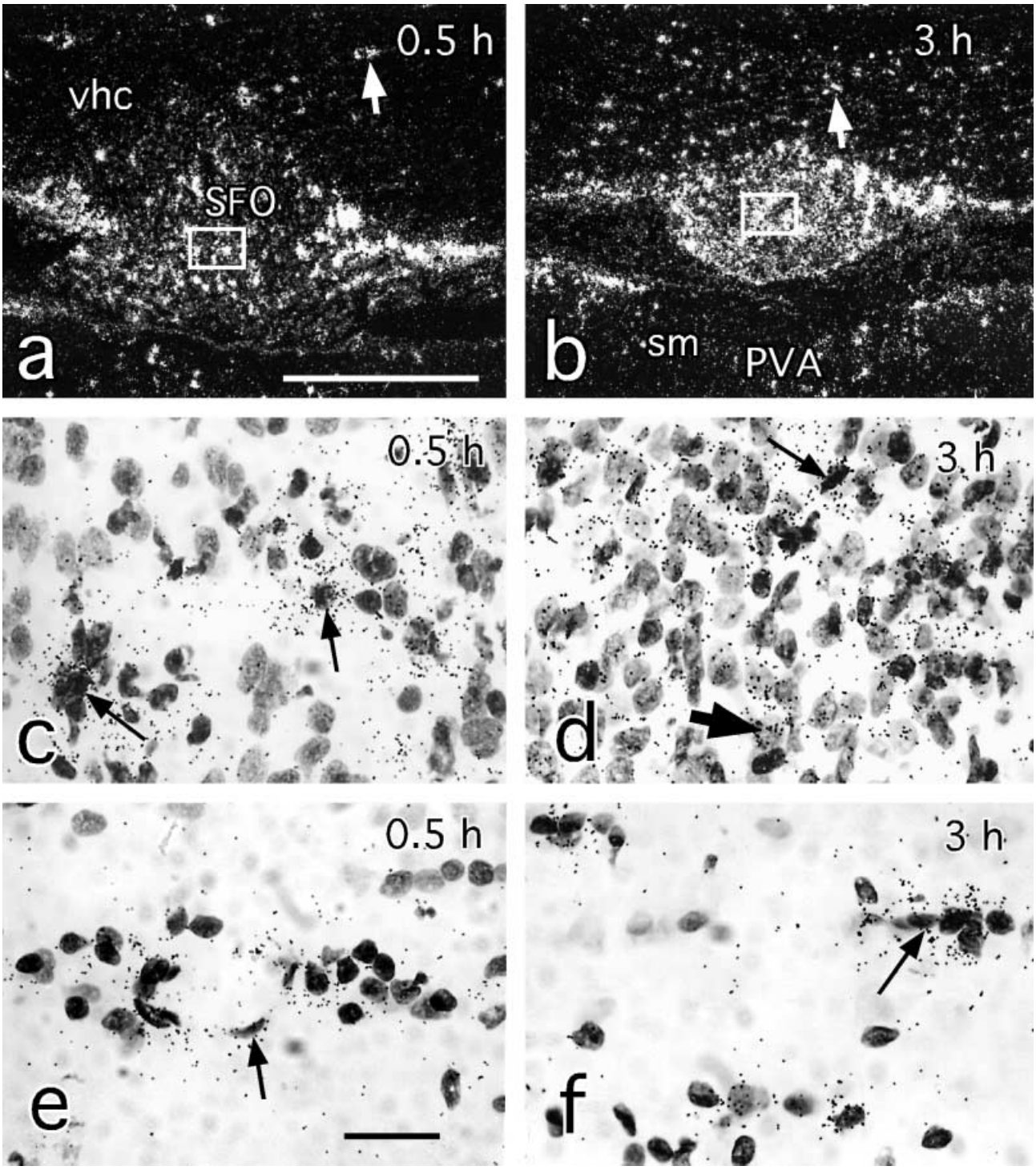

Fig. 5. Photomicrographs of *c-fos* mRNA labeling in the subfornical organ (SFO) and ventral hippocampal commissure (vhc) at 0.5 hour (**a,c,e**) and 3 hours (**b,d,f**) after i.v. interleukin 1β (IL-1) injection. Coronal section is at the level of -1.30 mm behind bregma (Paxinos and Watson, 1986). At 0.5 hour, labeling in the SFO is largely confined to very small cells which are typically part of blood vessels (arrows in c; location in SFO marked by box a). Cells of a blood vessel in the white matter are shown in e (arrow; location marked by large arrow in a). At 3 hours in the SFO, both very small cells (small arrow in d) and larger cells (large arrow in d) are labeled. These may be endothelial cells and unidentified neuronal or non-neuronal cells, respectively. Location of d is marked by box in b. In the white matter (f; located by arrow in b), small cells are labeled, and some may be oligodendroglia by their appearance (arrow in f). PVA, anterior paraventricular thalamic nucleus; sm, stria medullaris. Scale bars = 0.5 mm for a,b, 25 μm for c–f.

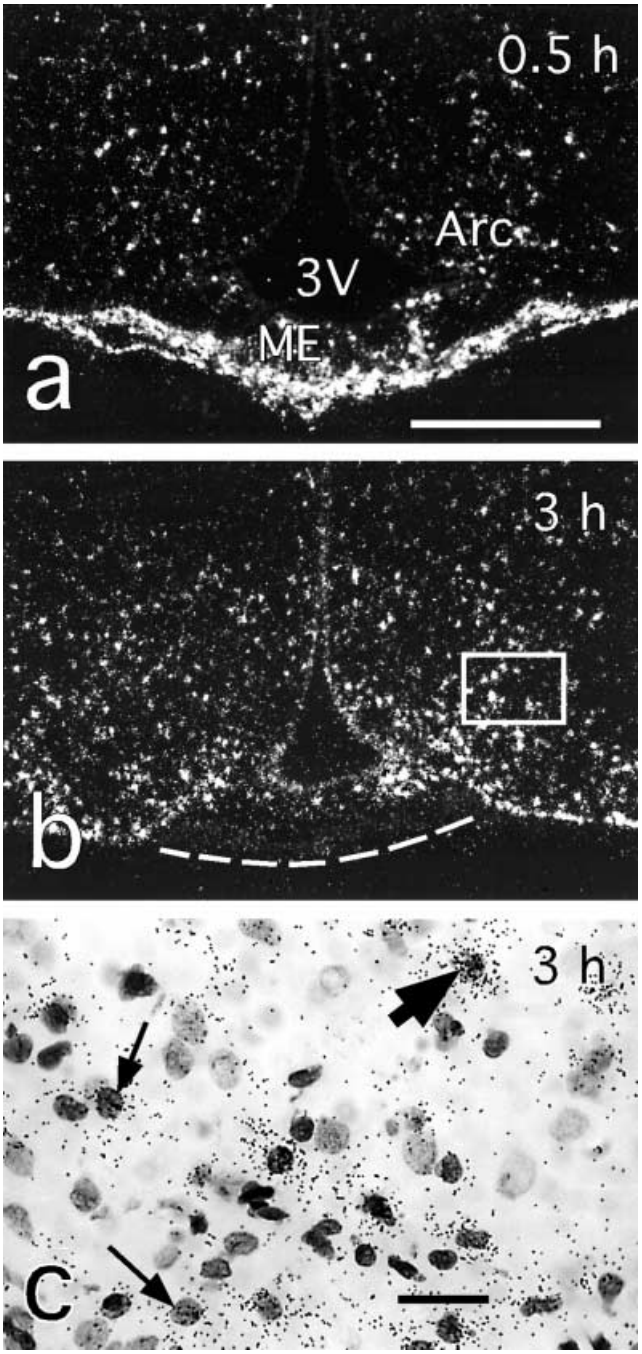

Fig. 6. Photomicrographs of *c-fos* mRNA labeling at the level of the median eminence (ME), third ventricle (3V), and arcuate nucleus (Arc) at 0.5 hour (**a**) and 3 hours (**b,c**) after i.v. interleukin 1β (IL-1) injection. The level is -2.30 mm from bregma (Paxinos and Watson, 1986). At 0.5 hour, prominent labeling appears in the meninges at the ventral surface of the brain. At 3 hours, labeling has shifted to locations inside the brain. In the Arc, labeled cells are small, non-neuronal cells (small arrows in c; location marked by large box in b) and some apparent neurons (large arrow in C). Dashed line in b marks the external border of the ME. Scale bars = 0.5 mm for a,b, 25 μm for c.

the organ and were of a uniform round, medium-staining type. The ME was unlabeled (Fig. 1b). The AP was slightly less intensely labeled than it had been at 0.5 hour. The cell types that were labeled remained the same, except that the meninges and blood vessels were unlabeled.

