A Functional Anatomical Analysis of Central Pathways Subserving the Effects of Interleukin-1 on Stress-related Neuroendocrine Neurons

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Systemic administration of the cytokine interleukin-1 (IL-1) results in increased secretion of ACTH and corticosterone in rats. The available evidence suggests that the acute effects of IL-1 are exerted ultimately at the level of the hypothalamus to increase corticotropin-releasing factor (CRF) secretion into the hypophyseal portal circulation, and hence the central drive on the pituitary–adrenal system. However, the route(s) and mechanism(s) by which circulating IL-1 gains access to central mechanisms governing pituitary–adrenal output remain poorly understood. In this study, we show that intravenous injection of IL-1β provokes time- and dose-dependent increases in the expression of the immediate-early gene c-*fos*, in identified CRF and oxytocin-producing cells of the paraventricular nucleus of the hypothalamus (PVH). Several cell groups known to be involved in central visceromotor regulation also displayed comparable time- and dose-related activation to systemic IL-1, including the bed nucleus of the stria terminalis, the central nucleus of the amygdala, the lateral parabrachial nucleus, and cell groups of the dorsomedial and ventrolateral medulla. Activation of circumventricular organs, which have been hypothesized to serve as central monitors of circulating IL-1, required doses roughly an order of magnitude above those required to activate CRF neurons in the PVH. Combined immunohistochemical and retrograde tracing experiments revealed many IL-1–responsive cells in the nucleus of the solitary tract and the ventrolateral medulla to be catecholaminergic and to project to the region of the PVH. Discrete and unilateral interruption of ascending catecholaminergic projections from the medulla attenuated IL-1–stimulated increases in Fos immunoreactivity and CRF mRNA in the PVH on the ipsilateral side. Disruption of descending projections from circumventricular structures associated with the lamina terminalis did not affect IL-1–mediated Fos induction in the PVH. We conclude that medullary catecholaminergic projections to the PVH play either a mediating or a permissive role in the IL-1–induced activation of the central limb of the hypothalamo-pituitary-adrenal axis.

[Key words: catecholamines, c-fos, corticotropin-releasing factor, cytokine, hypothalamo-pituitary-adrenal axis, interleukin-1, neurosecretory neurons, oxytocin, paraventricular nucleus, transcription factors]

Infectious and inflammatory processes evoke a broad spectrum of centrally mediated adaptive responses, including pyresis, somnolence, anorexia, and altered metabolic activities (Kuschner, 1982). Interleukin-1 (IL-1), a 17.5 kDa protein that is rapidly secreted from immunologically challenged cells primarily of macrophage lineage, appears to play a pivotal role in mediating such host responses in that most are mimicked by exogenous administration of the cytokine (for review, see Dinarello, 1991). Important components of the acute phase response are marked perturbations in neuroendocrine functions, paradigmatic of which is the increased activity of the hypothalamo-pituitary-adrenal (HPA) axis seen following central or peripheral administration of IL-1 (Besedovsky et al., 1986; Berkenbosch et al., 1987; Sapolsky et al., 1987; Harbuz et al., 1992). Glucocorticoids, the end product of the HPA cascade, are powerful immunosuppressants (Cupps and Fauci, 1982), and because various immune functions are rapidly inhibited in response to IL-1 *in vivo* (Sundar et al., 1990; Brown et al., 1991; Hori et al., 1991), these hormonal events have been suggested as comprising a substrate for negative feedback control of the immune by the neuroendocrine system (Besedovsky et al., 1986). Dysfunction of this posited neuroimmune regulatory network has been implicated as contributing to certain autoimmune disorders (Kroemer et al., 1988; Sternberg et al., 1989).

The mechanisms by which circulating IL-1 comes to modify pituitary–adrenal output are poorly understood, and potential sites of action at the levels of the hypothalamus (Berkenbosch et al., 1987; Sapolsky et al., 1987; Rivest and Rivier, 1991), pituitary (Bernton et al., 1987; Cambronero et al., 1992), and adrenal (Winter et al., 1990; Tominaga et al., 1991) have all been explored. The weight of the available evidence now tends to favor the hypothesis that the acute effects of IL-1 are exerted principally at the level of the hypothalamus to increase corticotropin-releasing factor (CRF) secretion into the hypophyseal portal circulation, and hence the central drive on the pituitary–adrenal system.

The existence of a blood–brain barrier to macromolecules poses a formidable question as to how circulating IL-1 gains

access to the brain parenchyma to exert its effects, directly or indirectly, on hypothalamic neurosecretory neurons. A frequently advocated hypothesis is that signaling by IL-1 may be transduced by one or more of the brain's circumventricular organs (Katsuura et al., 1990; Stitt, 1990), which lack a functional barrier, and are closely interconnected with the endocrine hypothalamus (Johnson and Loewy, 1990). Evidence suggestive of an involvement of central catecholaminergic neurons in the HPA response to exogenous IL-1 (Weidenfeld et al., 1989; Matta et al., 1990; Chuluyan et al., 1992) raises the possibility of peripheral neural transduction, since these pathways have been broadly implicated in the central processing of interoceptive information (Sawchenko and Swanson, 1982). Alternatively, it has been suggested that IL-1 may be subject to active transport across the barrier (Banks et al., 1989), thereby affording it direct and privileged access to intraparenchymal neuronal populations that express IL-1 receptors (Ban et al., 1991; Cunningham et al., 1992). The possibility also exists that IL-1 may interact with its own receptor on endothelial cells of the intracerebral vasculature (Cunningham et al., 1992) to allow penetration into the brain parenchyma of blood-borne substances that are capable of stimulating the synthesis and secretion of secondary signaling molecules (Katsuura et al., 1990; Kilbourn and Belloni, 1990; Watanabe et al., 1990), which may, in turn, trigger or modify local neuronal responses.

To begin to sort among these disparate mechanistic possibilities, we employed a functional anatomical approach that revolves in its initial phases about use of the immediate-early gene c-*fos* as a marker for functionally activated neurons. The general utility, sensitivity, and resolving power of c-*fos* as a marker for synaptically and/or transcriptionally activated cells have been widely discussed (Sagar et al., 1988; Morgan and Curran, 1991) and need not be reiterated here. We used this technique first to identify stress-related hypothalamic neurosecretory neurons that respond to intravenous injection of IL-1β by increased expression of c-*fos* mRNA and its protein product, Fos. Comparison of dose–response relationships and the time course of Fos induction in hypothalamic neuroendocrine target neurons and in potentially relevant afferents were used to identify candidate mediators/modulators of the IL-1 effect. This information was taken as an entry point from which to assess the connectivity and neurotransmitter specificity of select pools of IL-1–responsive neurons regarding their potential for regulating HPA axis activity. Finally, discrete surgical ablation of implicated afferents was carried out to directly assess their contribution to IL-1–induced Fos and CRF expression in hypothalamic neuroendocrine effector neurons.

Portions of these data have been presented in abstract form (Ericsson et al., 1992a, b).

Materials and Methods

Animals

Germ-free male adult Sprague–Dawley albino rats (280–320 gm; Harlan Sprague Dawley Inc, Indianapolis, IN) were housed individually under temperature- and light-controlled conditions (12:12 hr light:dark cycle with lights on at 06:00), with food and water available ad libitum. Rats were handled daily for a week prior to the onset of experimentation. All protocols were approved by the Institutional Animal Care and Use Committee.

Systemic administration of IL-1β

Recombinant human IL-1β was kindly provided by Dr. S Gillis, Immunex Research and Development Corporation (Seattle, WA). This protein corresponds to the 152 residue mature form of IL-1β, and had an original specific biological activity in excess of 1×10^5 units/μg protein [A375 assay (Nakano et al., 1988); 17 pg endotoxin/μg protein]. Upon receipt, the IL-1β protein was thawed on ice, diluted 1:1 in 200 mM Tris-HCl buffer, pH 7.4 at 25°C, containing 0.2% BSA, and then aliquoted in 1.5 μg batches and refrozen at −70°C. Thawed aliquots of IL-1β were stored at 4°C for no longer than 7 d.

Our procedure for systemic administration of IL-1β to rats has previously been described in detail (Ericsson and Sawchenko, 1993). Briefly, methoxyflurane-anesthetized rats were implanted with indwelling catheters (PE-50) containing sterile, pyrogen-free heparin-saline (500 U/ml) into the jugular vein; the sealed catheter was positioned with its internal Silastic tip at the atrium and was exteriorized at an interscapular position. After 2 d recovery, each experimental animal was connected, in its home cage, to a remote saline-filled catheter between 06:00 and 07:00, and either IL-1β (doses ranging between 0.21 and 3.58 μg/kg), 1.87 μg/kg of heat-inactivated IL-1β (70°C for 30 min), or vehicle alone was subsequently remotely delivered to freely moving rats in a total volume of 300 μl over 3 min at 1–4 hr prior to anesthetization and perfusion of the rats. The final composition of the vehicle was kept consistent at 0.01% BSA, 0.01% ascorbic acid, 10 mM Tris-HCl, 36 mM sodium-phosphate buffer, pH 7.4, at 25°C. The animals were routinely harvested by perfusion fixation between 12:30 and 14:00 to minimize the effects of diurnal variation in secretory and biosynthetic activities of the HPA axis.

Tissue processing and histology

Animals were deeply anesthetized with chloral hydrate (35 mg/kg, i.p.) and perfused via the ascending aorta with saline followed by 500–700 ml of 4% paraformaldehyde in 0.1 M borate buffer (pH 9.5 at 10°C). Brains were postfixed for 3–4 hr, and then cryoprotected in 10% sucrose in 0.1 M phosphate buffer overnight at 4°C. Frozen frontal sections were cut at 30 μm. Six 1-in-6 series through regions of interest, typically hypothalamus and medulla or whole brain, were collected in cold cryoprotectant (0.05 M sodium phosphate buffer, 30% ethylene glycol, 20% glycerol) and stored at −20°C until histochemical processing. Series of sections destined for *in situ* hybridization histochemistry were further postfixed in phosphate-buffered 4% paraformaldehyde overnight at 4°C before being transferred to cryoprotectant for storage.

Immunohistochemical labeling for Fos-like immunoreactivity (Fos-ir) was performed using an affinity-purified polyclonal antisera raised in rabbit against a synthetic peptide corresponding to residues 4–17 in the N-terminal portion of the human Fos protein (Oncogene Sciences). This antiserum displays no known cross-reactivity with any identified Fos-related antigen (Fra). Analysis for Fos-ir in IL-1β–injected and vehicle-injected rats was performed on free-floating sections using a conventional avidin-biotin-immunoperoxidase technique (Sawchenko et al., 1990). This included pretreating sections for 10 min each in 0.3% (v/v) hydrogen peroxide and in 1.0% (w/v) borohydride, with interposed rinses. Sections were incubated with the primary antiserum at dilutions ranging between 1:1000 and 1:7500, depending on the batch. The primary antiserum was localized using Vectastain Elite reagents, and the reaction product developed on ice using a nickel-enhanced glucose oxidase method (Shu et al., 1988). The specificity of the primary antisera was analyzed in separate control experiments by preabsorbing the antiserum overnight at 4°C with 50 μM of the corresponding immunogenic peptide.

c-*fos* mRNA was detected using a ³⁵S-labeled antisense cRNA probe transcribed from a 2.4 kilobase (kb) cDNA encoding the full-length rat c-*fos* mRNA (Curran et al., 1987). Control sections were hybridized with sense strand runoffs generated from the same cDNA clone. ³⁵S-labeled antisense and sense (control) cRNA probes transcribed from a 1.2 kb cDNA encoding the full-length rat prepro-CRF mRNA (kindly provided by Dr. K. Mayo; cf. Jingami et al., 1985) were used to localize neuronal CRF mRNA for qualitative and quantitative analyses.

