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Expression of c-Fos immunoreactivity in transmitter-characterized neurons after stress

(corticotropin-releasing factor/catecholamine neurons/paraventricular nucleus/medulla oblongata/colchicine)

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ABSTRACT **The effect of intracerebroventricular injection of the mitosis inhibitor colchicine and of immobilization stress, subcutaneous injection of capsaicin, and intraperitoneal injection of hypertonic salt solution on expression of c-Fos-like immunoreactivity was studied in the rat brain with immunohistochemistry. All the procedures induced c-Fos immunoreactivity in parvocellular neurons of the paraventricular nucleus, and many of these neurons also contained corticotropin-releasing factor immunoreactivity. c-Fos immunoreactivity was also observed, for example, in subpopulations of neurons in the locus coeruleus, the ventrolateral medulla oblongata, and the nucleus tractus solitarii. Many of these cells also expressed catecholamine-synthesizing enzymes. The results suggest that intraventricular injection of colchicine is a stressful stimulus and support the view that several catecholamine cell groups in the lower brainstem are part of the brain circuitry mediating stress reactions, as are the hypothalamic neurons that contain corticotropin-releasing factor.**

The c-*fos* gene (1, 2) is expressed in many tissues in response to growth factor stimulation (3–9). It has been suggested that induction of protooncogenes such as c-*fos* may be important in the establishment of prolonged functional changes in neurons (10). It has been demonstrated by immunohistochemical methods that various types of stimulation induce a c-Fos protein-like immunoreactivity in specific neuron populations in various brain regions (11–13). Thus, immunohistochemical analysis of expression of c-Fos protein in nervous tissue may represent a new tool in neurobiology for metabolic mapping at the cellular level (14). In fact, numerous papers based on this methodology have appeared during the last 2 years.

In the present study we have used immunohistochemistry to analyze to what extent various stressors may induce expression of c-Fos-like immunoreactivity in certain brain regions. In addition, antisera to neuropeptides and transmitter-synthesizing enzymes were used to further characterize c-Fos-activated neurons. Of particular interest to us has been the question whether or not intracerebroventricular (i.c.v.) injections of the mitosis inhibitor colchicine (15) may affect stress-related systems. Ever since colchicine was shown to inhibit axonal transport (16, 17) and to cause marked accumulation of amine storage granules in cell bodies (18), this drug has been used to improve histochemical visualization of transmitters, peptides, and related substances in neuronal cell bodies (19, 20). Our results demonstrate that several stress-inducing procedures as well as i.c.v. colchicine treatment, including the i.c.v. injection procedure *per se*, cause expression of c-Fos protein in restricted neuronal cell populations both in the hypothalamus, including the corticotropin-releasing factor (CRF)-positive neurons in the paraventricular nucleus (PVN), and in the lower brainstem, particularly in catecholamine neurons.

MATERIALS AND METHODS

Male Sprague–Dawley rats (250–300 g; Alab, Stockholm) were used. Five animals per group were studied. In addition to untreated animals, the following groups of rats were analyzed. (*i*) *Colchicine treatment.* The animals were anesthetized with chloral hydrate (1.2 g/kg of body weight, intraperitoneally), treated with colchicine (Sigma; 120 μg in 20 μl of 0.9% NaCl injected into the lateral ventricle), and killed for analysis 1, 3, 6, 12, or 24 hr later. (*ii*) *Immobilization stress.* Rats were immobilized in individual plastic tubes (length, 210 mm; internal diameter, 55 mm; with pores) for 1 hr and killed after 1 min or 2 hr. (*iii*) *Hypertonic stress* (21, 22). Rats were injected intraperitoneally with 2.5 ml of 1.5 M NaCl 3 hr before sacrifice. (*iv*) *Nociceptive activation* (23). Capsaicin (10 mg/kg, Sigma) was injected subcutaneously 3 hr before sacrifice. (*v*) *Control experiments.* (*a*) Animals were gently handled 3 hr before sacrifice. (*b*) A sham intraperitoneal injection (no fluid injected) was performed 3 hr before sacrifice. (*c*) Chloral hydrate was injected intraperitoneally 3 hr before sacrifice. (*d*) After anesthesia, a sham i.c.v. injection was performed 1, 3, 12, and 24 hr before sacrifice. (*e*) Twenty microliters of 0.9% NaCl was injected i.c.v. and the rats were killed 1, 3, 6, 12, or 24 hr later.

