

# ***c-fos* mRNA, Fos, and Fos-related Antigen Induction by Hypertonic Saline and Stress**

Frank R. Sharp, Stephen M. Sagar, Katy Hicks, Daniel Lowenstein, and Kinya Hisanaga

Department of Neurology, University of California at Francisco and the San Francisco Veterans Affairs Medical Center, San Francisco, California 94121

The induction of *c-fos* mRNA was assessed using Northern blots and *in situ* hybridization in adult rats administered hypertonic saline (HS) and isotonic saline (IS). HS induced *c-fos* mRNA in magnocellular paraventricular nucleus (PVNm), parvocellular paraventricular nucleus (PVNp), supraoptic nucleus (SON), and lamina terminalis (LMT). This occurred within 5 min, peaked at 30–60 min, and disappeared by 180 min. Fos protein, detected using a specific monoclonal antibody, was maximal at 1–2 hr and disappeared 4–8 hr after HS administration. This confirms observations showing that the *c-fos* gene response is transient even in the presence of a continuing stimulus.

In contrast, Fos-like immunoreactivity (FLI), detected using two polyclonal antisera, was observed in PVNm, PVNp, SON, and LMT for 1–24 hr during continuous osmotic stimulation. Moreover, FLI was observable in these structures for 7 d in rats administered HS and allowed to drink water ad libitum beginning 24 hr later. At times greater than 8 hr, FLI presumably represents Fos-related antigens (FRA), proteins immunologically and functionally related to Fos, whose expression is much more prolonged than authentic Fos following the osmotic stimulus.

In addition to induction of *c-fos* expression in regions specifically involved in osmotic regulation, HS injections also induced *c-fos* in many other forebrain regions. In order to assess the induction of *c-fos* mRNA due to the “stress” of the injections, rats injected with isotonic saline were compared to uninjected controls. Isotonic saline injections induced *c-fos* mRNA in the PVNp, anterior hypothalamus, suprachiasmatic nucleus, cingulate gyrus, neocortex, ventral lateral septal nucleus, piriform cortex, hippocampal pyramidal and dentate granule neurons, paraventricular and intralaminar thalamic nuclei, bed nuclei of stria terminalis, cortical and medial amygdaloid nuclei, and other structures. In accord with other work, we interpret this pattern of *c-fos* expression to result from the stress of handling and injections.

Since Fos and FRA probably bind to the promoters of target genes and regulate their expression, they likely mediate biochemical changes in the cells activated by the osmotic

and stressful stimuli. Whereas the Fos signal is transient, FRA may act on target genes for the duration of the stimulus or longer.

Fos is the 380-amino-acid protein product of *c-fos*. Fos and Jun complex with DNA to regulate transcription from specific target genes (Curran and Morgan, 1987; Curran, 1988; Sonnenberg et al., 1989c). Antibodies to some peptides of Fos recognize both Fos and related proteins termed Fos-related antigens (FRA). FRA are also induced by a variety of extracellular stimuli and have antigenic, structural, and DNA-binding properties in common with Fos (Sonnenberg et al., 1989a,b). Fos and FRA are proposed to act as “third messengers” that link extracellular signals to long-term alternations in gene expression (Verma and Sassone-Corsi, 1987; Curran, 1988).

Since ions, neurotransmitters, and growth factors induce *c-fos*, it is not surprising that many stimuli, including seizures, cutaneous stimulation, and cortical injury, induce Fos and FRA in brain (Morgan and Curran, 1986; Morgan et al., 1987; Dragunow and Robertson, 1987, 1988; Hunt et al., 1987; White and Gall, 1987; Sagar et al., 1988; Bartel et al., 1989; Draisci and Iadarola, 1989; Sharp et al., 1989a–c; Sonnenberg et al., 1989a,b). Induction of the *c-fos* gene in brain is mediated by many receptors including neuronal NMDA and opiate receptors (Szekely et al., 1987, 1989; Cole et al., 1989; Menetrey et al., 1989; Sonnenberg et al., 1989a; Hisanaga et al., 1991) as well as glial EGF and FGF receptors (Arenander et al., 1989; Condorelli et al., 1989; Hisanaga et al., 1990).