Hybridization histochemical localization of c-*fos* and CRF mRNA was performed as previously described (Simmons et al., 1989). Briefly, sections destined for *in situ* hybridization were subjected to additional postfixation in neutral-buffered 4% paraformaldehyde overnight at 4°C, mounted on gelatin and poly-L-lysine–coated slides, and desiccated under vacuum overnight. Sections were then postfixed with neutral-buffered 4% paraformaldehyde for 30 min, digested in 10 μg/ml proteinase K at 37°C for 30 min, acetylated for 10 min, and then dehydrated and vacuum dried overnight. Hybridization of the ³⁵S-labeled antisense cRNA probes (10⁶ cpm/100 μl/slide) was carried out in 247 mM NaCl, 8.2 mM

Tris-HCl (pH 8.0 at 25°C), 41% (v/v) formamide, 0.82× Denhardt's (1× Denhardt's is 0.002 % of BSA, Ficoll 400, and polyvinylpyrroli-done), 8.2 % (w/v) dextran sulphate, 411 mg/ml yeast tRNA, and 8.2 mM dithiothreitol (DTT) on coverslipped slides at 60°C for 16 hr. After initial rinses in 4× saline–sodium citrate (SSC), slides were incubated in RNase A (20 μg/ml, 37°C, 30 min), washed in 0.1× SSC, 10 mM DTT at 75°C for 30 min, and finally dehydrated and dried. Slides were exposed to Amersham β-max autoradiography film for 1–4 d, defatted in graded ethanols and xylene, and dipped in Kodak NTB-2 nuclear track emulsion. Slides were exposed for 7–14 d and developed in D-19 developer (Kodak) for 5 min at 14°C. Sections were counterstained with thionin, dehydrated, and coverslipped.

Procedures and analysis

Time course. To establish a time course of c-*fos* induction in the hypothalamus, 59 rats were injected with a moderate dose (1.87 μg/kg) of IL-1β or an equivalent volume of vehicle and perfused at hourly intervals for up to 4 hr. Immunohistochemical localization of Fos-ir and hybridization histochemical localization of c-*fos* mRNA were carried out on series of sections through the hypothalamus from each animal.

Histochemical identification of IL-1–responsive neuroendocrine neurons. To determine the neuropeptide phenotype of IL-1–responsive cells, immunoperoxidase localization of Fos-ir was combined with concurrent immunofluorescence staining for oxytocin (OT) or vasopressin, the principal peptide products of the magnocellular neurosecretory system, or with hybridization histochemical localization of CRF mRNA, in series of hypothalamic sections obtained from four rats injected with 1.87 μg/kg of IL-1β and killed at the time of maximal Fos protein induction (3 hr). Dual staining for Fos-ir and OT-ir or arginine vasopressin-ir (AVP-ir) was performed by initially preparing the free-floating sections for immunoperoxidase localization of Fos-ir as outlined above, except that the hydrogen peroxide pretreatment was omitted. Sections were subsequently incubated in rabbit anti-OT serum (Incstar, 1:2000) or rabbit anti-AVP serum (Chemicon, 1:8000) for 48 hr and then in affinity-purified, FITC-labeled goat anti-rabbit IgG for 1 hr at room temperature (1:200; Tago, Inc.). Specific staining with the primary antisera was abolished by preincubation with 50 μM of the homologous, but not the heterologous, synthetic nonapeptide. Cells displaying nuclear immunoperoxidase staining for Fos and cytoplasmic immunofluorescence staining for OT-ir or AVP-ir were identified.

The lesser relative abundance of CRF peptide in the paraventricular nucleus of the hypothalamus (PVH) precluded using a similar approach to determine the extent to which IL-1–stimulated Fos induction might be localized to CRF-expressing neurons. Instead, a combined immunoperoxidase (Fos) and *in situ* hybridization (CRF mRNA) protocol, detailed elsewhere (Watts and Swanson, 1989a), was employed. In brief, immunolocalization of Fos-ir was carried out as described above, save that the tissue was not pretreated in borohydride or hydrogen peroxide. In addition, the primary antibody reaction was performed in buffer with 2% (v/v) BSA and 5 mg/ml heparin as blocking agents (in place of nonimmune goat serum) and the reaction product was developed without nickel enhancement. Subsequent localization of CRF mRNA by *in situ* hybridization was then carried out as described above.

Dose–response relationships. A comparison of the minimum effective doses at which IL-1 induces Fos-ir in the PVH and extrahypothalamic cell groups was undertaken in order to identify candidate afferent mediators of cytokine effects on hypophysiotropic CRF neurons. Groups of three to eight animals each were implanted with jugular cannulas and injected with either 0.21, 0.46, 0.87, 1.87, or 3.58 μg/kg IL-1β or vehicle, anesthetized 3 hr later (the time point at which IL-1–stimulated Fos-ir was found to be maximal in the PVH), perfused, and prepared for demonstration of Fos-ir throughout the brain. Effective doses of IL-1 were determined by comparing counts of Fos-ir nuclei in the PVH and cell groups either that are known to provide afferents to it or that have been implicated as potential mediators of the IL-1 effect on the HPA axis in control and cytokine-treated animals. We adopted the arbitrary criterion of considering as an effective dose one that resulted in Fos induction in cells whose number equalled or exceeded the mean + 2 SD of vehicle-injected control values in a majority of animals that received a particular dose of cytokine.

Characterization of candidate afferents. To determine whether cells displaying Fos induction in response to intravenous IL-1 are anatomically related to the PVH, and to determine their neurotransmitter phenotype, a combined retrograde transport–multiple immunohistochemical labeling paradigm was used. Under anesthesia (10 mg/kg xylazine,

50 mg/kg ketamine, 2 mg/kg acepromazine), seven rats received stereotaxically guided crystalline implants of the retrogradely transported fluorochrome true blue aimed at the PVH (Lind, 1986). Four weeks later, tracer-injected rats were implanted with jugular cannulas and injected with 1.87 μg/kg IL-1β as described above. Complete and regularly spaced series of sections throughout the brains of animals with well placed injections were processed for immunoperoxidase detection of Fos-ir as described above, except that hydrogen peroxide pretreatment was omitted. The extent to which individual neurons manifested both IL-1–induced Fos-ir and retrograde labeling was determined. Because such doubly labeled cells were seen consistently in the dorsomedial and ventrolateral parts of the medulla, regions known to provide catecholaminergic projections to the PVH (Cunningham and Sawchenko, 1988; Cunningham et al., 1990), brainstem sections were treated as above (i.e., for demonstration of Fos-ir and retrogradely transported true blue) and further incubated in a cocktail of antisera raised against dopamine-β-hydroxylase (DBH; a marker for adrenergic and noradrenergic neurons) and phenylethanolamine-*N*-methyltransferase (PNMT; a specific marker for adrenergic neurons). The anti-DBH serum was a mouse-derived monoclonal antibody (Chemicon; diluted 1:1000) raised against rat adrenal DBH. The anti-PNMT serum (diluted at 1:2000), generously provided by Dr. M. C. Bohn (Univ. Rochester), was raised in rabbits against purified rat adrenal PNMT (Bohn et al., 1987). Primary antisera were localized using a mixture of FITC-labeled goat anti-rabbit IgG (1:200; Tago, Inc.) and rhodamine-labeled goat anti-mouse IgG (1:100; American Qualex). This procedure allowed determination of the extent to which medullary cells that are responsive to IL-1 and that project to the region of the PVH displayed the adrenergic or noradrenergic phenotype.

Experimental manipulation of candidate afferents. Ablation methods were used to evaluate the dependency of IL-1–stimulated increases in the expression of Fos and/or CRF mRNA on afferents implicated in the analyses outlined above. A group of rats received discrete, stereotaxically guided, unilateral transections, designed to interrupt ascending catecholamine projections near their origins in the medulla. Knife cuts were made in the coronal plane, using a retracting wire knife fashioned from a microliter syringe (Gold et al., 1973), as previously described (Sawchenko, 1988). Twelve days after surgery, the animals were implanted with jugular cannulas, randomly injected 2 d later with 1.87 μg/kg IL-1β or vehicle, and anesthetized and perfused 3 hr after injection. Adjacent series of horizontal sections though the brainstem were stained with DBH and thionin to aid in evaluation of the placement and effectiveness of knife cuts. Separate series of coronal sections through the hypothalamus were prepared for immunohistochemical demonstration of DBH-ir, Fos-ir, and hybridization histochemical demonstration of CRF mRNA. Knife cuts were judged to be effective if they resulted in a pileup of DBH-ir in fibers immediately proximal to the lesion site, and a significant (>60%) depletion of DBH-ir varicosities in the ipsilateral PVH, as judged by semiquantitative (rating scale) comparison of the strength of the DBH innervation of the PVH on the ipsilateral and contralateral sides by two independent observers (Sawchenko, 1988).

Nuclear staining for Fos-ir was compared qualitatively in the PVH on the ipsilateral and contralateral sides of animals bearing cuts judged to be effective. In addition, relative levels of CRF mRNA were assessed densitometrically from emulsion-dipped slides using a Leitz optical system coupled to Macintosh-based IMAGE software (version 1.42, W. Rasband, NIH). Optical density over the medial parvocellular subdivision of the PVH (PVHmpd), defined by redirected sampling from the corresponding bright-field images of thionin-stained sections, was determined under dark-field illumination on both sides of the brain. Optical densities were corrected for background, and calibrated to emulsion-dipped sections of brain paste standards containing serial dilutions of ^{35}S-UTP that were processed in parallel with the experimental material. Data were normalized to the contralateral side in lesioned and saline-injected rats and statistically analyzed by two-way ANOVA as a mixed design with one within (ipsi- vs contralateral sides) and one between (IL-1 vs saline) group variable. Duncan's multiple range test was used for post-hoc comparisons between individual group means.

As an additional control, a separate group of rats received unilateral transections designed to disrupt descending projections from lamina terminalis–associated circumventricular structures using a similar knife construction, with cuts 2 mm wide × 3 mm high placed in the coronal plane at a locus, extending from the midline just caudal to the vascular organ of the lamina terminalis (OVLT), which has been shown to be
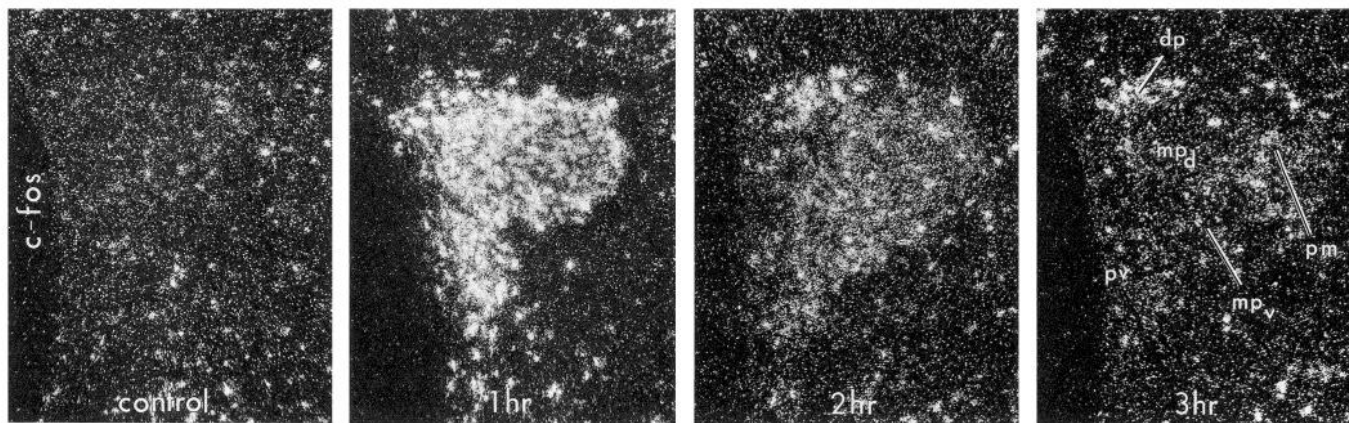
Figure 1. Time course of IL-1–stimulated c-*fos* mRNA induction in the PVH: dark-field photomicrographs of hybridization histochemical localization of a ^{35}S-labeled antisense c-*fos* probe at a comparable level through the PVH of rats injected 1, 2, or 3 hr previously with 1.87 µg/kg of IL-1β. *control* is representative of the low level of hybridization seen in rats that received injection of vehicle alone, 1 hr prior to perfusion fixation. Rapid induction of c-*fos* transcripts are evident at 1 hr after treatment, and localized predominantly to the dorsal aspect of the medial parvocellular subdivision (*mp$_d$*). Less widespread induction is also evident at the periphery of the magnocellular division (*pm*) of the nucleus, and in autonomic-related parts of the parvocellular division (*dp, mpv*). IL-1–stimulated c-*fos* induction is transient in neurosecretory populations, and more persistent in autonomic-related subdivisions. The third ventricle is at *the left* of all micrographs. *mp$_v$*, ventral aspect of the medial parvocellular subdivision. Magnification (all micrographs), 75×.

effective in disrupting osmotically driven alterations in CRF mRNA expression in both the magnocellular and parvocellular neurosecretory systems (Kovács and Sawchenko, 1993). In the absence of reliable histochemical markers for lamina terminals–PVH projections, the location of these lesions was evaluated histologically from thionin-stained sections. After implantation of venous cannulas, injection of IL-1 or vehicle, perfusion-fixation, and histology, the number of nuclei displaying Fos-ir and the relative strength of Fos-ir were compared on the sides ipsilateral and contralateral to the cuts.