Immunohistochemistry. According to published procedures (24), rats were fixed with picric acid-containing formalin, and frontal sections of the rostral hypothalamus, pons, and medulla oblongata were cut in a cryostat and processed for the indirect immunofluorescence method. The sections were incubated with rabbit antiserum to c-Fos (1:400 dilution; Medac, Hamburg, F.R.G.), rinsed, and incubated with rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulins (1:40; Boehringer Mannheim). Hypothalamic sections were then incubated with a rabbit antiserum to CRF (1:800; ref. 25) or a mouse monoclonal anti-oxytocin antibody (1:400; ref. 26) and sections from the lower brainstem with rabbit antiserum to rat tyrosine hydroxylase [TyrOHase; tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] (27) or rat phenylethanolamine *N*-methyltransferase (PNMTase; *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase, EC 2.1.1.28) (28, 29). The sections were then rinsed, incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (Boehringer Mannheim) or sheep anti-mouse antibodies (1:10, Amersham), respectively, and examined in a Nikon Microphot-FX epifluorescence microscope

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Abbreviations: CRF, corticotropin-releasing factor; i.c.v., intracerebroventricular(ly); NTS, nucleus tractus solitarii; PNMTase, phenylethanolamine *N*-methyltransferase; PVN, paraventricular nucleus; TyrOHase, tyrosine hydroxylase; VLMO, ventrolateral medulla oblongata.

equipped with appropriate filters. For control purposes the antisera were preadsorbed with the respective peptide (1 μM) and processed for indirect immunofluorescence. Normal rabbit serum served as control for antisera to c-Fos, TyrOHase, and PNMTase. Incubation with these control sera did not result in any of the staining patterns described below.

RESULTS

The analysis was focused on the rostral hypothalamus including the supraoptic, paraventricular, and arcuate nuclei and on the lower brainstem. No c-Fos-like immunoreactivity was observed in any region of untreated animals (Fig. 1*a*).

Colchicine Treatment. In colchicine-treated animals numerous c-Fos-immunoreactive cell nuclei were observed, mainly in the parvocellular part of the PVN, 1 hr after injection. Slightly more numerous and more strongly immunoreactive cells were seen at 3 hr (Fig. 1*b*) and 6 hr (Fig. 1*c*), followed by a moderate decrease at 12 hr (Fig. 1*e*) and 24 hr (Fig. 1*f*). Many of the c-Fos-positive cells in the parvocellular part were CRF-immunoreactive (Fig. 1*d*). c-Fos-positive cells were also observed, for example, dorsal to the PVN (Fig. 1*b*) and in the arcuate nucleus. At all time intervals after i.c.v. injection of colchicine, positive cells were seen in the pons, *inter alia*, in the locus coeruleus (Fig. 2*a*), the lateral parabrachial nucleus (Fig. 2*b*), the central gray matter, and the reticular formation as well as in the ventrolateral medulla oblongata (VLMO) (Fig. 2*c*) and the dorsal vagal complex, mainly in the nucleus tractus solitarii (NTS). Double-staining experiments demonstrated that many c-Fos-immunoreactive cells in the locus coeruleus and in the VLMO were, at rostral levels, PNMTase-positive (Fig. 2*d*) and TyrOHase-positive, whereas at caudal levels only TyrOHase-like immunoreactivity could be found. Also, in the dorsal medulla many c-Fos-positive cells were PNMTase- and TyrOHase-immunoreactive in the rostral parts, whereas in the caudal part many were TyrOHase-positive but PNMTase-negative.

In control animals given i.c.v. injections with 0.9% NaCl, c-Fos-positive cells were present in the PVN at 1 hr, 3 hr (Fig. 1*g*), and 6 hr after injection but not after 12 or 24 hr. The cells were less strongly immunoreactive and were fewer in number than in the colchicine-injected rats (compare Fig. 1*g* with 1*b*). In the lower medulla oblongata, weakly immunoreactive c-Fos-positive cells were seen in the VLMO and in the NTS.