Labeling patterns of *c-fos* mRNA in the barrier regions showed the most dramatic departure from the patterns seen at 0.5 hour. Specifically, message levels almost disappeared at the brain's edge (Figs. 1b,e, 7c,d, 8b). In the meninges, labeling over the outermost arachnoid was absent, though some residual labeling of the pia was still evident. Labeling in the choroid plexus was absent, as was most vascular labeling.

Labeling at 3 hours. At 3 hours after i.v. IL-1 injection, another dramatic change occurred in the pattern of *c-fos* mRNA labeling, and the change occurred in both neurons and non-neuronal cells. Neuronal labeling was very sparse in the PVN, Ce, and dorsolateral BST (Fig. 1c,f). At the brain's edge, which had been nearly unlabeled at 1 hour, a second wave of *c-fos* mRNA labeling appeared (Figs. 1f, 8c). However, the labeled cells were not the same as those that had been labeled at 0.5 hour. The arachnoid and pia were not labeled at most locations; instead, a row of cells just inside the pia was strongly labeled (Figs. 7e, 8e, 9b). The labeled cells were typically medium-sized, round, and lightly stained (Figs. 7f, 9b). Whereas the cells appeared by morphological criteria to resemble neurons, they were slightly darker staining than neurons, and they resided in the superficial glial membrane (glia limitans), which is the line of astrocytes just beneath the pia. In some of the recesses of subarachnoid cisterns, the labeled cells comprised the pia itself, whereas in other areas the labeled cells were adjacent to the small, darkly staining meningeal cells. For example, edge labeling on the dorsal surface of the thalamus was confined to the pial cells, but in the ventrolateral mesencephalic tegmentum, cells just inside the pia were labeled (Fig. 9c). Wherever fiber tracts were invested with meninges, labeling appeared in the superficial glial membrane just inside the pia (e.g., optic chiasm, Fig. 3b; ventral hippocampal commissure and stria medullaris, Fig 5b; and the spinal trigeminal and spinocerebellar tracts).

At 3 hours after i.v. IL-1, *c-fos* mRNA-labeled cells occupied the same circumscribed area of OVLT and adjacent ventromedial preoptic area (Fig. 4e) as had been labeled at the 1 hour time point (Fig. 4c). Labeling was apparently over both neuronal and non-neuronal cells (Fig. 4f). The SFO was more densely labeled than it had been at earlier time points (Fig. 5b); a greater percentage of cells was labeled, and small, dark-staining cells were labeled (Fig. 5d). Cells in the ventral hippocampal commissure dorsal to the SFO were also labeled These cells were not the vascular cells that had been labeled at 0.5 hour, and some appeared to be oligodendroglia because they were parts of strings of small, round cells arranged in the direction of the fiber flow (Fig. 5f). The ME showed some labeling of cells in the inner lamina, and these were coextensive with labeled cells in the arcuate nucleus (Figs. 1c, 6b). In the arcuate, many of the labeled cells were round and more darkly staining, suggesting that they were non-neuronal (Fig. 6c).

At the level of the arcuate nucleus and ME, ^{35}S-labeled *c-fos* and digoxygenin-labeled GFAP ribonucleotide probes were coapplied to brain sections from animals given i.p. IL-1 3 hours prior. Micrography revealed that a number of digoxygenin-reactive cells in the ME and arcuate, visible in brightfield illumination (Fig. 12a), also contained silver grains, visible in darkfield illumination (Fig. 12b), indicat-

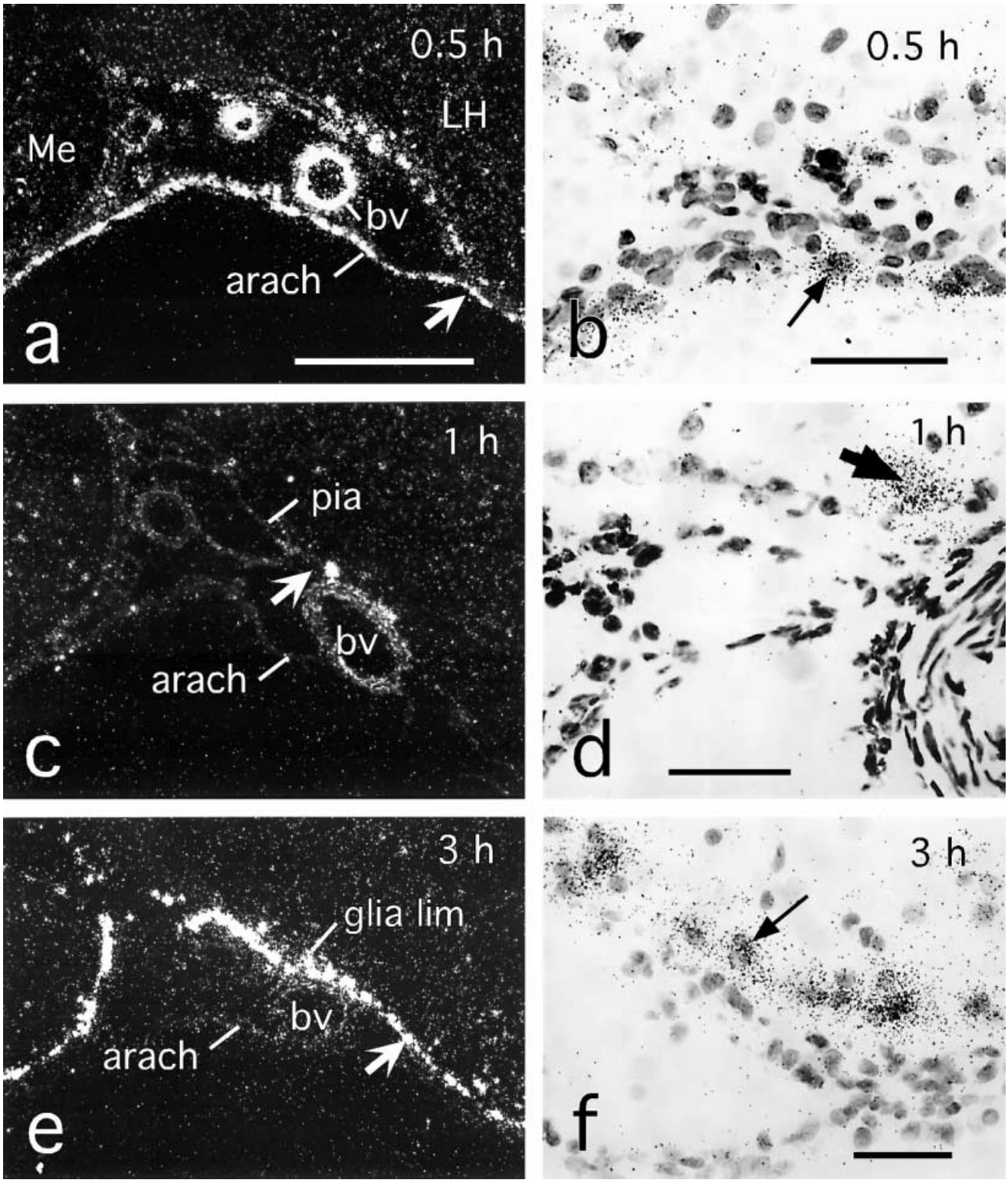

Fig. 7. Photomicrographs of *c-fos* mRNA labeling at the junction of the lateral hypothalamus (LH) and medial amygdala (Me) on the ventral surface of the brain at -2.56 mm from bregma (Paxinos and Watson, 1986). Midline is to the right. At 0.5 hour after i.v. interleukin 1β (IL-1; **a**,**b**), cell labeling appears along the outer surface of the arachnoid membrane and in large blood vessels, both arteries and veins, in the subarachnoid space. Arrow in b points to a labeled arachnoid cell. Its location is marked by an arrow in a. At 1 hour (**c**,**d**), no cells are labeled at the edge, except for a rare cell, such as the large cell, probably a neuron, seen in c and d (arrow). At 3 hours (**e**,**f**), the meninges and blood vessels are not labeled, but the cells of the glia limitans (glia lim) are labeled (arrow in f; location marked by arrow in e). arach, arachnoid; bv, blood vessel; pia, pia mater. Scale bars = 0.5 mm for a,c,e, 50 μm for b,d,f.