Results

Time course of IL-1–stimulated expression of c-fos in the PVH

Our initial experiment sought to determine whether, and over what time frame, transcription factor Fos may serve as a useful marker for identifying neuroendocrine neurons that are affected by systemic administration of IL-1β. Tissue harvested from rats injected with a 1.87 µg/kg dose of IL-1β via an indwelling jugular catheter, a paradigm previously shown to stimulate secretory activity of the HPA axis (Sapolsky et al., 1987; Rivier et al., 1989), reliably displayed an orderly sequence and topography of c-*fos* mRNA and cognate Fos protein induction. Hybridization histochemical localization of a ^{35}S-labeled rat c-*fos* riboprobe in complete series of sections through the hypothalamus revealed significant labeling for c-*fos* mRNA over cells primarily localized in the dorsal aspect of the PVHmpd at 1 hr after administration of IL-1β (Fig. 1). Labeling in this region, which is recognized as the seat of parvocellular neurosecretory CRF-expressing neurons, was diminished by 2 hr, and not discernably distinct from that seen in controls by 3 hr after injection. IL-1β injection also reliably provoked c-*fos* mRNA expression that followed a similar time course in topographically distinctive aspects of the magnocellular division of the PVH, and a more persistent induction in aspects of the PVH that project to autonomic centers in the brainstem and spinal cord. No specific labeling over the PVH was observed in adjacent series of sections exposed to a ^{35}S-labeled c-*fos* riboprobe transcribed in the sense direction, or using the antisense probe in tissue obtained from vehicle-injected controls (Fig. 1).

Series of sections from IL-1β–injected and vehicle-injected rats were also prepared for immunohistochemical localization of Fos protein. In cytokine-treated rats, nuclear staining for Fos-ir in the PVNmpd was clearly detectable at 1 hr, augmented at 2 hr, and maximal, in terms of both cell number and staining intensity, at 3 hr after injection (Fig. 2). The temporal expression of Fos-ir in the magnocellular division of the PVH was similar to that of the PVHmpd, though less robust than was evident in hybridization material. Interestingly, and in line with the hybridization data, strong labeling for Fos protein was detected in the (autonomic-related) dorsal parvocellular part of the PVH that persisted throughout the 4 hr time period examined. Preadsorption of Fos antiserum with synthetic immunogen abolished staining in the PVH of IL-1β–injected rats, and animals receiving vehicle alone displayed little or no specific labeling in the PVH (Fig. 2). Furthermore, rats injected with IL-1β that had been previously preheated to 70°C for 30 min, a procedure that will not inactivate potentially contaminating bacterial endotoxins (Bernton et al., 1987), failed to result in Fos-ir induction in the PVH.

To determine whether IL-1β–induced c-*fos* expression may be associated with an index of increased CRF biosynthesis, relative levels of CRF mRNA content in the PVH were compared densitometrically in animals killed 3 hr after systemic administration of IL-1β at 1.87 µg/kg or vehicle; the 3 hr time point was chosen as it represented the time of maximal Fos protein induction in this region. This analysis revealed a reliable, 47.6 ± 13.1% increase in CRF mRNA content in the PVHmpd at 3 hr after injection of IL-1β as compared to vehicle-injected control rats ($p < 0.02$, $n = 5$, two-tailed t test).

Histochemical identification of IL-1–responsive neuroendocrine neurons

The topography of cells displaying IL-1–stimulated c-*fos* induction in the PVH corresponded principally to the recognized distribution of parvocellular CRF-synthesizing and magnocellular OT-synthesizing neurosecretory neurons. To confirm the biochemical identity of IL-1β–responsive neurons in the PVH, we carried out combined immunohistochemical and *in situ* hybridization analysis for Fos-ir and CRF mRNA in the PVH of
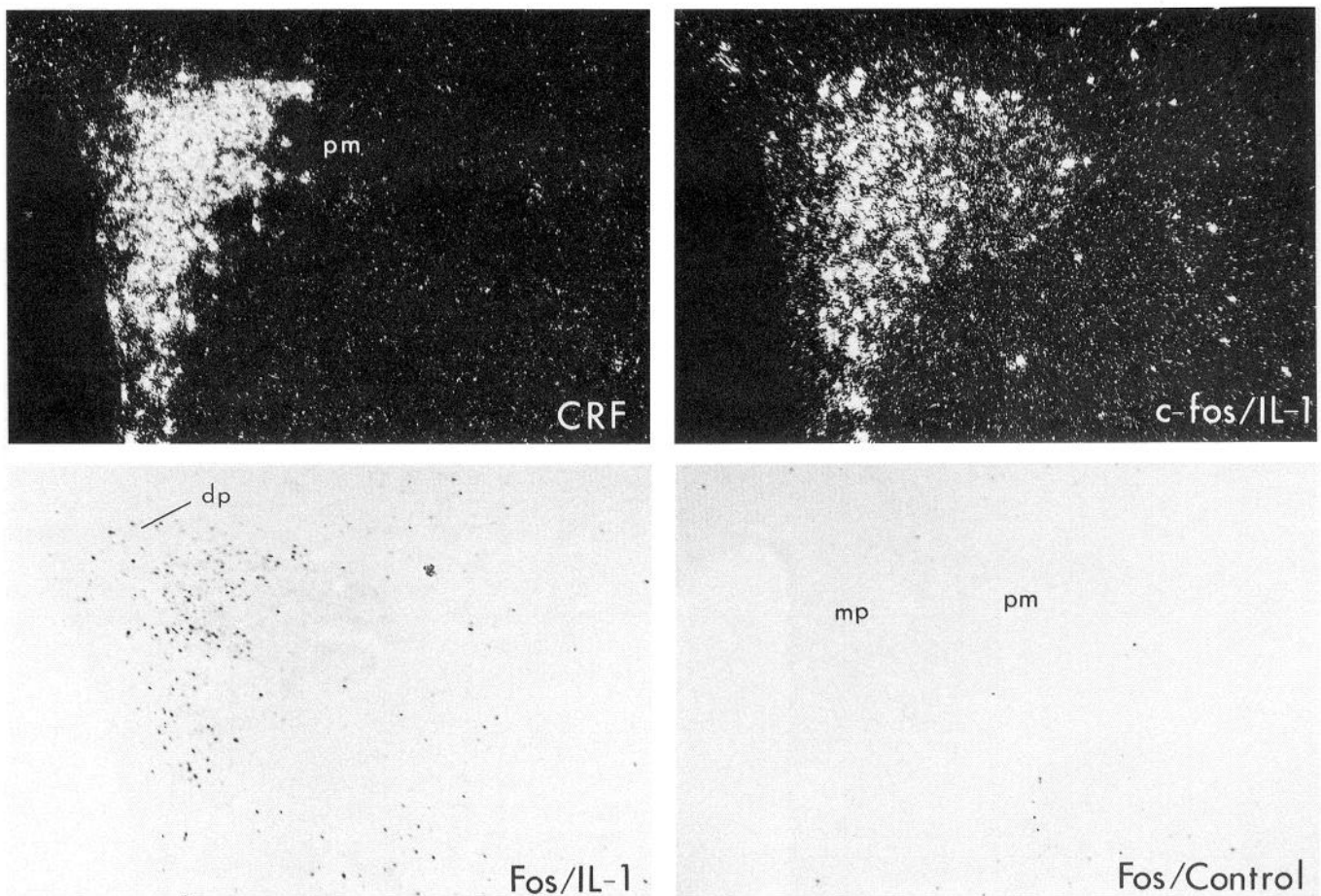
Figure 2. IL-1–stimulated patterns of c-*fos* mRNA and Fos protein induction, and their relationship to CRF-expressing neurons in the PVH: dark-field photomicrographs of hybridization histochemical localization of CRF and c-*fos* mRNAs and bright-field photomicrographs of immunoperoxidase localization of Fos protein in IL-1–injected or vehicle-injected (control) animals. Hypophysiotropic cells expressing CRF mRNA are predominantly localized to the dorsal aspect of the medial parvocellular part (*mp*) of the PVH. Systemic administration of IL-1β results in specific and comparable patterns of c-*fos* mRNA and Fos-ir induction in the PVH, whose topography overlaps extensively with that of cells expressing CRF mRNA. Rats were injected with 1.87 µg IL-1β/kg or vehicle (*Control*). IL-1–stimulated c-*fos* mRNA and Fos-ir are illustrated in animals killed at time points optimal for detection (1 and 3 hr postinjection, respectively). *Control* animal was also killed at 3 hr after injection of saline. *pm*, periphery of the magnocellular division. Magnification, 60×.

rats that had received 1.87 µg/kg of IL-1β 3 hr prior to perfusion. The results confirmed that the majority of Fos-ir cells in the PVHmpd of IL-1–injected rats express CRF mRNA (Fig. 3). Dual immunostaining methods revealed that the great majority of Fos-ir cells in the magnocellular division of the PVH displayed OT-ir; few, if any, Fos-ir neurons stained positively for vasopressin, the other major secretory product of the magnocellular neurosecretory system (Fig. 3). Corrected (Abercrombie, 1946) counts in one representative animal indicated that 71% of 1134 Fos-ir cells in the PVHmpd displayed a positive hybridization signal for CRF mRNA, defined as a grain density more than five time background overlying a cell-sized area centered about a Fos-positive nucleus. Counts in adjacent series from the same animal indicated that of 163 Fos-ir cells in the magnocellular division of the nucleus, 69% also stained positively for OT, while 7% were immunoreactive for vasopressin.

Extrahypothalamic sites of IL-1–induced Fos expression

Cell groups outside the PVH that are known to be involved in neuroendocrine regulation and/or to provide afferent projections to the PVH also displayed prominent and specific Fos

induction following intravenous injection of IL-1β. Rapid and robust induction of c-*fos* mRNA (not shown) and Fos-ir were observed in the oval subnucleus of bed nucleus of the stria terminalis (Fig. 4*A*; Ju and Swanson, 1989), the lateral division of the central nucleus of the amygdala (Fig. 4*B*), and specific subdivisions of the lateral parabrachial nucleus (Fig. 4*C*) after systemic administration of 1.87 µg IL-1β/kg. Likewise, specific IL-1β–mediated Fos induction was detected throughout much of the rostrocaudal extent of the medial and commissural subnuclei of the nucleus of the solitary tract (NTS; Fig. 5) as well as in the rostral ventrolateral medulla (Fig. 4*D*). A few cells in the dorsal motor nucleus of the vagus nerve also displayed IL-1–stimulated c-*fos* mRNA and Fos-ir.