Water deprivation also induces Fos and possibly FRA (Sagar et al., 1988) in brain regions known to respond electrophysiologically to osmotic stimuli (Kalimo, 1975; Brimble and Dyball, 1977; Swanson and Sawchenko, 1983). In this study, we administered hypertonic saline intraperitoneally in order to define the time course of Fos and FRA induction related to the onset and cessation of the osmotic stimulus.

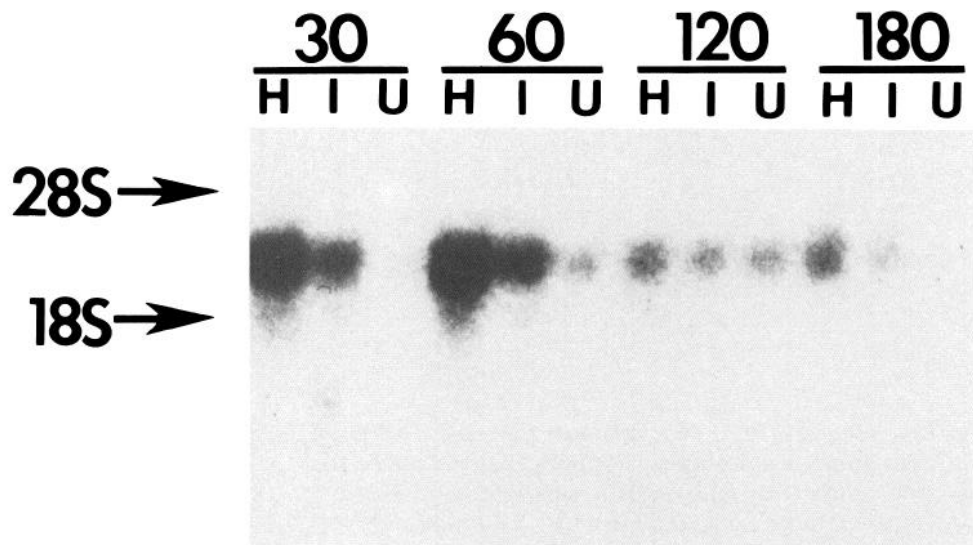
## **Materials and Methods**

Sprague–Dawley rats (250–350 gm) were injected intraperitoneally with 4 cc of isotonic (0.9%) NaCl or 4 cc of hypertonic (1.35%) NaCl. Following isotonic saline (IS), three subjects were allowed to survive for 5 min, two for 20 min, and seven for 60 min. Following hypertonic saline (HS), three subjects were allowed to survive for 5 min, two for 15 min, two for 30 min, five for 60 min, three for 120 min, two for 180 min, three for 240 min, and three for 24 hr. Opiate analgesics were not administered following HS because these drugs affect Fos and FRA. Subjects were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) and perfused with 100 cc of IS and heparin (10 U/cc) and 500 cc of 4% paraformaldehyde (PFA) in 0.1 M, pH 7.4, phosphate buffer. Six controls were anesthetized in the animal room without prior handling and perfused within 5 min. Brains were removed and postfixed

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**Figure 1.** Northern blots of *c-fos* mRNA from adult rat hypothalamus of uninjected control subjects (U), of subjects injected intraperitoneally with isotonic (I) saline, and of subjects injected intraperitoneally with hypertonic (H) saline. The *c-fos* mRNA responses ( $n = 3$  for each blot) at 30, 60, 120, and 180 min after saline injection are pictured.

overnight. 50- or 100- $\mu$ m-thick coronal sections were cut on a vibratome from the anterior commissure through the hypothalamus and placed in sterile tissue culture wells with DEP-treated phosphate buffer (PB).

**Fos immunocytochemistry.** Alternate sections were placed in 2% horse serum/0.2% Triton X-100/0.1% BSA (Hors) for 15 min. Sections were incubated for 72 hr at +5°C on a slow shaker in primary antibody diluted 1:3000 in Hors. The antibody was a monoclonal raised to a synthetic peptide (Fos 4–17) from the N-terminal end of Fos (LA041; Microbiological Associates, Bethesda, MD). This peptide has little homology with known Fos-related antigens, and the antibody produces one band on Western blots (De Togni et al., 1988). Therefore, LA041 is presumed to be specific for Fos. Sections were immunostained as previously described (Sharp et al., 1990) using an Elite Vectastain kit (Burlingame, CA). Control sections, incubated in the presence of 2  $\mu$ g/ml of synthetic N peptide, showed no nuclear staining.