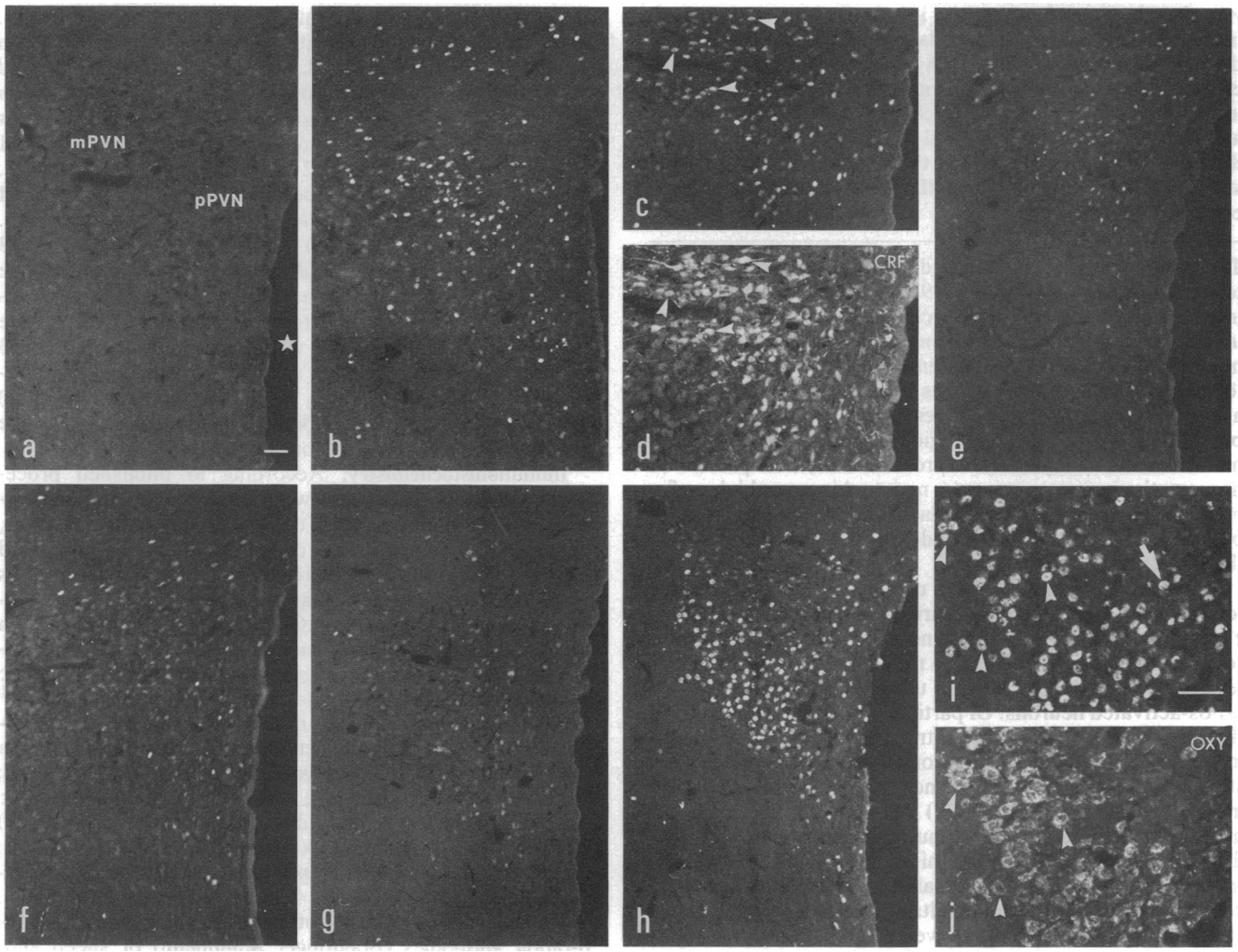

FIG. 1. Immunofluorescence micrographs of the PVN of control rat (*a*) and rats treated with i.c.v. colchicine for 3 hr (*b*), 6 hr (*c* and *d*), 12 hr (*e*), or 24 hr (*f*), or with i.c.v. 0.9% NaCl for 3 hr (*g*), or by intraperitoneal injection of hypertonic salt solution (*h–j*). Sections were processed with antiserum to c-Fos (*a–c* and *e–i*), CRF (*d*), or oxytocin (*j*). The *c,d* and *i,j* pairs show sections processed for double staining. *i* represents a higher magnification of *h*. (*a–f*) Maximum number of c-Fos-positive cells is seen in the parvocellular PVN (pPVN) 3 and 6 hr after colchicine injection. Arrowheads in *c* and *d* point to double-stained cells (c-Fos plus CRF). CRF reaction in *d* is weak because of short colchicine time. (*g*) c-*fos* activation is seen (as c-Fos immunoreactivity) in the pPVN. (*h–j*) Strongly c-Fos-positive cells are seen, especially in the magnocellular PVN (mPVN), and many of these cells are oxytocin-positive (arrowheads in *i* and *j*). Arrow in *i* points to c-Fos-positive, oxytocin-negative cell. Star in *a* indicates third ventricle. (Bar in *a* = 50 μm in *a–h*; bar in *i* = 50 μm in *i* and *j*.)