Significantly, little or no specific staining for c-*fos* mRNA or Fos protein was observed in circumventricular organs at the dose of IL-1β utilized over 1–4 hr after injection. Aspects of these structures, which include the OVLT, subfornical organ, median eminence, and area postrema, lie outside the blood–brain barrier, and have been suggested as likely CNS monitors of circulating IL-1 (Katsuura et al., 1990; Stitt, 1990). In addition, we reliably failed to detect IL-1–stimulated Fos induction
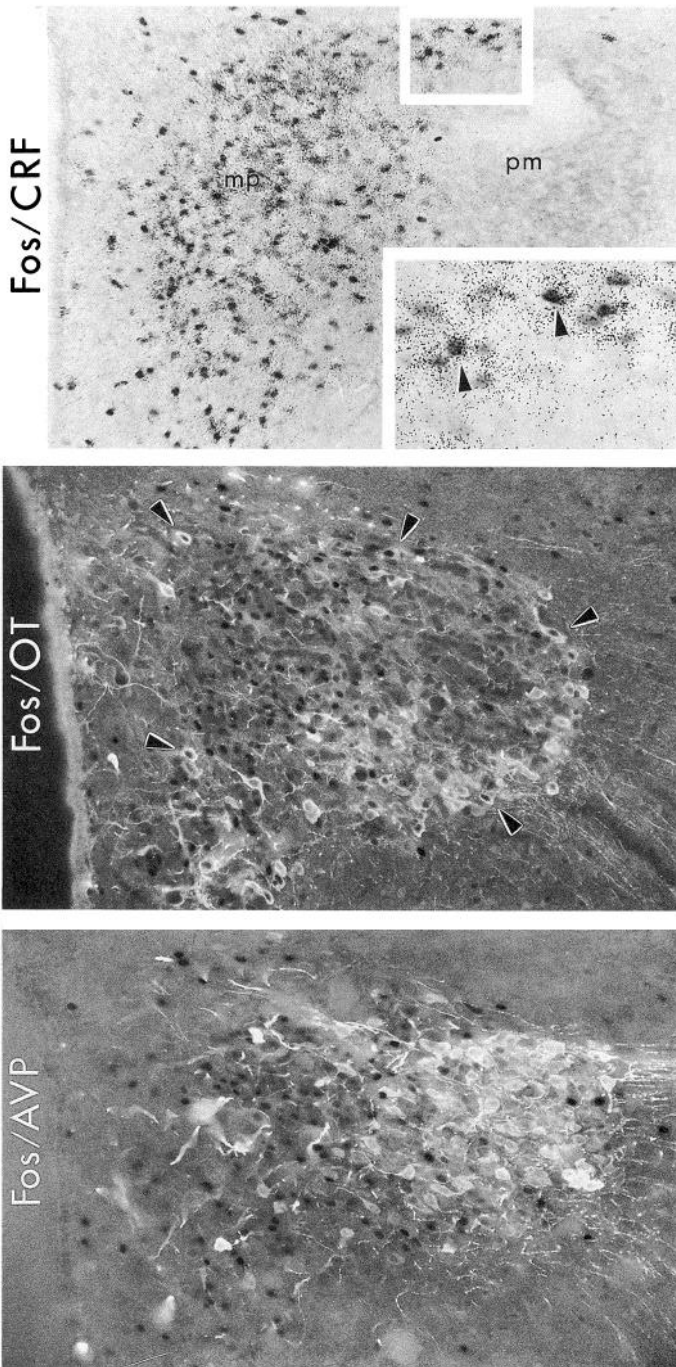
Figure 3. Biochemical characterization of IL-1–responsive neurosecretory neurons in the PVH: bright-field (*top*) or fluorescence (*middle, bottom*) photomicrographs through a comparable level of the PVH showing combined immunoperoxidase localization of IL-1–stimulated nuclear Fos-ir with markers for major neurosecretory cell types. Animals were killed at 3 hr after injection of 1.87 µg IL-1β/kg. The *top panel* shows a section cohybridized for CRF mRNA. Extensive association of reduced silver grains indicative of CRF mRNA signal and Fos-ir neurons is apparent in the parvocellular division of the PVH. See *inset* for higher magnification of *boxed region*; *arrowheads* indicate examples of doubly labeled cells. The *middle* and *bottom panels* show combined immunoperoxidase localization of Fos-ir and immunofluorescence staining for oxytocin (*OT*) or vasopressin (*AVP*), respectively. *OT*-ir and *AVP*-ir are localized to topographically distinct aspects of the more laterally disposed magnocellular division of the nucleus. In the magnocellular compartment, IL-1–stimulated Fos-ir is preferentially associated with OT-ir neurons. Magnification, 150× (*inset*, 360×).

at intra- and extraparenchymal loci known to harbor IL-1 receptors and/or ligand binding sites (Ban et al., 1991; Cunningham et al., 1992), including the dentate gyrus (Fig. 4*E*), choroid plexus (Fig. 4*F*), and the mesencephalic raphe nuclei. One exception was a weak induction of c-*fos* mRNA throughout much of meninges at 1 hr after injection; Fos-ir was detected inconsistently in these membranes at 2–3 hr after injection, suggesting a low and variable level of Fos induction in these membranes. No specific Fos induction was detected at any time point in the locus coeruleus, a structure that might be taken to serve as an indicator of generalized stress or arousal (Foote et al., 1983).

Finally, several structures within the CNS displayed c-*fos* mRNA and Fos-ir in both IL-1β–injected and vehicle-injected animals. These areas included aspects of the cingulate and piriform cortices, several midline thalamic nuclei, including the paraventricular nucleus (Fig. 4*F*), the habenula, and scattered cells in the preoptic region and in the anterior and lateral hypothalamic areas. Labeling for c-*fos* mRNA and Fos-ir was also observed in the suprachiasmatic nucleus, a structure known to contain a pacemaker that drives circadian rhythms in HPA axis activity (Watts and Swanson, 1989b). To avoid potential complications of oscillatory influences on IL-1β–responsive neurons, animals were killed consistently over a 90 min portion of the light:dark cycle.

Dose–response relationships

Structures listed above as displaying specific IL-1–induced c-*fos* expression represent potential mediators of cytokine-induced activation of the HPA axis. To pare the list of candidates, we compared Fos induction in extrahypothalamic sites and the PVHmpd over a range of IL-1β doses, reasoning that cell groups displaying a sensitivity similar to that of CRF target neurons in the PVH would be most apt to be mechanistically involved in the hypothalamic response. Animals were killed, again at 3 hr, following injection of IL-1β at doses ranging from 0.21 to 3.58 µg/kg (corresponding roughly to administration of 60–1000 ng of cytokine to a 300 gm rat). The arbitrarily defined (see Materials and Methods) effective dose for eliciting Fos induction differed markedly among the regions studied (Figs. 5, 6). Thus, few if any Fos-ir cells were detected in the PVNmpd in response to the lowest (0.21 µg/kg) dose tested (Fig. 6). However, beginning at 0.47 µg/kg (Figs. 5, 6), above criterion labeling was detected in this cell group, which progressively increased in number and staining intensity at higher dosage levels. At the highest dose (3.58 µg/kg), very strong labeling was observed throughout the PVHmpd, as well as in OT-rich aspects of the magnocellular division of the PVH (Figs. 5, 6). Among extrahypothalamic regions, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, the lateral parabrachial nucleus, as well as the NTS, displayed above criterion labeling even at the lowest dose of IL-1β given (Fig. 6). The intensity and number of Fos-labeled cells increased progressively to a point where a large number of cells in these structures were strongly labeled for Fos-ir at the highest dose of IL-1β studied, 3.58 µg/kg (Fig. 5). Reliable labeling in ventrolateral medulla required roughly 0.47 µg/kg IL-1β (Fig. 6), and the supraoptic nucleus, a pure magnocellular neurosecretory cell group, required at least 0.87 µg/kg IL-1β to obtain a clear induction of Fos-ir (Fig. 6).

Interestingly, no reliable induction of Fos-ir was observed in the circumventricular organs at the minimum dose of IL-1β
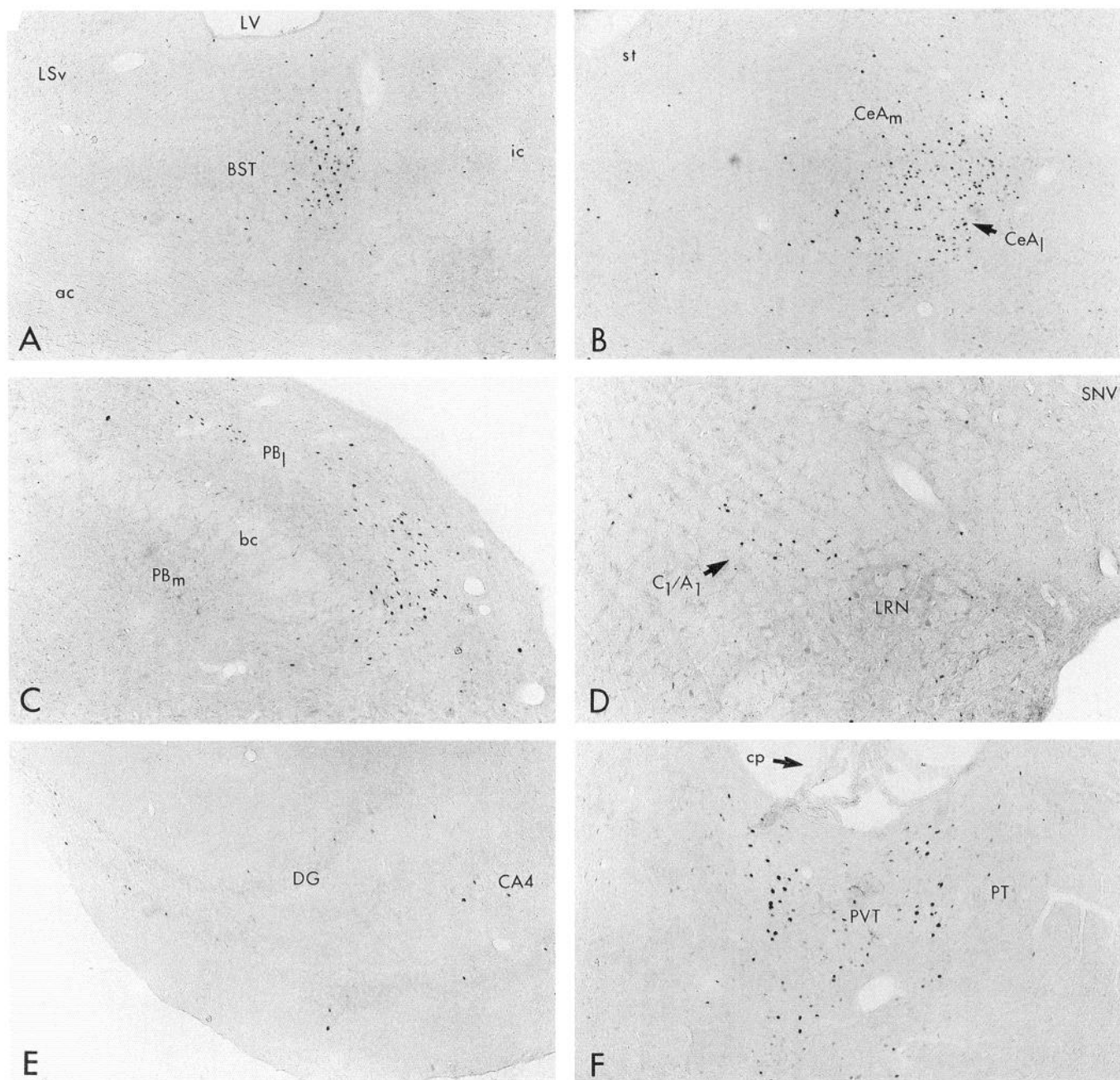
Figure 4. Extrahypothalamic sites of IL-1–stimulated Fos induction: bright-field photomicrographs of immunoperoxidase preparations, showing nuclear Fos induction in various rat CNS regions following intravenous administration of 1.87 μg IL-1β/kg 3 hr prior to perfusion fixation. Specific induction of Fos-ir was detected in the bed nucleus of the stria terminalis, particularly in the oval subnucleus (*A*), the lateral division of the central nucleus of amygdala (*B*), the lateral division of the parabrachial nucleus (*C*), and the rostral ventrolateral medulla, in the vicinity of the C_1 and A_1 catecholamine cell groups (*D*). No specific Fos induction was detected in the dentate gyrus of the hippocampal formation (*E*) or the choroid plexus (*F*) in IL-1–injected rats as compared to vehicle controls. Constitutively high levels of Fos-ir was observed in the paraventricular nucleus of the thalamus in both IL-1–injected and vehicle-injected controls (*F*). *ac*, anterior commissure; *bc*, brachium conjunctivum; *BST*, bed nucleus of the stria terminalis; *CA4*, pyramidal cell layer of hippocampus; *CeA$_{m,l}$*, central nucleus of the amygdala, medial, lateral parts; *cp*, choroid plexus; *DG*, dentate gyrus; *ic*, internal capsule; *LRN*, lateral reticular nucleus; *LSv*, ventral nucleus of the lateral septum; *LV*, lateral ventricle; *PB$_{m,l}$*, parabrachial nuclei, medial, lateral; *PT*, parataenial nucleus; *PVT*, paraventricular nucleus (thalamus); *SNV*, spinal trigeminal nucleus; *st*, stria terminalis. Magnification, 75×.