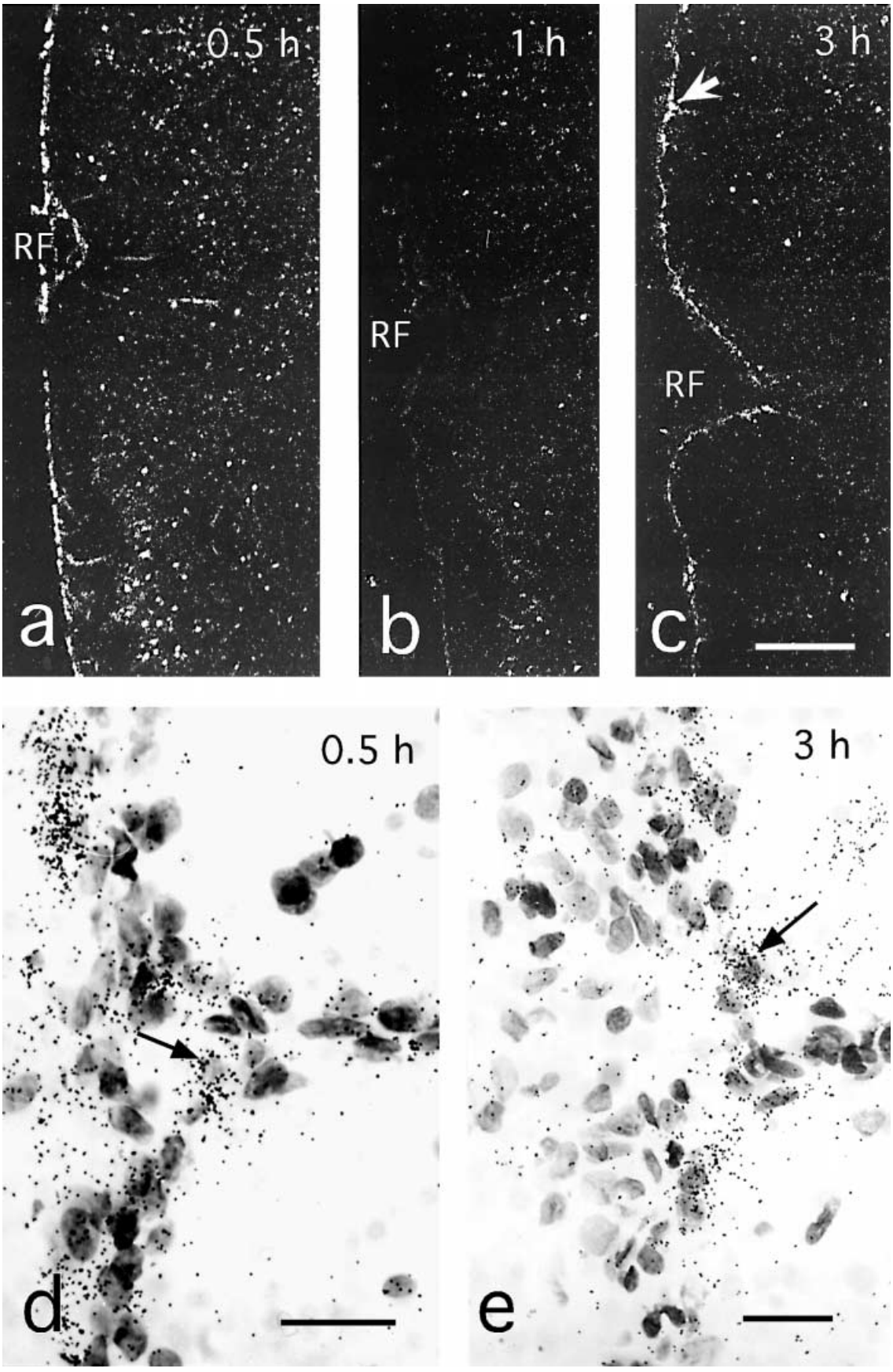

Fig. 8. Photomicrographs of *c-fos* mRNA labeling in cerebral cortex at 0.5 (**a**,**d**), 1 (**b**), and 3 hours (**c,e**) after i.v. interleukin 1β (IL-1) injection, at approximately -3.1 mm behind bregma. At 0.5 hour, outer meninges and small surface and penetrating blood vessels are labeled. Arrow in d points to a labeled non-neuronal cell alongside a small vessel. At 1 hour, labeling is absent from the surface (b). At 3 hours, labeling appears in the pia and in perivascular cells; arrow in e marks perivascular cell, in the location marked by arrow in c. Note somewhat complementary patterns of cell labeling in d and e. RF, rhinal fissure. Scale bars = 0.5 mm for a–c, 25 µm for d,e.

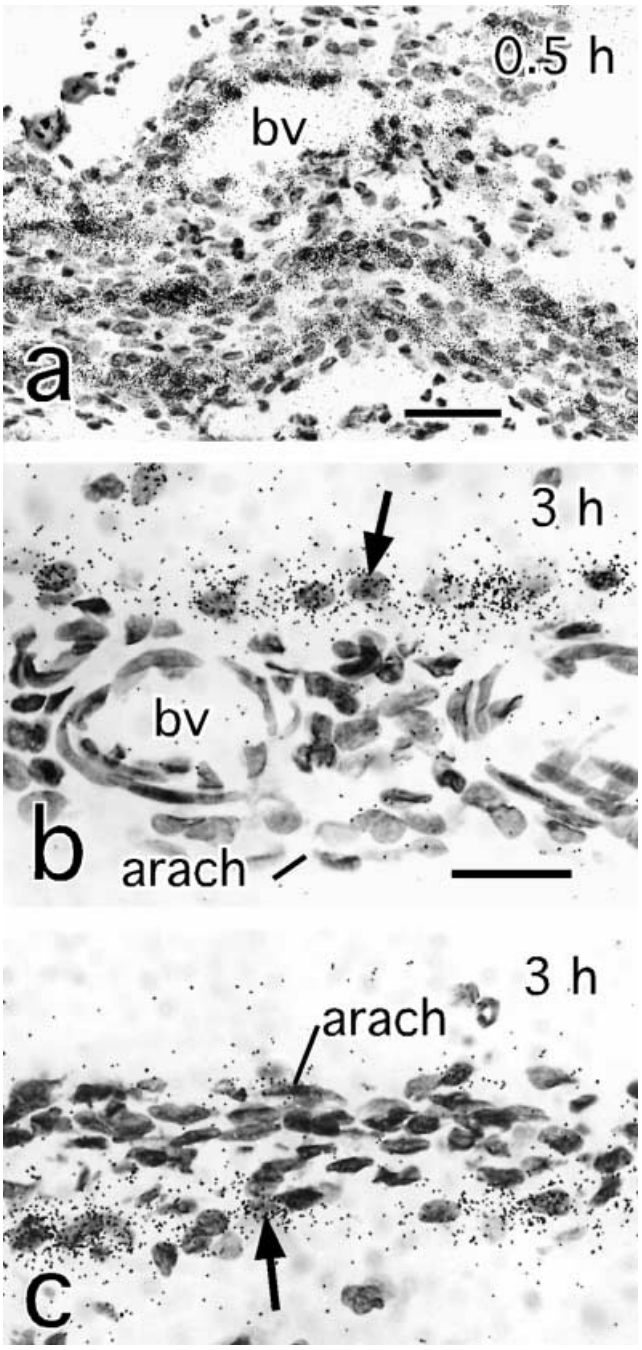

Fig. 9. Brightfield photomicrographs of meningeal labeling at 0.5 hour (**a**) and of perimeningeal labeling (**b,c**) at 3 hours after i.v. interleukin 1β (IL-1) injection. A striking example of the selectively responsive cells in the meninges is shown in a, which is taken from the lateral surface of the medulla (-12 mm behind bregma) where the meninges appear as folded sheets. Labeled cells comprise a portion of the sheet, including the walls of the vein at the top of the picture. At 3 hours, the cells of the glia limitans are clearly labeled at the level of the medial amygdala, approximately -2.56 mm behind bregma (b) and ventrolateral mesencephalon (c; mesencephalon is at the bottom, hippocampus is at the top of picture, at -6.04 mm behind bregma). Arrows in b and c point to labeled cells of the glia limitans. In b, note that blood vessels and the subarachnoid trabeculae are not labeled. Scale bars = 50 μm for a, 25 μm for b,c.