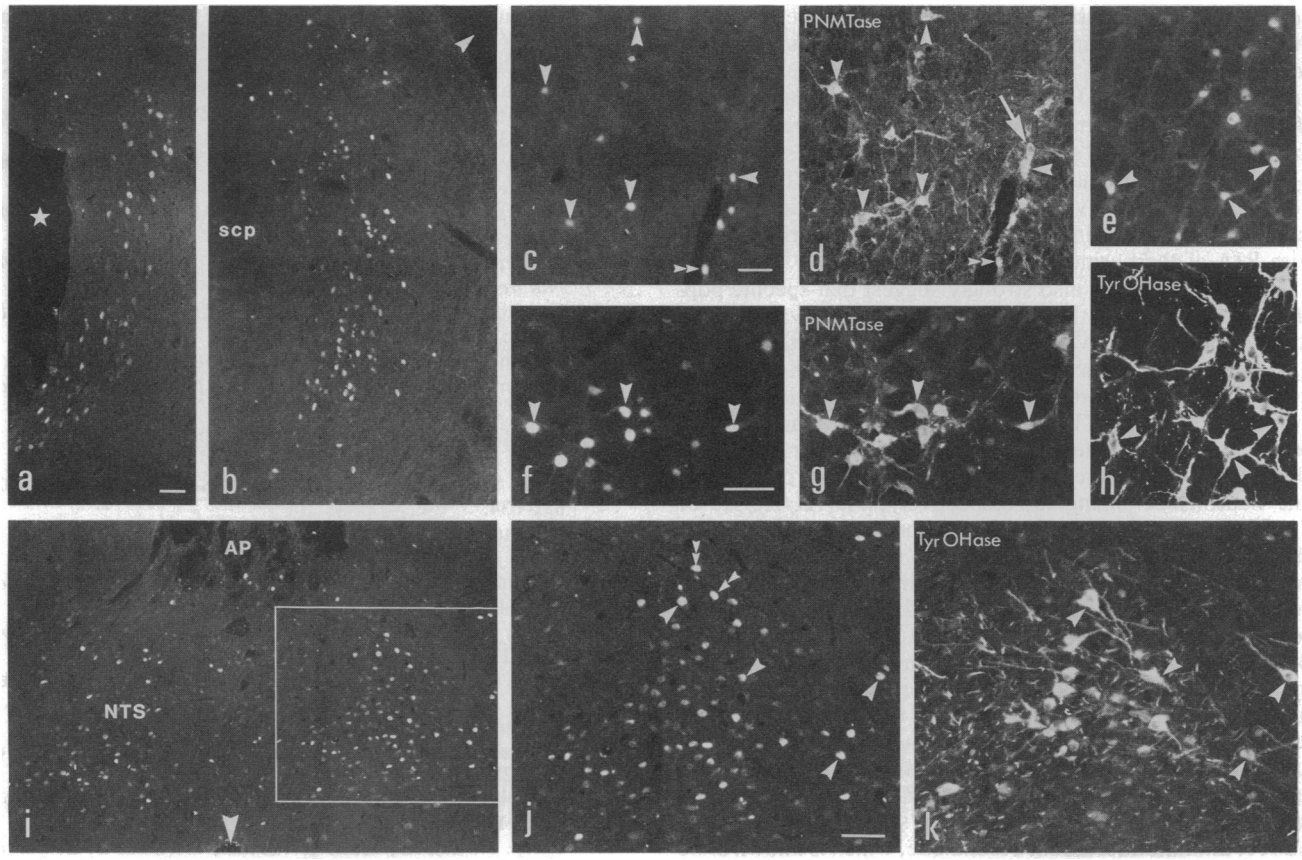

FIG. 2. Immunofluorescence micrographs of the locus coeruleus (*a*), parabrachial nucleus (*b*), rostral VLMO (*c, d, f,* and *g*), caudal VLMO (*e* and *h*), and dorsal vagal complex (*i–k*) of rat treated with i.c.v. colchicine for 3 hr (*a–d*), capsaicin (*e–h*), or hypertonic salt injection (*f, g,* and *i–k*). Sections were processed with antiserum to c-Fos (*a–c, e, f, i,* and *j*), PNMTase (*d* and *g*), or TyrOHase (*h* and *k*). The *c,d; e,h; f,g;* and *j, k* pairs represent sections processed for double staining. *k* represents higher magnification of area indicated by rectangle in *i*. (*a* and *b*) Numerous cells are c-Fos-positive. Star indicates fourth ventricle; scp, superior cerebellar penduncle. Arrowhead in *b* points dorsally. (*c–h*) Most c-Fos-positive cell bodies contain catecholamine-synthesizing enzymes (arrowheads). Arrow in *d* points to PNMTase-positive, c-Fos-negative cell body. (*i–k*) Numerous c-Fos-positive nuclei are seen, mainly in the NTS, but a few are seen in area postrema (AP), some of which are TyrOHase-positive (arrowheads in *j* and *k*). Double arrowheads denote c-Fos-positive, TyrOHase-negative cells. Arrowhead in *i* points to central canal. (Bars = 50 μm for the groups *a, b* and *i; c* and *d; e–h*; and *j* and *k*.)

Sham i.c.v. injection resulted in activation at 1 and 3 hr, but not at 12 and 24 hr. Neither handling nor sham i.p. injection induced expression of detectable c-Fos-like immunoreactivity.