(0.47 μg/kg) required to activate neurons in the PVNmpd (Figs. 5, 6). Extensive Fos induction was detected in these regions only at the upper extreme of the dosages tested (Figs. 5, 6). It may be significant, however, that small Fos-ir cells were occasionally

observed at this and higher doses at the ventral and lateral margins of the OVLT region, at the rostralmost level of the optic chiasm (Fig. 5). Based on cell size and contiguity with the meninges, we interpret these as indicative of Fos induction in mem-
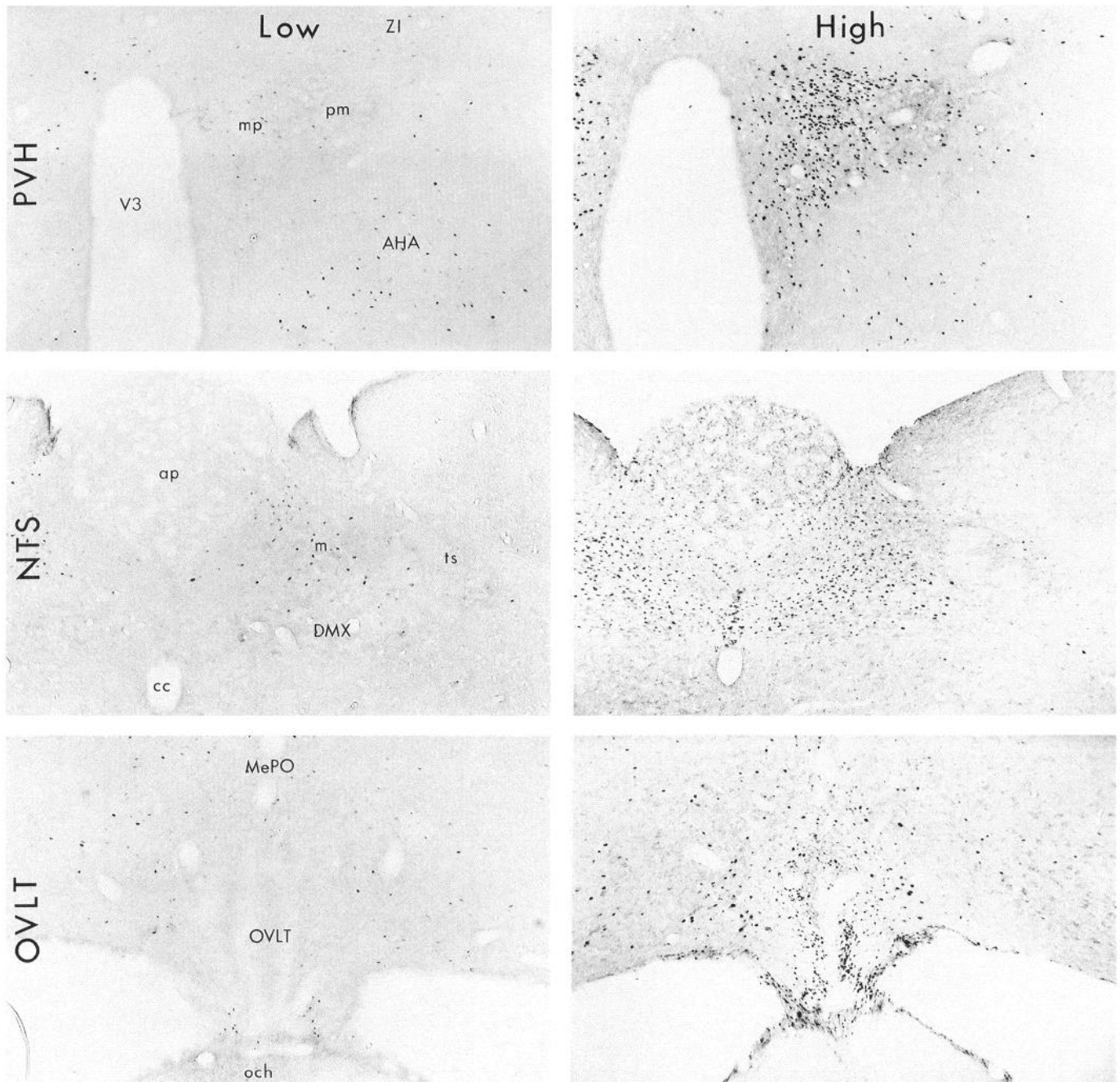
Figure 5. Dose dependence of IL-1–stimulated Fos induction: bright-field photomicrographs of immunoperoxidase preparations to compare IL-1–stimulated Fos-ir expression at doses of the cytokine that were minimal for reliable induction of Fos-ir in the parvocellular PVH at 3 hr postinjection (0.47 µg/kg; *Low*), or at the upper end of the range examined (3.58 µg/kg; *High*). In the PVH (*top row*) the lower dose resulted in only a few weakly Fos-ir nuclei in the parvocellular division (*p*), which was nonetheless reliably discriminated from that observed in vehicle-injected controls. The higher dose gave rise to much more robust expression throughout the parvocellular division, and at the OT-rich periphery of the magnocellular division, of the nucleus. In the NTS (*middle row*) distinct Fos induction was reliably observed in the medial division (*m*) at the lower dose level, while the higher dose gave rise to a much more widespread pattern of induction that included the dorsal motor nucleus of the vagus (*DMX*) and the area postrema (*ap*), a circumventricular organ. In contrast, no specific Fos induction was detected in the vascular organ of the lamina terminalis (*OVLT*) at the 0.47 µg/kg dose of cytokine. We interpret the small Fos-ir cells at the ventral margin of the OVLT as representing an extension of the meninges, which showed an inconsistent Fos response at the lower end of the range of dosages tested (see Discussion). *AHA*, anterior hypothalamic area; *CC*, central canal; *MePO*, median preoptic nucleus; *och*, optic chiasm; *ts*, solitary tract; *V3*, third ventricle; *ZI*, zona incerta. Magnification, 75×.

branous infoldings. No detectable Fos induction was apparent in the hippocampal formation or the mesencephalic raphe nuclei over the range of dosages examined.

Characterization of candidate afferents

Combined axonal transport–immunohistochemical studies were used to determine which among the candidate afferent media-

tors of IL-1 effects identified above might be anatomically related to the PVH, and to determine their neurotransmitter/modulator phenotype. Animals bearing deposits of a retrogradely transported fluorescent tracer (true blue) were injected with IL-1β at 1.87 μg/kg and anesthetized for perfusion 3 hr later. Four experiments were obtained in which tracer injections were centered in the PVH, with minor and inconsistent involvement of the adjoining nucleus reuniens of the thalamus and/or the anterior hypothalamic area. Consistent with the results of previous studies (e.g., Sawchenko and Swanson, 1982, 1983), injections that involved only the ventral thalamus failed to result in significant labeling of the brainstem cell groups described below, while injections centered in the anterior hypothalamic area ventral and/or caudal to the PVH gave rise to less robust labeling in the caudal brainstem and patterns of labeling in the forebrain that were largely distinct from that seen following deposits centered in the PVH. Only data from the four animals whose injections involved primarily the PVH are considered below.

Complete series of sections from these animals prepared for concurrent demonstration of the retrograde tracer and Fos-ir revealed consistent and substantial double labeling in only two broad regions, the NTS and the rostral ventrolateral medulla; no more than an occasional retrogradely labeled Fos-ir cell was detected in components of the lamina terminalis or any of the other cell groups alluded to above that have been implicated as mediators of IL-1 effects on the HPA axis. Catecholaminergic neurons in both brainstem regions (A_2 noradrenergic and C_2 adrenergic cells of the NTS and C_1 adrenergic neurons of the rostral ventrolateral medulla) have previously been shown to project to the parvocellular division of PVH (Cunningham and Sawchenko, 1988; Cunningham et al., 1990), and have been implicated in the regulation of CRF synthesis and release (Plotsky et al., 1989). To evaluate the extent to which cells in these regions that project to the region of the PVH and manifest Fos induction in response to IL-1 correspond to medullary catecholaminergic neurons, additional sections from these experiments were prepared for concurrent visualization of DBH (a marker for adrenergic and noradrenergic neurons), PNMT (a specific marker for adrenergic neurons), as well as Fos-ir and transported true blue. These preparations revealed that a vast majority of retrogradely labeled NTS neurons displaying IL-1β–induced Fos-ir were catecholaminergic, with caudally situated
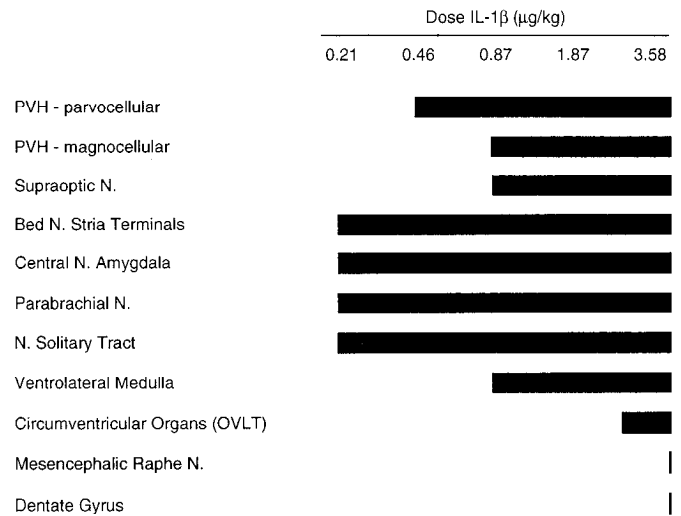
DOSE-DEPENDENT FOS INDUCTION IN CNS REGIONS

Figure 6. Dose-dependent induction of Fos-ir in various regions of the rat brain. *Bars* represent dosage levels at which a majority of animals displayed IL-1–stimulated nuclear Fos-ir in a number of cells that were >2 SD above the mean number detected in vehicle-injected controls. n = 3–8 per condition.

noradrenergic (A_2) neurons predominating (Table 1). A relatively small number of C_2 adrenergic cells of the rostral NTS displayed Fos induction and retrograde labeling (Fig. 7). Fos induction was not observed in dorsomedial medullary catecholamine neurons outside the NTS, that is, in the C_3 adrenergic cell group. It is important to point out that many IL-1β–responsive neurons in the NTS were noncatecholaminergic, though few, if any, of these were retrogradely labeled by tracer injections centered in the PVH.

In the ventrolateral medulla, where a majority of all Fos-ir neurons were retrogradely labeled, rostrally situated PNMT-positive, DBH-negative neurons predominated, indicating a preferential targeting of the C_1 adrenergic cell group (Fig. 7). Nevertheless, a small number of cells largely restricted to the A_1/C_1 transition zone, where C_1 adrenergic and A_1 noradrenergic neurons are intermixed, were reliably triply labeled for true blue, Fos-ir, and DBH-ir, but not PNMT-ir. Overall, these experi-

Table 1. Mean (±SEM) number of cells[a] displaying IL-1β-induced Fos-ir and/or enzyme markers for catecholamine neurons and/or a retrograde tracer following injections in the region of the PVH

	Fos[b]	Fos + Retro	Fos + DBH	Fos + Retro + DBH	Fos + DBH + PNMT	Fos + Retro + DBH + PNMT
Ventrolateral medulla	128 ± 10	4 ± 2	137 ± 27	39 ± 7	319 ± 33	170 ± 22
			A_1		C_1	
Nucleus of the solitary tract	361 ± 99	6 ± 1	204 ± 47	129 ± 14	62 ± 6	32 ± 6
			A_2		C_2	

[a] Counts are derived from four animals and were corrected for double-counting errors using the method of Abercrombie (1941).