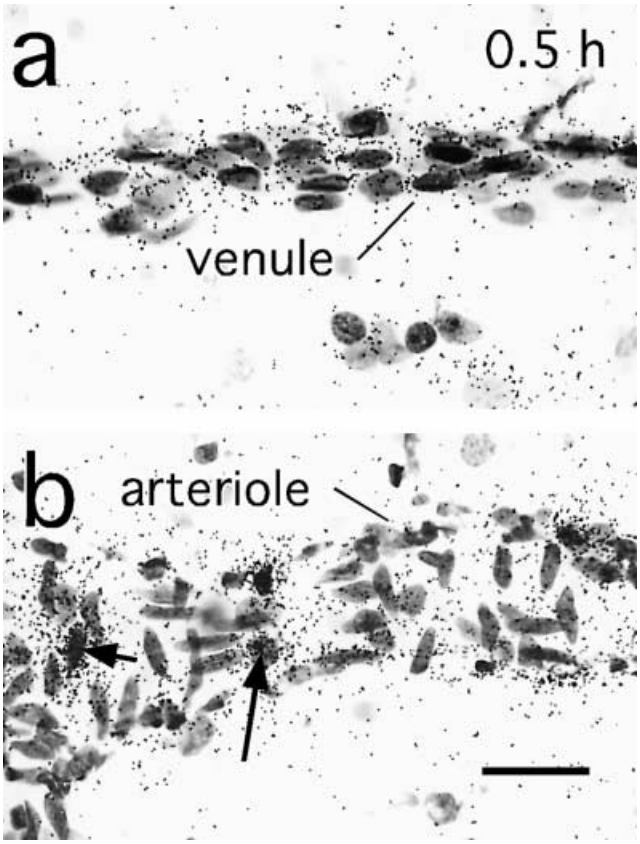

Fig. 10. High-magnification brightfield photomicrographs of *c-fos* mRNA-positive endothelial cells in blood vessels inside the brain at 0.5 hour after i.v. interleukin 1β (IL-1) injection. Both venules (**a**) and arterioles (**b**) are labeled. In the arteriole, both elongated smooth muscle (small arrow) and round endothelial cells (long arrow) are labeled. Scale bar = 25 μm for a,b.

ing the presence of *c-fos* mRNA in astrocytes. At high magnification, most of the GFAP mRNA-positive cells in the ventral portion of the arcuate were also *c-fos* mRNA-positive, and vice versa (Fig. 13b). Using an arbitrary criterion of fairly strong labeling intensity for both markers, it was determined by cell count analysis that approximately 80% of the *c-fos* mRNA-positive cells were also strongly GFAP mRNA-positive. In the dorsomedial and dorsolateral portions of the arcuate, the colocalization was reduced to about 40% due to the fall off of strongly GFAP mRNA-positive cells. Additionally, numerous GFAP mRNA-positive cells lined the edge of the brain, extending laterally to the optic tract, and most of these were also *c-fos* mRNA positive (Fig. 13a).

The AP showed greatly reduced levels of labeling at 3 hours after i.v. IL-1 (Fig. 2b). The region of the caudal NTS surrounding the AP contained labeled cells occupying a larger territory than was seen at the earlier time points (Fig. 2b). Cells were positive throughout the NTS and also in the adjacent nuclei (dorsal motor nucleus of the vagus, hypoglossal nucleus, and gracile nucleus). The labeled cells in the NTS appeared to be fairly darkly staining and small to medium- sized (Fig. 2e). Similarly, in the gracile nucleus, labeled cells were mostly of the small, darkly staining variety and did not appear to be neurons (Fig. 2d).

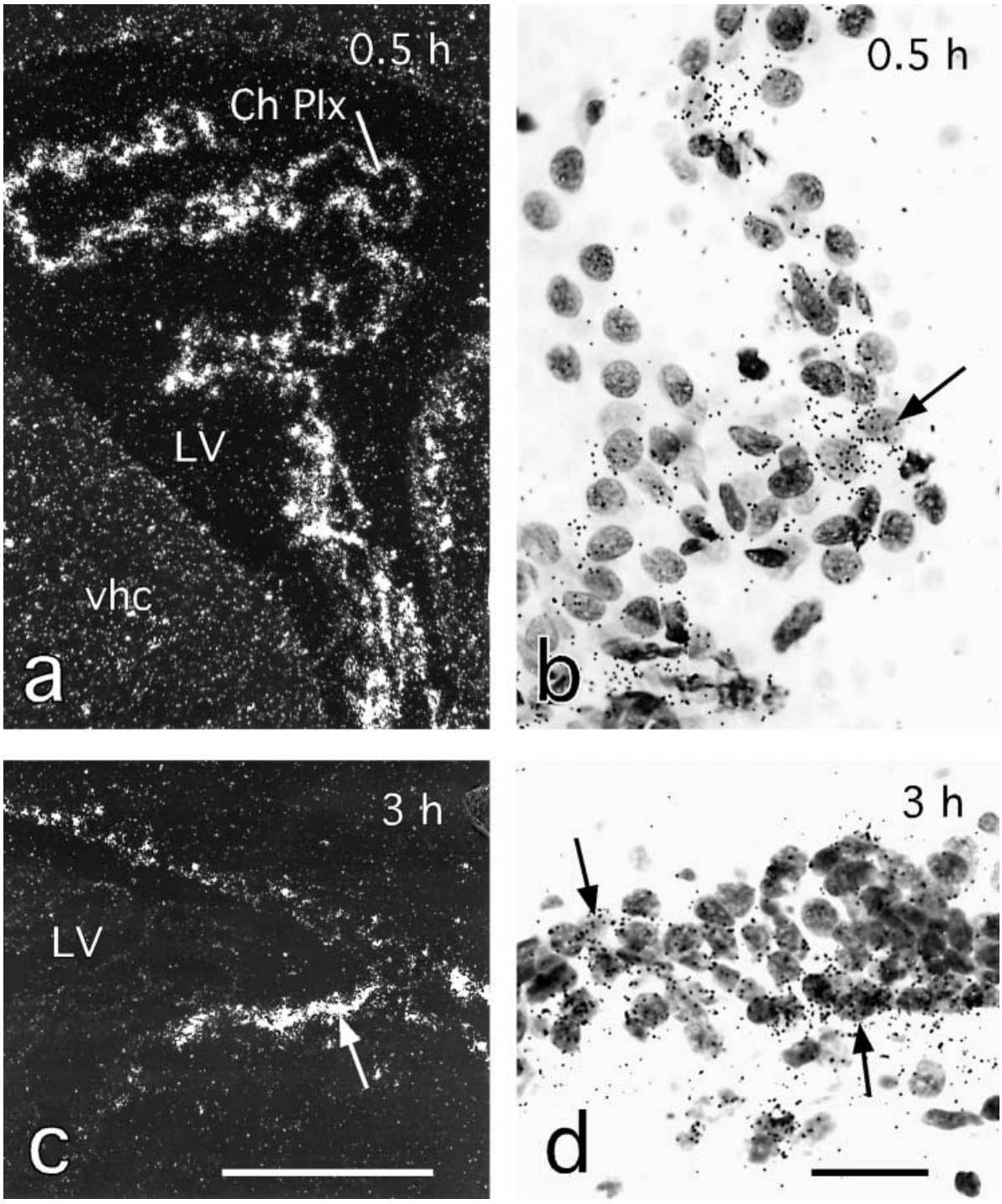

Fig. 11. Photomicrographs of *c-fos* mRNA labeling in the choroid plexus (Ch Plx) at the level of the ventral hippocampal commissure (vhc) 0.5 hour after i.v. IL-1 injection (**a**,**b**) and in the ependyma of the lateral ventricle (LV) at 3 hours (**c**,**d**). At 0.5 hour, labeled cells in the Ch Plx appear to be vascular endothelia and choroidal epithelia (arrow in b). At 3 hours, labeled ventricular cells are marked by arrows in d (location of cells marked by large arrow in c). Scale bars = 0.5 mm for a,c, 25 μm for b,d.

An additional non-neuronal cell type, the ependymabecame labeled with *c-fos* mRNA at 3 hours. Positive ependymal cells were found only in a few locations, namely lining the third ventricle at the level of the ME (Figs. 1c, 6b, 12b) and in the lateral ventricle, mainly its lateral part (Fig. 11c,d). Labeling of the choroid plexus was not apparent at 3 hours (Fig. 11c), and vascular labeling had largely disappeared.

At 3 hours, a new neural structure became labeled for the first time, the ventral BST (Fig. 3b,c). The ventral BST was labeled in all 3 hour cases examined (n = 7), but the intensity of labeling (expressed as numbers of labeled