Stress Procedures. After all three procedures, immobilization stress, capsaicin treatment (Fig. 2*e*), and intraperitoneal injection of hypertonic salt solution (Fig. 1 *h* and *i*; Fig. 2 *f, i,* and *j*), c-Fos-positive nuclei were seen in the parvocellular part of the PVN (Fig. 1 *h* and *i*), in the VLMO (Fig. 2 *e* and *f*) and in the NTS (Fig. 2 *i* and *j*), with a similar distribution as seen after colchicine treatment. Also cells in, *inter alia*, the locus coeruleus and the parabrachial nucleus were c-Fos-positive. The number of c-Fos-positive cells and the strength of the immunoreactivity were lower in rats subjected to immobilization stress than in colchicine-treated rats but were higher in capsaicin- and in salt-treated rats. With the two latter treatments, c-Fos immunoreactivity extended outside the NTS—for example, into the area postrema (Fig. 2*i*). In contrast to colchicine treatment, particularly capsaicin and salt injection induced c-Fos in many magnocellular neurons of the PVN (Fig. 1*h*). Double staining revealed that in this nucleus many, but not all, magnocellular c-Fos-positive cell bodies contained oxytocin immunoreactivity (Fig. 1*j*). In the medulla oblongata the cells in the rostral VLMO and NTS were often both TyrOHase- and PNMTase-positive (Fig. 2*g*), whereas many caudal cells contained TyrOHase immunoreactivity (Fig. 2*h*) but no PNMTase immunoreactivity. However, it should be emphasized that many positive cells, especially after capsaicin and hypertonic salt treatment and particularly in the dorsal medulla oblongata, lacked these enzymes (compare Fig. 2*j* with 2*k*).