[b] Categories are noninclusive, and indicate estimates of the number of cells in the NTS and ventrolateral medullary reticular formation displaying Fos-ir nuclei only (Fos), Fos-ir and the retrograde tracer (Fos + Retro), Fos-ir and dopamine-β-hydroxylase (Fos + DBH), and so on. It should be noted that no cells were identified that stained positively for PNMT but not DBH. Values indicative primarily of counts obtained from the A_1 and A_2 noradrenergic cell groups of the caudal medulla and from the C_1 and C_2 adrenergic cell groups of the rostral medulla are underlined for purposes of clarity.
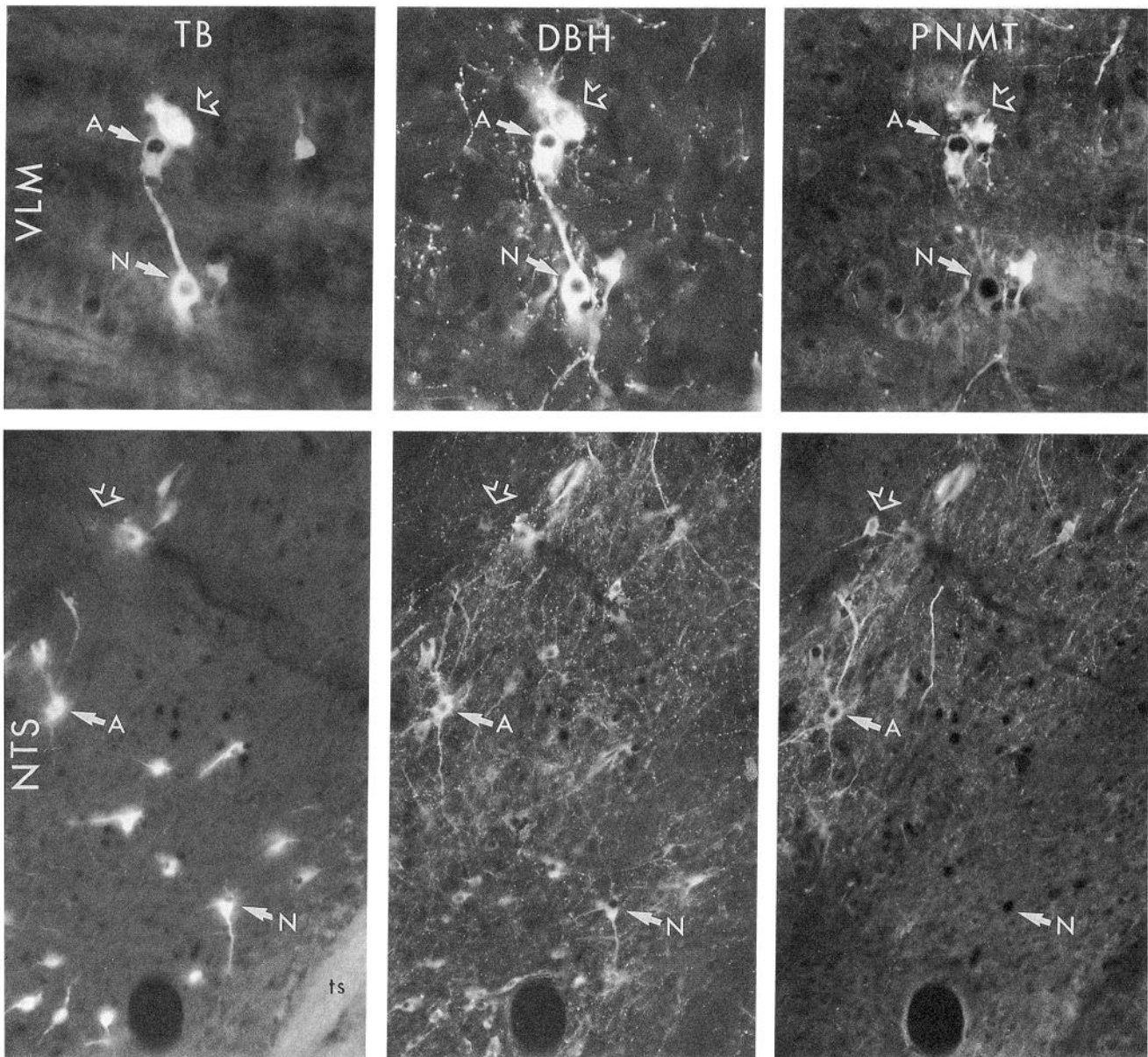
Figure 7. Anatomical and biochemical identification of IL-1–responsive medullary neurons: fluorescence photomicrographs of horizontal sections through the ventrolateral medulla (*VLM*) and the NTS from an animal that received an implant of the retrogradely transported fluorescent tracer, true blue (*TB*), into the PVH, was subsequently challenged with 1.87 μg/kg IL-1, perfused 3 hr later, and prepared for concurrent fluorescence detection of tracer, immunoperoxidase detection of Fos-ir, and immunofluorescence localization of immunoreactive DBH and PNMT. Similarly oriented *solid white arrows* in each set of three micrographs identify examples of cells displaying IL-1–stimulated nuclear Fos-ir, the retrograde tracer, and either the noradrenergic (*N*) or adrenergic (*A*) phenotype. For contrast, *open arrows* identify Fos-negative neurons labeled for one or more of the other markers. *ts*, solitary tract. Magnification: *top row*, 300×; *bottom row*, 150×.

ments suggest that medullary neurons that are responsive to systemic IL-1 and project to the PVH are predominantly catecholaminergic, with a relative regional contribution of $C_1 >$ $A2 \gg C_2 \approx A_1$ (see Table 1).

Experimental manipulation of candidate afferents

To test the hypothesis that medullary catecholamine neurons are involved in mediating IL-1β effects on parvocellular neurosecretory neurons in the PVH, we attempted to unilaterally disrupt ascending aminergic projections near their origins in the medulla and compare cytokine effects on Fos-ir and CRF mRNA on the ipsilateral and contralateral sides. Cuts judged to be effective by virtue of the appearance of accumulated DBH-ir in

axons immediately proximal to the lesion, and a depletion of DBH-ir terminals in the ipsilateral PVH (Fig. 8), lay between the rostrocaudal levels of the trigeminal and facial motor nuclei, and thus would be expected to spare projections from pontine cell groups known to contribute afferents to the PVH, such as the locus coeruleus, mesencephalic raphe, and the parabrachial nuclei (Swanson and Sawchenko, 1983). These transections resulted in a marked attenuation in the number of cells that displayed nuclear Fos-ir in the ipsilateral PVHmpd, seen in response to injection of 1.87 μg/kg of IL-1β 3 hr prior to death (Fig. 8). In addition, these knife cuts also disrupted Fos induction in the magnocellular division of the PVH (Fig. 8) and in the supraoptic nucleus on the ipsilateral side. Fos induction in
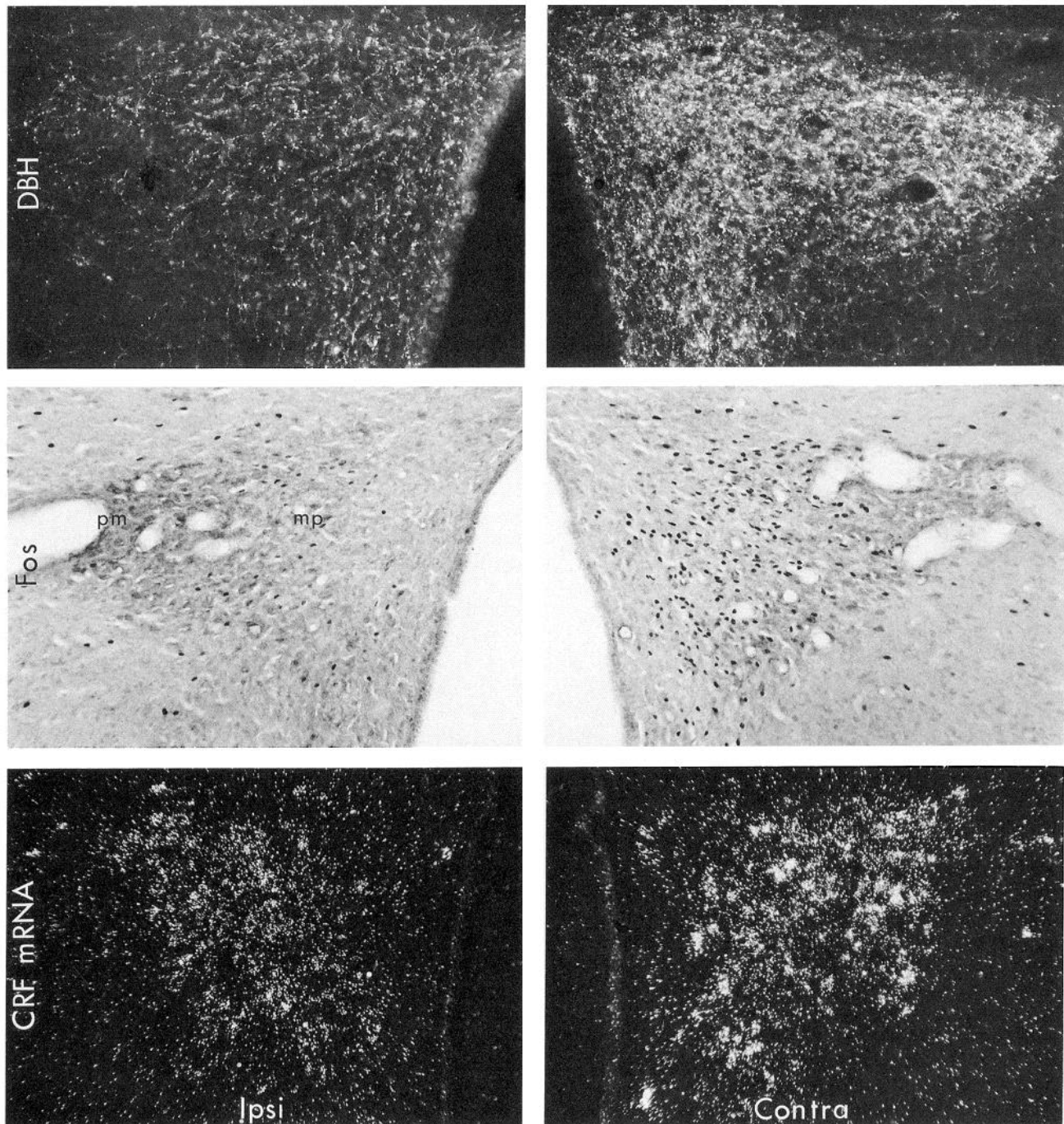
Figure 8. Transection of ascending catecholamine projections interferes with IL-1–stimulated Fos and CRF mRNA expression in the PVH: fluorescence photomicrographs of DBH-ir, bright-field photomicrographs of Fos-ir, and dark-field photomicrographs of CRF mRNA signal in the PVH on the sides ipsilateral (*Ipsi*) and contralateral (*Contra*) to a medullary knife cut intended to disrupt the catecholaminergic innervation of the PVH. The animal was injected with 1.87 μg of IL-1β/kg 3 hr prior to perfusion fixation. Cuts that were effective in reducing the DBH-ir innervation of the PVH dramatically attenuated IL-1–stimulated induction of Fos-ir and increases in CRF mRNA on the ipsilateral side. On the contralateral side, the aminergic innervation and cytokine-stimulated expression of Fos-ir and CRF mRNA were similar to those observed in nonlesioned animals. Abbreviations are as in previous figures. Magnification, 150×.

the contralateral PVH and supraoptic nucleus remained robust in knife-cut, IL-1–injected animals, and comparable to that observed previously under similar treatment conditions in nonlesioned animals. Lesioned animals injected with vehicle showed the very low basal levels of Fos expression throughout the hypothalamus that is characteristic of nonmanipulated rats, with no apparent lateralized differences.