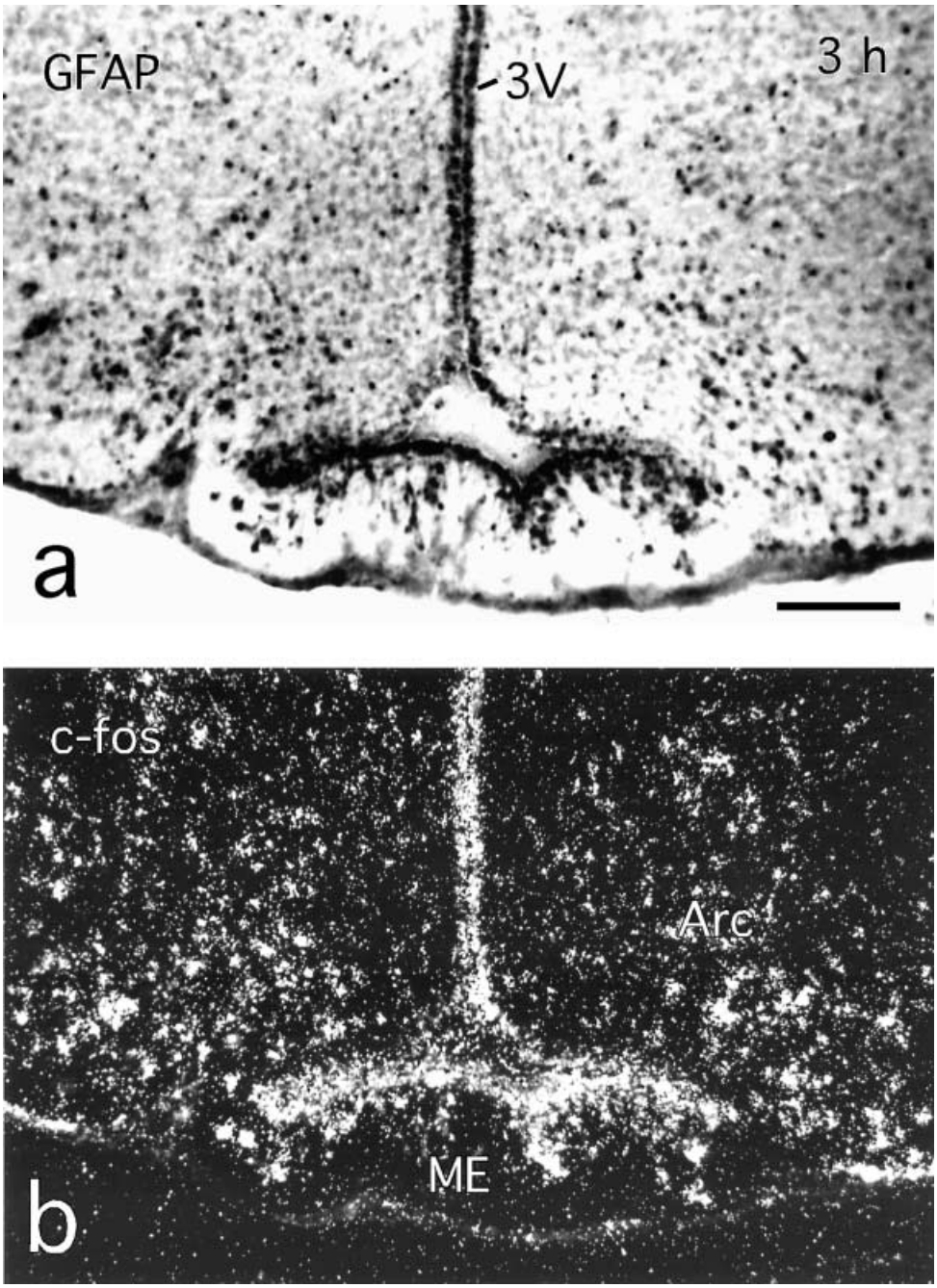

Fig. 12. Photomicrographs of the median eminence and arcuate nucleus in adjacent sections processed for glial fibrillary acidic protein (GFAP) mRNA using a digoxigenin-labeled ribonucleotide probe (**a**) and for *c-fos* mRNA using a ^{35}S-labeled ribonucleotide probe (**b**) at 3 hours after i.v. interleukin 1β (IL-1) injection. Illumination is brightfield in a and darkfield in b. Scale bar = 200 μm for a,b.

cells) varied from case to case. The level of intenisty shown in Figure 3 is representative. The labeled cells in the ventral BST were neurons, identified by the large diameter of the grain clusters and the very faint-to-nonexistent underlying Nissl staining. Smaller and more infrequently appearing labeled cells could not be specifically assigned a phenotype.

Labeling at 5 hours. At 5 hours after i.p. IL-1, almost no labeling differences between experimental (n = 6) and control (n = 6) animals were observed. Sporadic *c-fos* mRNA-positive cells in the glia limitans at the base of the hypothalamus were seen in the IL-1-injected animals (not shown).

Dose effects of i.v. IL-1

Dose study at 0.5 hour. An i.v. IL-1 dose range of 0.05 to 2.0 μg/kg was examined at the 0.5 hour survival time. The doses of 0, 0.5, 1.0, and 2.0 μg/kg were examined in the first experiment (n = 6 per group; total = 24), and the

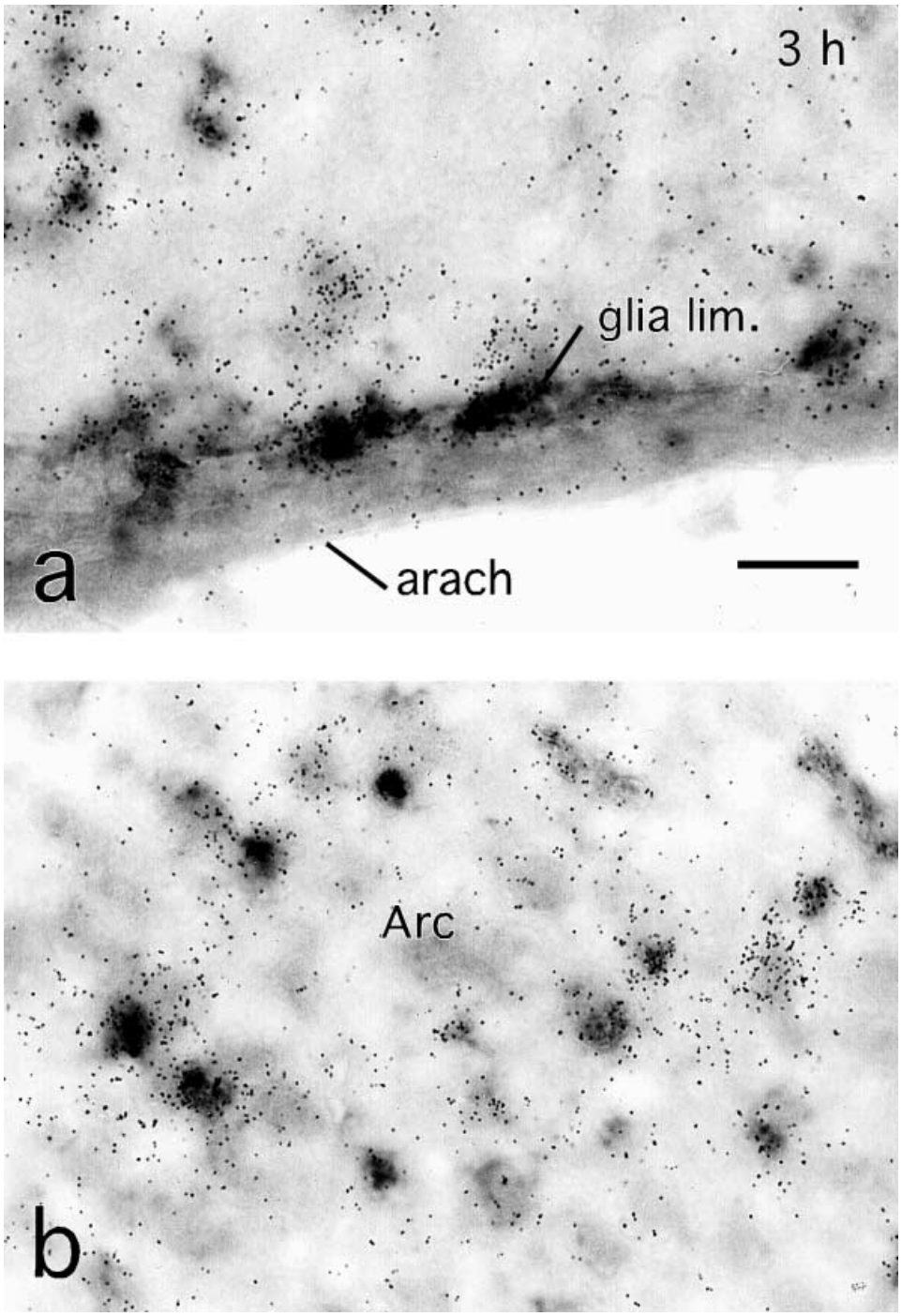

Fig. 13. High-magnification photomicrographs of the meninges at the ventral surface of the hypothalamus (**a**) and of the ventromedial arcuate (arc) nucleus (**b**) showing *c-fos* mRNA induction in astrocytes 3 hours after i.v. interleukin 1β (IL-1) injection. The tissue was processed simultaneously for glial fibrillary acidic protein (GFAP) mRNA using a digoxigenin-labeled probe and for *c-fos* mRNA using a ^{35}S-labeled probe. Double-labeled cells are those that show both dark reaction product and accumulation of silver grains. arach, arachnoid; glia lim., glia limitans. Scale bar = 25 μm for a,b.

doses of 0, 0.05, 0.10, 0.25, and 0.5 μg/kg were examined in the second experiment (n = 3–4 per group; total = 17). The dose that was common to both experiments, 0.5 μg/kg, produced strong *c-fos* mRNA and plasma ACTH and corticosterone elevations in the first experiment, and weak *c-fos* mRNA induction and no elevation in plasma levels of ACTH and corticosterone in the second experiment (Fig. 14). The reasons for this difference in sensitivity between the two experiments are not known. Induction of *c-fos* mRNA occurred at doses lower than 0.5 μg/kg, but not in