DISCUSSION

The present results demonstrate that several experimental procedures induce rapid expression of nuclear c-Fos protein in specific regions of the hypothalamus and lower brainstem. At the hypothalamic level the neurons involved are mainly localized in the PVN, which is well known to be an important link in the brain–pituitary–adrenal axis. In the PVN a large population of parvocellular neurons contain CRF immunoreactivity (30), and these neurons also contain the glucocorticoid receptor (31). As shown here, immobilization stress, intraperitoneal injection of hypertonic salt solution, or subcutaneous injection of capsaicin induces c-Fos immunoreactivity in neurons containing CRF, the hypothalamic releasing hormone for adenocorticotropic hormone (32). These various procedures show similar patterns of activation, although the degree of activation differs, with the most intense reaction after capsaicin treatment or salt injection. Interestingly, the CRF neurons are also activated by i.c.v. injection of colchicine, as well as by the i.c.v. injection procedure *per se*. However, the colchicine activation is long-lasting and can be seen also 24 hr after injection, which is not the case with the various control experiments. Thus all these procedures may represent stressful stimuli of different intensity. There are,

however, some apparently qualitative differences between the effects of the various stress procedures and those of colchicine treatment. Thus, whereas only a few magnocellular neurons in the PVN contain c-Fos immunoreactivity after colchicine treatment and immobilization stress, capsaicin or intraperitoneal salt injection induces expression of the c-*fos* gene in many magnocellular neurons (22). The activation of magnocellular vasopressin neurons by noxious stimuli has been shown both in electrophysiological experiments and with RIA of the hormones (33, 34).

The present results also strongly suggest that specific populations of neurons in the lower brainstem are activated by the stressful stimuli as well as by colchicine injection. Thus, subpopulations of neurons in the pons, VLMO, and NTS exhibit c-Fos immunoreactivity under these conditions. Moreover in the VLMO, NTS, and locus coeruleus, many of these neurons can also be shown to contain TyrOHase or TyrOHase plus PNMTase, thus representing norepinephrine or epinephrine neurons belonging to, respectively, the A1/A2/A6 or C1/C2 groups (35). These results support many earlier studies of an involvement of catecholamines in stress reactions as measured with biochemical and histochemical (formaldehyde fluorescence) methods. Thus acute and/or chronic stress has been shown to affect norepinephrine (36–45), epinephrine (46–48), and catecholamine-synthesizing enzymes (45–51). In agreement, both norepinephrine and epinephrine medullary neurons have been shown to contain the glucocorticoid receptor (52). Also, our results show many c-Fos-immunoreactive neurons in the lower brainstem that are not catecholamine neurons, for example, in the parabrachial nucleus. Finally, we emphasize that the present study is based on the indirect immunofluorescence technique, which is known to be less sensitive than the peroxidase-antiperoxidase method and its modifications. It may therefore be that we have mainly revealed strongly activated systems.

In conclusion, the present study demonstrates that colchicine induces prolonged expression of c-Fos immunoreactivity in discrete neuronal populations in the rat brain, to a large extent the same systems that express c-Fos immunoreactivity after rats are exposed to osmotic and noxious stimuli and restraint stress. Therefore, it seems likely that i.c.v. administered colchicine represents a powerful stress stimulus, a conclusion that may be of importance in the design of experiments involving this drug. However, the results also show differential activation; for example, colchicine seems to influence magnocellular hypothalamic neurons only to a small extent, whereas two of the other stimuli markedly affect these systems. A more detailed analysis may therefore further characterize the neuronal circuitry underlying mediation of stressful events. The results also support the view, expressed in the pioneering papers cited above (11–14), that c-Fos histochemistry provides a powerful method for mapping activated neuronal subsystems at the cellular level. As shown here the combination of c-Fos immunostaining with immunohistochemical visualization of transmitters, neuropeptides, and related molecules allows further chemical characterization of the c-Fos-positive neurons. Finally, a recent study of pain-induced c-*fos* activation at the spinal cord level combined with retrograde tracing (53) demonstrates another extension of the applicability of c-Fos immunohistochemistry.

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