Quantitative densitometric assessments of relative levels of CRF mRNA in additional series of sections through the PVHmpd provided compatible results (Figs. 8, 9). That is, no significant side-to-side differences in CRF mRNA content were detected in vehicle-injected rats ($p > 0.10$), while injection of IL-1β at 1.87 μg/kg to lesioned rats resulted in a significant increase in CRF mRNA on the contralateral side ($p < 0.025$ vs the con-
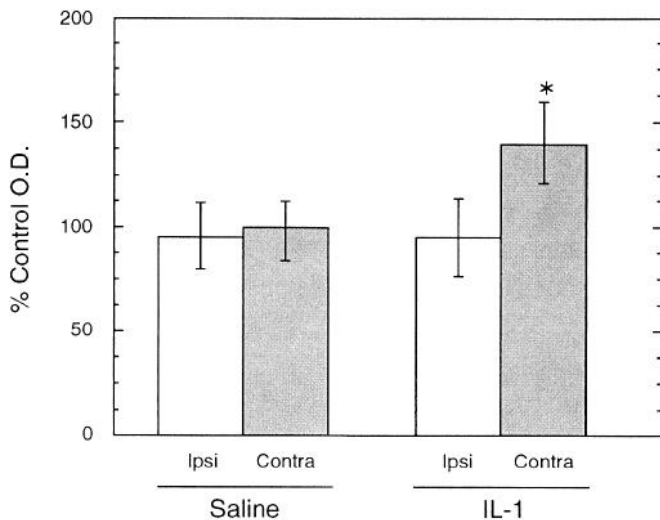
Figure 9. Unilateral knife-cut effects on relative levels of CRF mRNA in IL-1–injected and vehicle-injected rats. Data were obtained by densitometric assessments of emulsion-dipped autoradiograms, and are presented as mean ± SEM percentage of values obtained in the control condition (*Saline, contra*). Knife cuts abolish the expected IL-1–stimulated increase in CRF mRNA on the ipsilateral, but not the contralateral, side. $n = 5$ per group; *, $p < 0.025$.

tralateral side of lesioned, vehicle-injected animals), whose magnitude (40.1 ± 20.5%) was similar to that noted above in comparably treated nonlesioned animals. On the ipsilateral side, however, CRF mRNA levels did not differ significantly from values obtained on either side of the brain of lesioned, vehicle-injected controls (p values > 0.10), suggesting that the knife cuts functionally ablated the response to IL-1 of CRF-producing neurons on the ipsilateral side.

To provide a relevant surgical control, a separate group of five animals were administered unilateral stereotaxic knife cuts in the preoptic region that were designed to interrupt projections to the PVH from cell groups comprising the lamina terminalis of the third ventricle, whose circumventricular aspects have been implicated as possible central monitors of circulating IL-1. These cuts, which were similar in size and placement to those shown previously to abolish osmotically driven responses of parvocellular and magnocellular neurosecretory neurons in the PVH (Kovács and Sawchenko, 1993), did not affect IL-1β–induced Fos-ir in the PVH, which was comparably robust on both sides of the brain.

Discussion

In situ morphological methods were used to identify and characterize stress-related neuroendocrine neurons that are responsive to systemic IL-1β, as well as extrahypothalamic afferents that might be mechanistically involved in the HPA response to the cytokine challenge. Predictions generated from this analysis were tested experimentally, and the results support the view that the integrity of ascending catecholaminergic projections is required for functional activation of hypophysiotropic CRF neurons by IL-1 (see Fig. 10). These findings, in turn, define testable hypotheses as to how IL-1, an important mediator of interactions between the immune and neuroendocrine systems, may be transduced to influence stress-related, as well as other, functional circuitries within the CNS.

Methodological considerations

The present analyses relied heavily on the assumed general applicability of c-*fos* as a marker for functionally activated neurons. While it remains to be determined how widespread the capacity for stimulation-induced c-*fos* expression may be among central neurons, and failures of patterns of Fos induction to conform with other indices of synaptic and/or transcriptional activation have been reported (Dragunow and Faull, 1989; Labiner et al., 1993), this immediate-early gene has proven to be a reliable activity marker for stress-related neuroendocrine neurons, as well as functionally related afferents, across a range of acute and chronic challenge paradigms (see Ceccatelli et al., 1989; Chan et al., 1993, and citations therein). Fewer data are available that speak to the question of whether neurons in different cell groups that may be recruited in a challenge-specific manner to comprise functional circuits need necessarily manifest c-*fos* induction with roughly equivalent sensitivities. This issue is important, as a comparison of dose–response relationships in different cell groups was used to restrict an initially sizeable list of candidate afferent mediators of IL-1 effects to a testable subset. In the few relevant instances in which this notion has been tested, reasonable congruence in the sensitivity of target and afferent cell groups to express c-*fos* have been found (Chan et al., 1993), but the generality of these findings remain to be established. Nonetheless, the fact that an hypothesis generated, in part, from the dose–response data was supported by direct experimental evaluation prompts us to suggest that comparison of time course and dose–response relationships may prove to be a powerful and generally applicable strategy for honing analyses of polysynaptic circuits to a point where relatedness can be assessed directly. In this manner, patterns of c-*fos* expression are not utilized as end points unto themselves, but as tools with which to define a time frame over which stimulation-induced alterations in target gene expression may be expected, and to focus and refine subsequent experimentation.

Along similar lines, the dose dependency of Fos induction is also relevant to the critical question of whether exogenous IL-1 injection may mimic cytokine levels encountered during infectious and inflammatory processes. Intravenously injected IL-1β has a half-life in the rat circulation of approximately 2.9 min (Reimers et al., 1991). Based on this finding, a dose of 0.47 μg/kg, which provoked a reliable induction of Fos-ir in the PVH and the NTS, would result in an initial plasma concentration on the order of 20 nM. While this is similar to peak plasma levels of IL-1β (about 30 nM) reported following systemic administration of bacterial cell wall lipopolysaccharide (LPS; Zuckerman et al., 1989), a widely employed model for systemic bacterial infection, elevated levels of cytokine are sustained for at least 24 hr in LPS-injected mice, but would be expected to fall precipitously in rats injected acutely with IL-1 (Reimers et al., 1991). It is also worthy of note that naturally occurring immune challenges, as well as LPS injection, are associated with elevated levels of additional cytokines, including tumor necrosis factor-α and interleukin-6, that are well known to act synergistically within the immune system, and are independently capable of stimulating the HPA axis (Naito et al., 1988; Bernardini et al., 1990). These considerations, coupled with the very limited number of cell groups evincing Fos induction over the lower end of the range of dosages employed here, suggest that the acute injection paradigm is relevant at least to an established animal model for sepsis. We consider it an open question as to whether
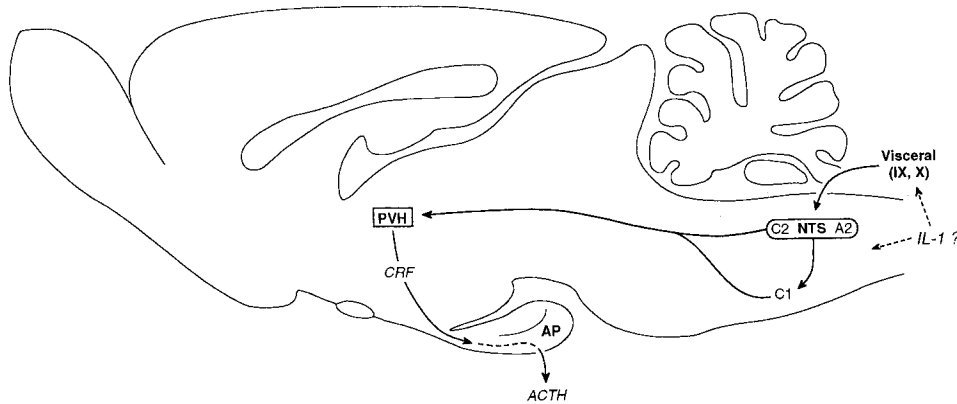
Figure 10. Schematic drawing of a sagittal section through the rat brain to summarize the suggested dependence of the IL-1–induced activation of the HPA axis on medullary catecholaminergic projections to the PVH. The NTS is the principal recipient of primary visceral sensory information carried by the vagus (*X*) and glossopharyngeal (*IX*) nerves. The fact that the C_1, C_2, and A_2 aminergic cell groups implicated in the present study as being involved in the hypothalamic response to IL-1 either reside in, or are synaptically related to, the NTS raises the possibility that circulating IL-1 may be transduced peripherally by sensory components of the cranial nerves, or centrally, at the level of the aminergic neurons themselves, to effect changes in the synthesis and/or secretion of CRF at the level of the PVH. *AP,* anterior pituitary.

the recruitment to Fos expression of additional cell groups seen at higher dosage levels represent specific responses to IL-1.

Hypothalamic targets of systemic IL-1

Since the seminal experiments of Besedovsky et al. (1975, 1986), a wealth of evidence has accrued to document that central or peripheral administration of IL-1 activates the HPA axis. The weight of evidence indicates that this effect is mediated predominantly through increased synthesis and secretion of hypothalamic CRF (Berkenbosch et al., 1987; Sapolsky et al., 1987; Rivest and Rivier, 1991), though participatory sites of action at downstream levels of the axis (i.e., in median eminence, pituitary, and adrenal) have been suggested (Bernton et al., 1987; Matta et al., 1990; Winter et al., 1990; Tominaga et al., 1991; Cambronero et al., 1992). The present findings provide the first report of time- and dose-dependent increase in c-*fos* mRNA and protein in identified CRF-producing cells of the neuroendocrine hypothalamus in response to intravenous administration of IL-1β (cf. Ju et al., 1991; Rivest et al., 1992), thus suggesting that these neurons rapidly increase cellular activity in response to increased circulating IL-1β. This was reflected in a moderate, but reliable, increase in the CRF mRNA content in these cells 3 hr after IL-1β injection, suggesting increased synthesis of CRF in the hypophysiotropic zone of the PVH to this stimulus (see also Suda et al., 1990; Harbuz et al., 1992). The minimum effective dose of IL-1β for Fos induction in parvocellular CRF neurons was about an order of magnitude above that reported to be required for an ACTH secretory response (Rivier et al., 1989). It is not clear whether this disparity may be indicative of some threshold for manifestation (or detection) of Fos synthesis in activated neurons, or of a capacity of IL-1 to stimulate ACTH release via mechanisms independent of the central pathways considered here (see Rivest et al., 1992, and discussion below).

Even within the PVH, neurons exhibiting IL-1β–mediated Fos induction were not limited to parvocellular CRF cells. Cytokine-induced c-*fos* mRNA and protein were reliably detected in cells in the magnocellular division of the PVH, as well as in the supraoptic nucleus, at dosage thresholds only slightly above those observed in the PVHmpd. On the basis of their topography and direct costaining experiments, these were found to corre-

spond preferentially to OT neurons. The literature is conflicting as to whether IL-1β provokes secretion of OT, vasopressin, or both hormones (Sapolsky et al., 1987; Berkenbosch et al., 1989; Naito et al., 1991; Hansen and Christensen, 1992). OT and vasopressin are present in high concentration in hypophyseal-portal plasma and are capable of interacting with CRF to stimulate ACTH secretion (Plotsky, 1991). While the mechanism(s) by which the magnocellular nonapeptides may gain access to the portal circulation is unclear, activation of magnocellular neurons by IL-1 could augment the stimulatory effects of CRF on pituitary–adrenal output.

In addition, a third major visceromotor cell type in the PVH, one that gives rise to long descending projections to cell groups in the brainstem and spinal cord that are associated with sensory and motor functions of the autonomic nervous system (Swanson and Sawchenko, 1983), displayed a rapid and persistent Fos induction in response to IL-1. Whether activation of this cell group may be related to the increased peripheral sympathetic activity that has been reported following IL-1 injection (Berkenbosch et al., 1989; Rivier et al., 1989; Saito et al., 1991) is not yet clear. Though we did not attempt to determine the neurotransmitter phenotype of the autonomic-related PVH neurons manifesting IL-1–stimulated Fos induction, it is of interest to note that CRF and OT are among the neurochemical markers that have been identified for this population (e.g., Sawchenko, 1987; Cechetto and Saper, 1988).