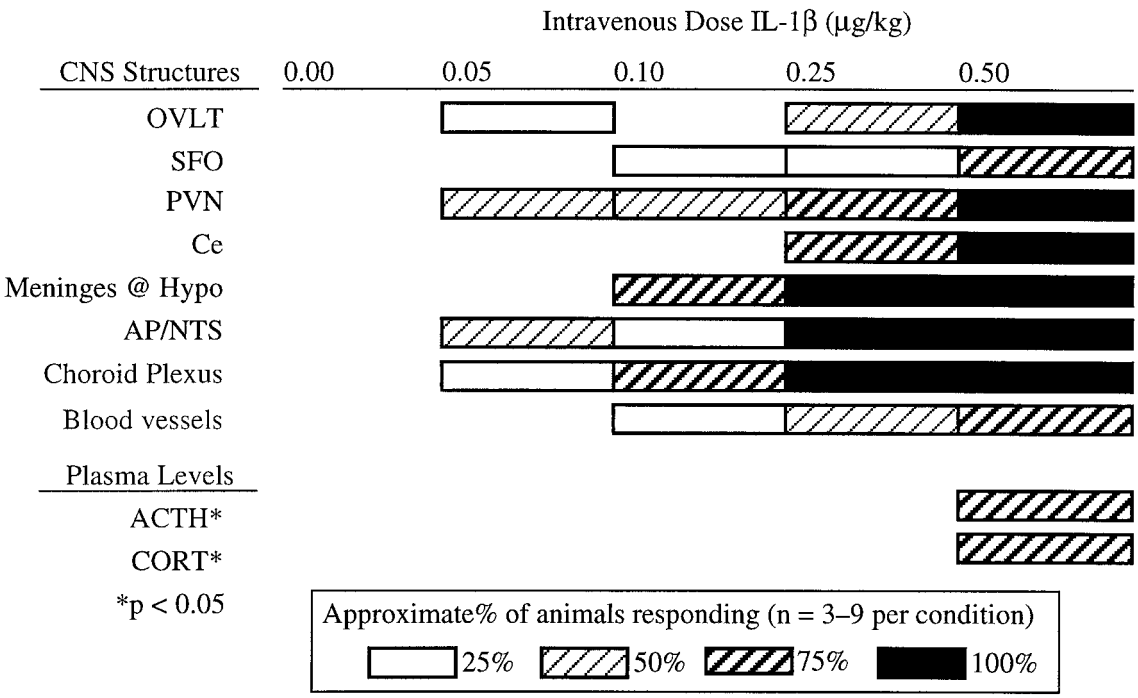

Fig. 14. Relationship of interleukin 1β (IL-1) dose to *c-fos* mRNA induction in various regions and cell types in the brain. A criterion of induction or no induction was established by microscopic examination of the regions or cell types in question and comparing the message levels with those in control animals. A positive response was scored if the message levels were consistently higher than those in controls in all sections containing the structure in question (or in representative sections in the case of blood vessels and choroid plexus). For plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) determinations, statistical analysis was performed on group data to determine significance, and then the number of animals showing the elevations was determined by individual analysis of data. AP/NTS, area postrema/nucleus of the solitary tract; Ce, central nucleus of amygdala; Hypo, hypothalamus; OVLT, organum vasculosum of the lamina terminalis; PVN, paraventricular nucleus of hypothalamus; SFO, subfornical organ.

all animals, and the intensity of labeling was barely above background. At the lowest dose given (0.05 μg/kg), there were almost no hybridization responses that were different from the saline control; 2 of 4 animals showed slightly elevated *c-fos* mRNA labeling in the PVN and AP/NTS region, and one showed some labeling of the meninges at the OVLT and the region of the lateral medulla. At 0.1 μg/kg, the choroid plexus and meninges were sparsely labeled in 3 of 4 animals. At 0.25 μg/kg, all animals showed meningeal and choroid plexus labeling, and all but one showed detectable message increases in the PVN and AP/NTS. At 0.5 μg/kg, the OVLT, PVN, Ce, and AP/NTS were consistently labeled in all 9 animals. Given the level of variability of response across animals at the lowest doses, it was not possible to determine whether any particular structure or cell type showed a different threshold for responding to i.v. IL-1. Both neuronal and non-neuronal labeling was sparsely present in some animals at the lowest dose examined (0.05 μg/kg). A summary of the 0.5 hour labeling patterns at the i.v. doses examined is given in Figure 14.

Dose study at 3 hours. In a final i.v. IL-1 experiment, doses of 0 (n = 1), 0.5 (n = 4), and 1.0 μg/kg (n = 1) were examined at the 3 hour time point. Only portions of the 3 hour pattern were visible in animals given either IL-1 dose. Neuronal labeling was not evident. The strongest relative labeling was in the SFO, where scattered cells were moderately positive, but no cells around the SFO were labeled. Barrier labeling was absent in most locations; it persisted at the OVLT and along the base of the medial amygdala. The arcuate nucleus and NTS were lightly labeled at 3 hours.

Comparison of *c-fos* and IL-1R1 mRNA patterns

To assess whether the pattern of cellular induction of *c-fos* mRNA could be explained by the presence of type 1 receptors for IL-1 on the activated cells, the IL-1R1 mRNA pattern in normal animals was examined (n = 3). IL-1-treated animals were not examined because IL-1R1 message levels are down-regulated in these cell types by prior IL-1 administration (Ericsson et al., 1995; Quan and Herkenham, unpublished data). Striking similarities and differences were noted. Confirming another report (Ericsson et al., 1995), IL-1R1 mRNA was discretely distributed in barrier cells, including cells of the choroid plexus, blood vessels, and meninges. Labeling was also present in ependymal cells. Among blood vessels, arterioles were not labeled, but venules were labeled with IL-1R1 mRNA. Among CVOs, only the AP showed labeling over non-vascular and non-meningeal cells. Thus, in the ME, positive cells were in blood vessels and meninges, and in the OVLT and SFO, they were in the small vessels. In the meninges of the subarachnoid cistern at the base of the hypothalamus, IL-1R1 mRNA-labeled cells were abundant on the innermost lining of the arachnoid membrane and in the arachnoid trabeculae (Fig. 15). Thus, the IL-1 receptor-

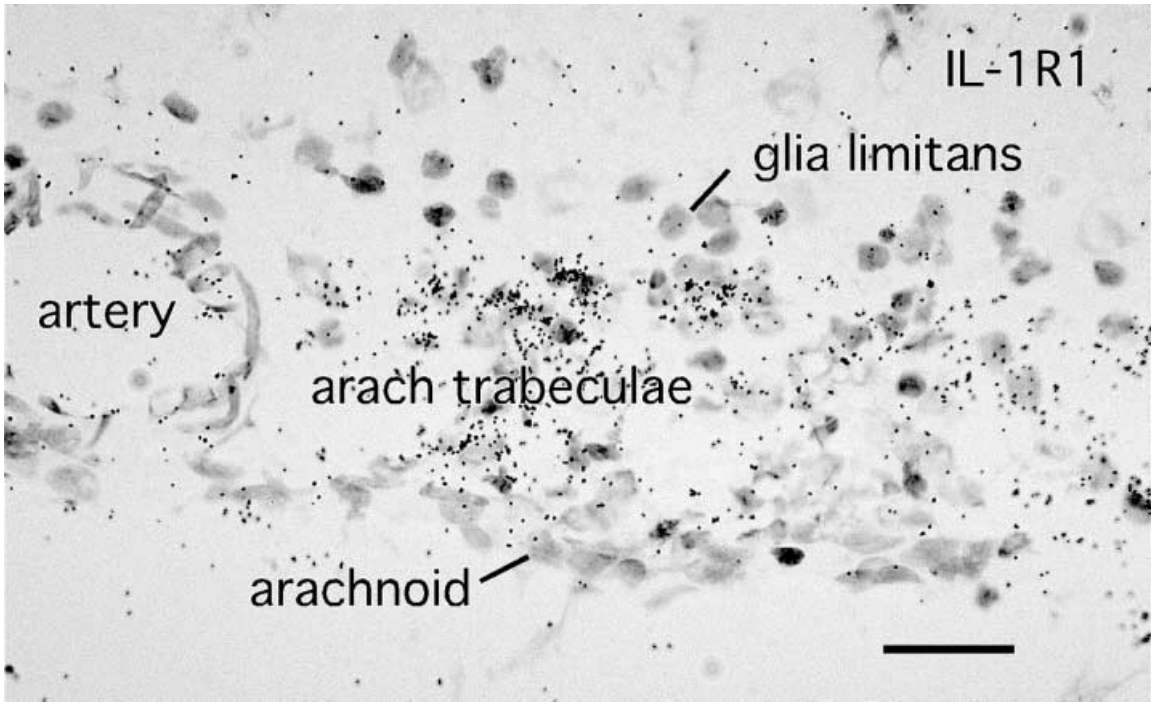

Fig. 15. Photomicrograph of interleukin 1β (IL-1) R1 mRNA labeling in the meninges at the base of the hypothalamus in a normal rat. Labeled cells are located mainly to the inner side of the arachnoid membrane and in the arachnoid trabeculae. Note the absence of labeling in the outer arachnoid membrane, glia limitans, and an artery. Compare with the distribution of *c-fos* mRNA-labeled cells at various times after i.v. IL-1 injection, shown in Figure 7. Scale bar = 100 μm.

bearing cells were sandwiched between the outermost arachnoid cells that were *c-fos* mRNA-positive at 0.5 hour and the glia limitans cells that were *c-fos* mRNA-positive at 3 hours.