**Immunohistochemistry with polyclonal antisera.** Subjects were given HS intraperitoneally and divided into three groups. The first group was not allowed to drink water. Following survivals of 1, 2, 3, 4, and 24 hr ( $n = 2$  at each time), they were anesthetized and perfused with PLP fixative (2% paraformaldehyde, 0.1 M lysine, and 0.2% periodate in PB). The second group began drinking water ad libitum 1 hr after intraperitoneal HS. The third group began drinking water ad libitum 24 hr after intraperitoneal HS. For the second and third groups, two subjects were anesthetized and perfused with PLP fixative 1, 2, 3, and 4 hr and 1, 2, 3, 4, and 7 d after administration of the HS. Following perfusion, brains of subjects in all groups were removed, postfixed 1–4 hr in PLP, and cut on a vibratome. Sections were immunostained for Fos and FRA using one of two polyclonal antibodies. One antibody to the M peptide (Fos 127–152) recognizes authentic Fos and two lower molecular weight FRA on Western blots (Sonnenberg et al., 1989a,b). The second polyclonal antibody, R1B6, was raised in our laboratory to the M2 peptide (Fos 132–154). It also recognizes Fos and two lower molecular weight FRA on Western blots (Aronin et al., 1990). The ABC immunocytochemical method was employed to visualize Fos and FRA as previously described (Sagar et al., 1988; Sharp et al., 1989a–c; Sagar and Sharp, 1990). Control sections incubated in the presence of the appropriate M

or M2 peptide (1  $\mu$ g/ml) showed no staining. The relative amount of immunostaining using the monoclonal (see Table 1) and polyclonal antibodies (see Table 3) was scored as undetectable (0), trace (Tr), and up to maximal (3+).

**In situ hybridization.** Free-floating sections in tissue culture wells were processed as previously described (Sharp et al., 1990). Hybridization was performed for 8 hr at 65°C in 4 $\times$  SSC, 50% formamide, 1 $\times$  Denhardt's reagent, 1 mg/ml salmon sperm DNA, 1 mg/ml yeast tRNA, 20 mM dithiothreitol, 5 mM EDTA, and 2.5  $\times$  10<sup>6</sup> cpm/ml complementary <sup>35</sup>S-radiolabeled RNA probe shortened to an average length of about 150 bases by alkaline hydrolysis. The RNA probe was synthesized using a plasmid kindly provided by Dr. Curran (Roche, Nutley, NJ). The plasmid contains the full-length *c-fos* cDNA cloned into pSP65 in the antisense direction relative to the SP6 promoter. Once hybridized, washed, and treated with RNAase, sections were mounted onto gelatinized slides that were placed in x-ray film cassettes with Kodak SB5 film for 5 d. Following this, the slides were dipped in Kodak NTB2 nuclear emulsion. After a 5-week exposure, they were developed in D19 for 3 min, washed, fixed, washed, dried, and coverslipped. Control sections from selected subjects were hybridized with sense probe.