Extrahypothalamic sites of IL-1–induced Fos expression

Dose-dependent induction of c-*fos* expression by IL-1β was also observed in a restricted number of extrahypothalamic cell groups, many of which are recognized as nodal points in central neuroendocrine and autonomic regulation (Loewy, 1990). These include the lateral portion of the central nucleus of the amygdala, the oval subnucleus of the bed nucleus of the stria terminalis, several subnuclei in the lateral division of the parabrachial nucleus, and catecholaminergic neurons of the NTS and ventrolateral medulla. It is of special interest that many IL-1β–responsive cells were found in the NTS, and that some of these were shown to be catecholaminergic and to project to the PVH. The fact that some cells in the caudal NTS displayed a distinct Fos induction at the lowest dose and the earliest time point

examined suggests that this cell group may play a pivotal role in the coordinate regulation of IL-1–mediated activation of CRF secretory activity in the endocrine hypothalamus. The NTS is the principal central terminus of primary visceral sensory information conveyed by the vagus and glossopharyngeal nerves, and projects to each of the major sites of IL-1–stimulated Fos induction described above (Loewy, 1990). Despite the fact that neurons in discrete aspects of amygdala, bed nucleus of the stria terminalis, and the parabrachial complex displayed among the lowest thresholds to IL-1–stimulated Fos induction of the cell groups that we examined, both the topography of Fos-ir neurons and the results of combined retrograde tracing and immunohistochemical analyses suggested that the vast majority of IL-1–activated neurons in these regions are not anatomically related to the PVH. By contrast, IL-1–responsive catecholaminergic neurons of the NTS (A_2 and C_2 cell groups) and ventrolateral medulla (C_1 cell group) were found to project to the region of the PVH. This is consistent with previous findings that each of these cell groups projects preferentially to the parvocellular division of the PVH (Cunningham and Sawchenko, 1988; Cunningham et al., 1990), where they have been implicated in regulating CRF secretion principally via $\alpha1$-adrenoceptor mechanisms (Plotsky, 1987). By contrast, the A_1 cell group of the caudal ventrolateral medulla, which provides the bulk of the aminergic innervation of vasopressinergic magnocellular neurosecretory neurons (Cunningham et al., 1988), was relatively unresponsive to systemic IL-1, an observation consistent with the paucity of magnocellular vasopressin neurons in the PVH that displayed Fos induction to IL-1.

Some of our negative results warrant comment. Induction of Fos-ir in the circumventricular organs, which have been implicated as central transducers of circulating IL-1, required a dose of IL-1β at the upper end of the range tested, approximately 5–10 times higher than the minimum needed to induce reliably Fos-ir in CRF neurons of the PVH. However, nuclear labeling for Fos-ir was sometimes observed at lower doses of IL-1β in small cells at the margins of the OVLT, where it adjoins the optic chiasm. Although this labeling was inconsistent at the lower doses administered, it is potentially relevant to IL-1 effects on the HPA axis. However, these cells, which appear to be meningeal, are not situated within the OVLT proper and were not retrogradely labeled following tracer deposits in the PVH region. Moreover, unilateral transections designed to disrupt descending projections from the lamina terminalis failed to affect the IL-1β–mediated Fos induction in the PVH. Thus, our results fail to support an involvement of cells in the lamina terminalis region in driving IL-1–induced activation (i.e., Fos induction) of hypothalamic neurosecretory neurons.

In addition, we failed to detect IL-1β–mediated induction of c-*fos* expression in the hippocampal formation, the mesencephalic raphe nuclei, the chorioid plexus, and postcapillary venules, all structures in the CNS that have been shown to express high levels of the type 1 IL-1 receptor and/or to be capable of IL-1 ligand binding (Ban et al., 1991; Cunningham et al., 1992). This would suggest that these cells are unresponsive to systemic IL-1β injection, at least over the range of doses and time points examined here. It is important to bear in mind, however, that the lack of a Fos response does not necessarily preclude involvement of a cell group in a functional circuit. In addition to the above-mentioned caveats as to the uncertain generality and sensitivity for recruitment to Fos induction among central neurons, we are particularly concerned that neither Fos, nor any

other immediate-early gene yet examined as a potential activity index in the CNS, provides a marker for cells presented with net *inhibitory* synaptic and/or transcriptional drives (Chan et al., 1993). Thus, while the failures to detect IL-1–stimulated c-*fos* expression in candidate mediators of cytokine effects on the HPA axis are suggestive, they are tempered by demonstrated or potential limitations of Fos-based activity mapping approaches.

Mechanisms underlying IL-1 effects on the HPA axis

In support of the hypothesized involvement of brainstem catecholamine neurons in the hypothalamic response to IL-1β, histochemically verified unilateral ablations of these ascending projections near their origins in the medulla were found to significantly attenuate cytokine-stimulated Fos induction, and to abolish the concomitant increase in CRF mRNA, in the ipsilateral PVH. Aminergic projections from the medulla are known to be predominantly ipsilateral, with a minor crossed component of similar topography (e.g., Sawchenko and Swanson, 1982; Palkovits et al., 1992). Transections like those employed here have been shown to exert lateralized effects on aspects of parvocellular neurosecretory function, while leaving intact the same parameters on the contralateral side (Sawchenko, 1988). Thus, the residual aminergic innervation on the side ipsilateral to such lesions seems insufficient to support normal function, while the minor denervation of the contralateral side is insufficient to disrupt them. It is unlikely that interference with IL-1 effects were due to nonspecific sequelae of the lesions because the magnitude of the CRF mRNA response to IL-1 on the contralateral side was similar to that observed in nonlesioned animals. Moreover, surgical controls that involved unilateral disruption of lamina terminalis projections to the hypothalamus had no impact on IL-1–induced Fos expression in the PVH. It is also worthy of note that with the exception of a relatively small population of peptidergic neurons centered in the caudal part of the NTS (Sawchenko et al., 1992), medullary catecholamine neurons constitute the only known source of afferents to the PVH that arise distal to the level of the transections employed here. The fact that our combined axonal transport–multiple immunohistochemical labeling experiments revealed most Fos-ir neurons in the NTS that were retrogradely labeled from the PVH to be catecholaminergic supports the view that aminergic neurons comprised the principal substrate whose disruption interfered with the hypothalamic responses to IL-1.

In assaying for the dependence of Fos and CRF mRNA responses on the integrity of particular afferents, the present results do not speak directly to the substrates underlying cytokine effects on secretory responses of hypothalamic neuroendocrine neurons. An involvement of catecholaminergic afferents in the hypothalamic response to IL-1 is supported by the results of previous studies where systemic IL-1β was shown to stimulate norepinephrine metabolism in the hypothalamus (Dunn, 1988; Kabiersch et al., 1988). Furthermore, complete deafferentation of the mediobasal hypothalamus (Ovadia et al., 1989) or neurotoxin-induced depletion of hypothalamic catecholamines (Weidenfeld et al., 1989; Matta et al., 1990; Chuluyan et al., 1992) has been shown to block or significantly attenuate ACTH and corticosterone secretory responses to central or peripheral injection of IL-1. Although studies that have examined the extent to which adrenoceptor antagonists may interfere with the secretory responses of the HPA axis to IL-1 have failed to reach a consensus (Rivier et al., 1989; Weidenfeld et al., 1989), it is

worthy of note that medullary catecholaminergic afferents to the PVH are known to express a number of additional synaptically active molecules (Sawchenko et al., 1992), any of which could serve as cotransmitters or modulators in these projections. Collectively, these data support the view that the integrity of medullary aminergic afferents is required for the IL-1 stimulation of CRF synthesis in the endocrine hypothalamus; the likelihood that this dependence may generalize, at least in part, to cytokine-stimulated CRF secretion seems high, but remains to be evaluated directly. While the present data provide no insight as to whether this involvement may be mediating or merely permissive, support for the former is found in the observation that hypothalamic amine depletion blocks pituitary–adrenal secretory responses to IL-1, but not other stressors (Chuluyan et al., 1992).

Systemic administration of IL-1β has, at doses similar to those employed here, been shown to provoke a number of systemic effects, including transient increases in body temperature (A. Morimoto et al., 1990), heart rate (K. Morimoto et al., 1990), serum triglycerides (Feingold et al. 1991), and blood flow to several vascular beds (Dascombe et al., 1989). This raises the question of whether the central neuronal responses observed following peripheral administration of IL-1 reflect responses targeted directly to specific neuroendocrine, among other, control systems, or are merely an indirect result of nonspecific stress and/or sympathetic activation. The weight of the available evidence argues for a substantial degree of targeting. For example, IL-1 affects minimally, if at all, secretory responses of neuroendocrine axes controlling growth hormone, prolactin, and melanotropin secretion (Berkenbosch et al., 1989). The sympathomimetic effects of the cytokine are generally modest (Berkenbosch et al., 1989; Rivier et al., 1989; Saito et al., 1991), and can be blocked by doses of systemic adrenergic antagonists that leave intact the secretory responses of the HPA axis (Rivier et al., 1989). Regarding pyretic effects, analogs of IL-1 have been identified that do not affect core temperature, but that do elicit ACTH and corticosterone secretion (Naito et al., 1990). Finally, the restricted number of cell groups displaying IL-1–induced Fos expression over at least the lower end of the range of dosages we employed and the absence of Fos induction in structures like the locus coeruleus, which might serve as generic indices of activation/arousal (Foote et al., 1983), lend additional support to the notion that the central effects of the cytokine are not simply a result of nonspecific stress.

While the data presented here identify a potential afferent mediator of IL-1 effects on the central limb of the HPA axis, the question remains as to how and where the circulating cytokine gains initial access to the CNS. In view of the strong evidence implicating aminergic neurons in the NTS and ventrolateral medulla as important stations in visceral sensory information processing (Loewy, 1990; Sawchenko et al., 1992), the possibility must be entertained that levels of circulating IL-1 may be recorded by the PNS and conveyed synaptically to the CNS. This hypothesis finds some support in preliminary evidence to suggest that infusion of picomolar quantities of IL-1 into the hepatic portal vein provokes increased firing in sensory fibers of the hepatic branch of the vagus nerve (Nijima, 1992). We are currently attempting to determine whether transection of various branches of the abdominal vagus nerve can mitigate IL-1β–mediated cellular activation in the brainstem and hypothalamus.

Alternatively, a more substantial body of evidence suggests

that IL-1 may exert actions directly within the brain parenchyma. This is indicated by the fact that IL-1 is a more potent stimulant of the HPA axis when administered intracerebroventricularly than systemically (Rivier et al., 1989). In addition, immunoreactive IL-1 has been detected in astrocytes, microglia, and neuronal elements within the CNS (Breder et al., 1988; van Dam et al., 1992), and, as noted above, IL-1 receptors are broadly expressed in brain (Ban et al., 1991; Cunningham et al., 1992). The question of whether the cytokine can traverse the blood–brain barrier remains unresolved, with some reports claiming that IL-1 is unable to enter the brain from the circulation (Coceani et al., 1988; Ferreira et al., 1988), and others providing support for facilitated transport across the barrier (Banks et al., 1989). An emerging alternative hypothesis holds that circulating IL-1 may interact with IL-1 receptors expressed on endothelial cells of the cerebral vasculature, thereby stimulating synthesis of secondary signaling molecules, such as IL-1 itself (Schindler et al., 1990), nitric oxide (Kilbourn and Belloni, 1990), and/or prostaglandins (McCarron, 1992), which may, in turn, be released locally to influence the activity of intraparenchymal neurons. In line with this notion are reports that IL-1–mediated activation of the HPA axis may be attenuated by pretreatment with prostaglandin synthesis inhibitors (Katsuura et al., 1990; Watanabe et al., 1990). It remains to be determined whether transmission through medullary catecholaminergic neurons may be affected, directly or transneuronally, by such IL-1–stimulated paracrine mechanisms.

In conclusion, by combining powerful c-fos–based activity mapping with histochemical characterization in an experimental setting, the present analysis has served to better define hypothalamic stress-related neurosecretory neuron pools that are affected by systemic IL-1β, and to identify specific medullary catecholamine cell groups as candidate afferent mediators of the hypothalamic response (Fig. 10). Experimental evaluation of predictions generated from hodological analyses revealed that the integrity of these ascending afferents is at least required, and may play a mediating role, in the cytokine-stimulated cellular activation, and concomitant increase in CRF synthetic machinery, within hypophysiotropic neurons of the PVH. These data provide a focus for further study of the site(s) of transduction of circulating cytokine, and of the manner in which they evoke integrated neuroendocrine and autonomic activity to maintain homeostasis following acute and chronic immunologic challenges.

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