DISCUSSION

The induction of *c-fos* mRNA or Fos protein after peripheral IL-1 or LPS injection has been described in several previous studies. A common finding after i.p. (Brady et al., 1994; Day and Akil, 1996) or i.v. (Ericsson et al., 1994) injection of IL-1 or after i.p. (Wan et al., 1993; Rivest and Laflamme, 1995; Lacroix and Rivest, 1997) or i.v. (Wan et al., 1994) injection of LPS is induction of *c-fos* mRNA or Fos protein in cells of the NTS, PVN, and several other neural structures, such as the Ce and dorsolateral BST. Some of these studies have also noted immediate-early gene activation in CVOs and barrier cells along the brain's edge (Brady et al., 1994; Ericsson et al., 1994; Rivest and Laflamme, 1995; Sagar et al., 1995; Day and Akil, 1996; Lacroix and Rivest, 1997), but a systematic and detailed analysis of this response has not been made. The data from the present study provide further information on cellular and temporal differentiation of *c-fos* mRNA induction patterns following i.v. administration of IL-1. The results shed new light on the interpretation of the brain *c-fos* mRNA response to peripherally injected IL-1.

Time course of IL-1 effects: Pathways of entry

The non-neuronal *c-fos* mRNA response pattern involving the brain's barrier cells shows a unique pathway by which peripheral IL-1 communicates with brain. Previously, non-neuronal responses were described at 1 and 3 hours after i.p. IL-1 (Brady et al., 1994) and, more recently, at 0.5 and 1 hour after i.p. IL-1 (Day and Akil, 1996). By combining the three time points into one study of *c-fos* mRNA activation after i.v. IL-1, the data reveal the existence of two discretely different patterns of cellular activation, suggesting a biphasic cascade of non-neuronal cellular responses occurring over the 3 hour time period.

The first non-neuronal response pattern is seen at 0.5 hour after i.v. IL-1, when cells that comprise the outermost cells of the BBB are *c-fos* mRNA-positive. In addition, cells of the CVOs are activated, though in some of the CVOs the labeled cells do not constitute CVO cells proper but rather meningeal or vascular cells within the organs. Then, at 1 hour after injection, the barrier cells are quiescent, whereas the *c-fos* mRNA response in the CVOs is further elaborated. At 3 hours after injection, a second wave of activation occurs in cells residing for the most part internally adjacent to the barrier cells that showed the early response. The magnitude of activation and the distance of penetration into the brain of this second wave is dependent on a number of characteristics, including proximity to the CVOs and magnitude of the initial response, as will be described in greater detail below. It can also be noted that the patterns of *c-fos* mRNA labeling seen at 1 and 3 hours after i.v. IL-1 are similar to those seen at the same time points after i.p. IL-1 (Brady et al., 1994) and after i.p. LPS, as briefly described later in the Discussion.

The IL-1-induced activation pattern at the BBB begins with *c-fos* mRNA induction in endothelial, choroidal, and meningeal cells. In the arachnoid membrane, *c-fos* mRNA-

positive cells are typically located at the outermost edge (Fig. 7a,b). This location is surprising because it suggests that the activating signal is present in the subdural fluid, which is a serous fluid that is in communication with the periphery, notably the deep lymphatic vessels of the neck and groin, and is not in direct communication with the subarachnoid CSF located on the brain side of the BBB (Warwick and Williams, 1973). Thus, it is possible that significant amount of circulating signal may enter the subdural fluid following its i.v. administration.

Induction of *c-fos* mRNA in cells elsewhere in the arachnoid and in the pia suggests that a bioactive signal already exists in the CSF at 0.5 hours after i.v. IL-1. It is not clear how the signal gets here. The signal could be the injected IL-1 itself, leaked into the CSF, or it might be a different molecule induced by IL-1. When IL-1 is injected directly into the CSF, a similar pattern of cellular activation is observed. The *c-fos* mRNA labeling 0.5 hour after intracerebroventricular (i.c.v.) IL-1 administration is localized in ventricular ependyma, pia, and arachnoid lining major subarachnoid spaces, choroid plexus, and blood vessels (both arteries and veins; Day and Akil, 1996). This pattern might be predicted on the basis of knowledge of flow of tracer substances in the CSF after i.c.v. injections; it has been shown that the subarachnoid spaces are rapidly reached by radiolabeled inulin injected into the cisterna magna (Hutto et al., 1987). Furthermore, *c-fos* mRNA labeling of blood vessels near the ventricular walls after i.c.v. injections may reflect paravascular flow pathways documented for flow of CSF though brain parenchyma (Cserr, 1975; Rennels et al., 1985).

At 1 and 3 hours after i.v. IL-1, the pattern of *c-fos* mRNA labeling shifted from barrier cells to a population of cells that included the pia and those of the adjacent superficial glial membrane (glia limitans). In the regions of the CVOs, signal spread into the brain. Only a few brain nuclei retained *c-fos* mRNA labeling at 3 hours. In most of these nuclei, the labeled cells appeared to be non-neuronal. An exception to this was the BST, which underwent a complete shift of site of labeling, from the dorsolateral subnucleus at 0.5 hour to the ventral subnucleus at 3 hours. The dorsolateral BST is part of the extended amygdala, in association with the Ce. These two structures showed parallel *c-fos* mRNA activation patterns across time. The ventral BST is anatomically distinct. One noteworthy feature of this location is the very dense adrenergic innervation (Swanson and Hartman, 1975) that is coextensive with the area showing *c-fos* mRNA labeling. This area receives a direct projection from tyrosine hydroxylase-positive cells in the region of the NTS (Sofroniew, 1983; Riche et al., 1990) that contains *c-fos* mRNA labeling at 3 hours. Perhaps this pattern emerges as a result of late synaptic driving from the adrenergic and noradrenergic cells in the NTS that remain activated at 3 hours after IL-1.

Nature of the activating signal

An obvious hypothesis is that cells initially responsive to i.v. IL-1 would be those that bear functional receptors for IL-1, i.e., the IL-1R1 (Simms et al., 1994). Cells that express IL-1R1 mRNA in the rat reside in blood vessels, choroid plexus, meninges, and the AP (Yabuuchi et al., 1994; Ericsson et al., 1995). All of these locations show *c-fos* mRNA labeling at 0.5 hours. However, levels of IL-1R1 mRNA are dense in the venules and choroid plexus, but the *c-fos* mRNA levels are comparatively weak in these locations. Close inspection in other regions reveals that the cells expressing *c-fos* mRNA at 0.5 hour and those containing IL-1R1 mRNA are not in register. Thus, the IL-1-induced *c-fos* mRNA is present in both arterioles and venules, whereas the IL-1R1 is not present in arterioles. In the basal hypothalamus, where the arachnoid, pia, and arachnoid trabeculae can be easily distinguished, the IL-1R1 is localized to cells comprising inner parts of the meninges, conspicuously the subarachnoid trabeculae (Fig. 15). These cells are not *c-fos* mRNA-positive at any of the time points in this study, though we have seen these cells express induced mRNA for NGFI-A, another immediate-early gene and transcription factor (Milbrandt, 1987), in our tissues from the i.p. IL-1 administration study at the 3 hour time point (data not shown). This observation serves to illustrate the necessary cautionary point that a negative result with a marker such as *c-fos* mRNA does not mean that the cells in question are unresponsive. It may be the case that an inhibitory signal is being transmitted to these cells (which would not result in *c-fos* mRNA induction), or that the signal is below the threshold for a *c-fos* mRNA response. Alternatively, the time point selected may not be optimal to see a response that is occurring, and the fact that cells of the arachnoid trabeculae were NGFI-A mRNA-positive at a time when they were *c-fos* mRNA-negative supports this interpretation.

Close inspection of the data in the study by Day and Akil (1996) suggests that *c-fos* mRNA-labeled cells 0.5 hour after ventricular injections of IL-1 are situated adjacent to the blood vessels in the subarachnoid spaces, apparently at the same locations where the IL-1R1 mRNA bearing cells reside. Further work with high resolution techniques is needed to confirm this possible relationship, but the implication is that IL-1 of central but not peripheral origin gains access to these cells. This seems implausible in light of the high likelihood that blood-borne IL-1 is leaking into the CSF at multiple sites, notably through the choroidal vasculature (Broadwell and Brightman, 1976). However, if true, this arrangement might help to explain why very low doses of IL-1 are effective when given i.c.v.

The comparative analysis reveals that the location of IL-1R1 does not always correlate with the site of the early *c-fos* mRNA response to i.v. IL-1. The discrepancy suggests that even at early time points, at certain locations, the *c-fos* mRNA responses in the brain are caused by signals other than IL-1. It is interesting to note that many of the observed *c-fos* mRNA activation patterns are also found after peripheral i.p. LPS injection at doses of 0.25 and 2.5 mg/kg (Lacroix and Rivest, 1997). We have observed both the early (in the meninges at 1 and 2 hours) and late (in the glia limitans at 4 hours) patterns of *c-fos* mRNA induction after i.p. LPS (2.5 mg/kg), but the signal strength is not as great as that observed after i.v. IL-1 (Quan and Herkenham, data not shown). Methodological as well as mechanistic factors could explain the differences in magnitude and timing, so the salient point is that LPS is capable of inducing *c-fos* mRNA responses in the arachnoid, vascular ependyma, and choroid plexus at early time points and the glia limitans at a later time point. These observations, together with the observations that the cells responsive to IL-1 are not necessarily IL-1R1-bearing cells, suggest that the brain *c-fos* mRNA response pattern reflects the stimulation of brain cells by multiple signal molecules after a single, bolus injection of IL-1.

Cell types activated by IL-1 and the signal molecules they may produce

Non-neuronal cells activated at 0.5 hour are located in the pia, arachnoid, endothelia, and choroid plexus. Cells activated at 3 hours appear to be small perivascular (Fig. 8e) and larger glial cells, mostly astrocytes. Astrocytic labeling is most likely to be the case for *c-fos* mRNA-positive cells located in the superficial glia membrane, a thin strip of moderately staining, rounded cells lining the brain's edge, and in the arcuate nucleus where double-labeling of *c-fos* mRNA and GFAP mRNA was confirmed (Fig. 13). Ependymal cells were positive in a few locations, such as in the lateral ventricle (Fig. 11c) and third ventricle dorsal to the ME (Figs. 6b, 12b).

A well-known function of IL-1 is activation of vascular endothelial cells. This phenomenon may occur in the brain vasculature as well, as indicated by the examples of specific *c-fos* mRNA labeling over surface and some penetrating blood vessels. We and others have documented a strong induction of inducible cyclooxygenase (COX 2) mRNA in brain vascular endothelia and, to a lesser extent, perivascular monocytes after i.p. or i.v. LPS (Cao et al., 1995; Elmquist et al., 1997a; Lacroix and Rivest, 1998; Quan et al., 1998b) or i.p. IL-1 (Cao et al., 1996), suggesting the possibility that its enzymatic product PGE_2 can diffuse from these sites in extracellular fluids throughout the brain parenchyma. Another signal induced in widespread endothelial cells after peripheral LPS challenge is mRNA for IκB, the molecule that regulates the activity of the transcription factor NF-kB (Quan et al., 1997). Thus, a wide range of bioactive molecules could be generated in endothelial cells after peripheral IL-1 injection, and these may trigger the late *c-fos* mRNA response pattern in neighboring cells. A similar transduction event might occur in the cells of the choroid plexus, which could produce a signal that flows in the CSF to activate ventricular ependymal cells.

The *c-fos* mRNA response may presage the later occurring and longer lasting responses to peripheral immune challenge, especially if the same cells that had been transiently *c-fos* mRNA- positive are shown at later times to express cytokine messages. The appearance of IL-1 mRNA (Quan et al., 1998a) and IL-1 immunoreactivity (Van Dam et al., 1995) in endothelial cells after i.p. LPS has a clear correlate with the *c-fos* mRNA expression in these cells at 0.5 hour after i.v. IL-1. The appearance of IL-1 mRNA and protein in brain after peripheral LPS injection follows a fairly long time course, beginning at 1.5 hours and peaking at 8–12 hours (Van Dam et al., 1992; Buttini and Boddeke, 1995; Van Dam et al., 1995; Wong et al., 1997; Quan et al., 1998a). Most of the cells labeled with IL-1 mRNA or IL-1β immunoreactivity after i.p. LPS are identified as microglia (Van Dam et al., 1992; Buttini and Boddeke, 1995; Van Dam et al., 1995). In addition, Van Dam et al. (1992) noted the induction of IL-1β immunoreactivity in perivascular cells and macrophages alongside the meninges at 4 and 8 hours after injection. We have no evidence for the induction of *c-fos* mRNA in microglia, but the presence of *c-fos* mRNA-positive perivascular cells (Fig. 8e) and inside the meninges at the 3 hour time point may correlate somewhat with the localization of IL-1-immunoreactive perivascular microglia and macrophages, respectively.

Role of the CVOs

The *c-fos* mRNA response in some CVOs began early and was strongly maintained at 3 hours after i.v. IL-1. In these, *c-fos* mRNA-positive cells spread beyond the boundaries of the CVOs. A striking example is seen in the territory surrounding the AP, which at 3 hours after i.v. IL-1 displayed *c-fos* mRNA labeling in cells in the NTS and several other nuclei in a pattern that strongly suggests a spreading wave of activation moving uniformly away from the AP (Fig. 2b). Another apparent spread is found in the territory around the ME, which includes the arcuate nucleus and ventromedial hypothalamic nucleus at 3 hours (Figs. 1c, 6b). Somewhat less striking are the SFO, whose surrounding tissue is the white matter of the ventral hippocampal commissure, and the OVLT, whose surrounding area is not deeply penetrated by *c-fos* mRNA signals. The cell types expressing *c-fos* mRNA in these locations at 3 hours appear for the most part to be non-neuronal. Cells in the same general locations show localized induction of cytokine mRNAs following i.p. LPS. Within several hours after LPS injection, IL-1β mRNA (Quan et al., 1998a) and IL-6 mRNA (Vallières and Rivest, 1997) are rather selectively induced in the CVOs and choroid plexus. It remains to be determined whether the *c-fos* mRNA-positive cells are the same cell types or, indeed, the same cells that also express cytokine mRNAs following peripheral immune challenge.

Lessons from the dose study

The original intent of the dose study was to functionally dissect apart the various response patterns to the IL-1 challenge. That goal was not met, in part because all *c-fos* mRNA induction patterns tended to disappear at the same dose level and in part because of the problem of inter-animal and interstudy variability of response to i.v. IL-1. Therefore, we cannot say whether the neuronal response, such as that seen in the NTS, Ce, and PVN was reliably elicited at a dose lower than the non-neuronal responses, though there was some slight indication of this possibility. Interestingly, the NTS response was always associated with a concomitant AP response, and thus the two structures are paired in Figure 14.

These results differ somewhat from the results of a similar dose-response study performed by Ericsson et al. (1994). The most likely explanation for the apparent greater overall sensitivity in the present study is the use of ISHH rather than immunohistochemistry and the use of unfixed rather than perfusion-fixed tissues, as mentioned in above.

The problem of variability was one which was not overcome by using i.v. rather than i.p. IL-1 administration (we had observed this variability in our earlier studies using the i.p. route). All experiments were performed with prealiquoted IL-1 from the same original lot, and all materials were stored, prepared, and injected in the same manner. The problem of variability in part influenced our decision to switch to LPS in our subsequent experiments, and we have not encountered the same level of variability with LPS. We propose, therefore, that the variability of response associated with IL-1 is biological and not technical in nature.

Summary: Models of transduction at the BBB

The routes of cytokine entry across the BBB leading to activation of cells inside the brain appear to be numerous. One route is access to the meninges leading to subsequent activation of the adjacent glia limitans. The functional consequences of this pattern of activation are not known. A second route may be activation of brain endothelial cells that respond by producing prostaglandins and NF-kB-mediated signal molecules including perhaps IL-1 and IL-6. This mechanism might lead to the presence of a host of bioactive molecules distributed widely throughout the brain parenchyma by extracellular diffusion. A third may involve activation of epithelial cells in the choroid plexus which may in turn generate cytokines that are released into the ventricular CSF to spread throughout the brain via CSF flow in the ventricles and subarachnoid spaces. A fourth is direct entry into the brain at the CVOs, primarily the AP and ME. The peripheral molecules may diffuse into the brain and activate local cells that in turn generate a second set of active molecules. Evidence from the present study supports all of these avenues of entry and suggests further the ability of transduced signal molecules to effect a functional response at these locations. The identity of the transduced signal molecules has yet to be determined.

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