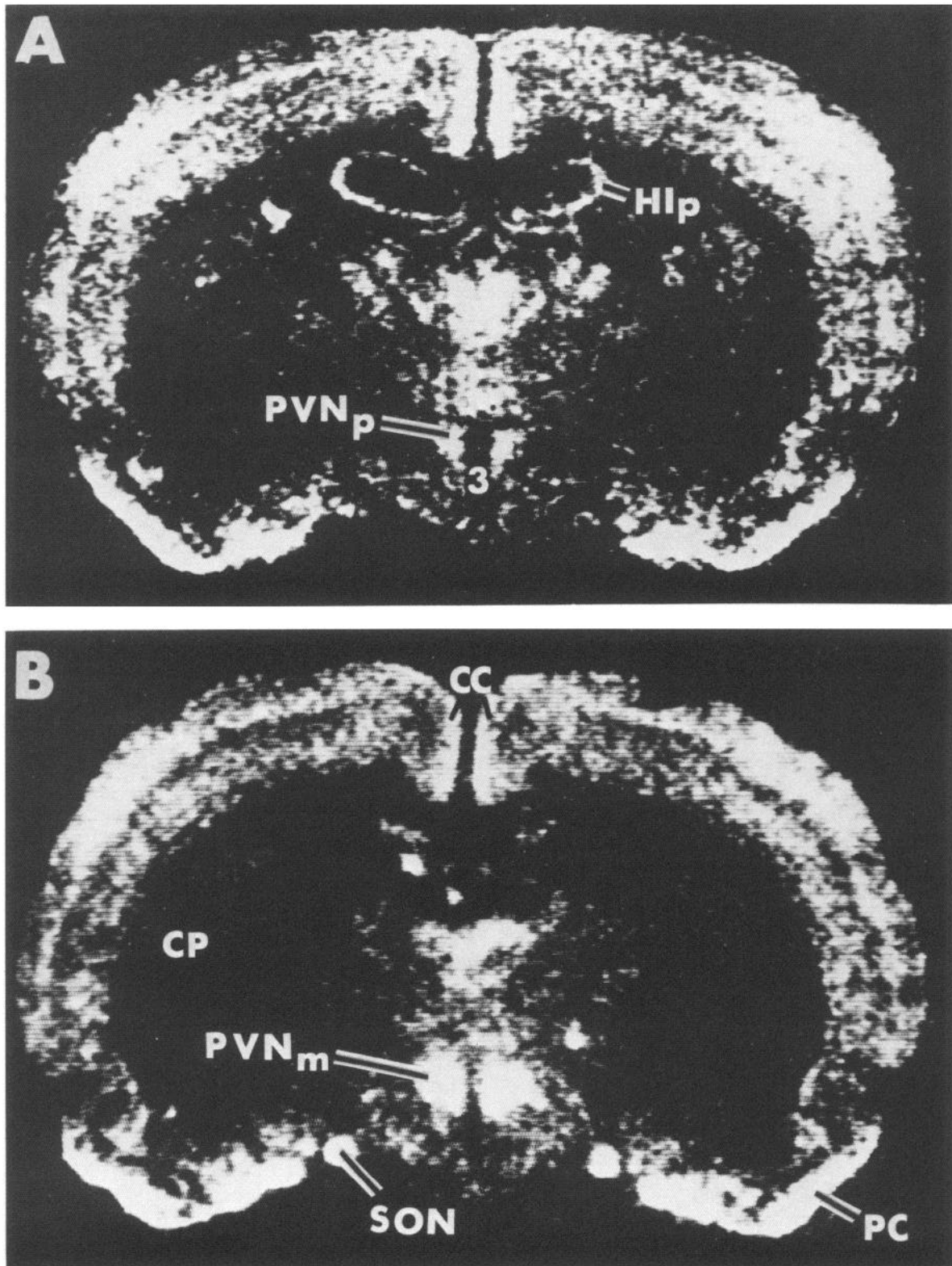
To quantify the hybridization signal, x-ray film autoradiographs were digitized on a video camera/IBM computer-based image analysis system (MCID, Canada). The optical densities of areas of interest were measured on at least three sections from each subject. The average optical density for each region of each subject was divided by the average optical density of the internal capsule to obtain an optical density ratio (ODR) for each region. Significant differences between groups were determined using a Student's *t* test ( $p < 0.05$ ).

**Northern blotting.** Northern blots were performed from a separate group of subjects allowed to survive 30, 60, 120, and 180 min ( $n = 3$  at each time) after each of the following manipulations. Subjects were administered either 4 cc of IS intraperitoneally or 4 cc of HS intraperitoneally or were left alone to serve as uninjected controls. Subjects were narcotized with CO<sub>2</sub> and decapitated. The hypothalamus was dissected and frozen on dry ice. Total RNA was extracted in 5 M guanidinium isothiocyanate followed by precipitation in 3 M LiCl. Following pro-

**Table 1.** Fos protein immunostaining obtained with the LA041 monoclonal antibody in PVNm neurons from three adult rats following intraperitoneal administration of 4 cc of hypertonic (1.35%) saline

Time (hr)	0	1	2	3	4	5	6	8
Immunostaining	0	2+	3+	2+	1+	1	Tr	0
Osmolarity	291	340	338	333	328	326	325	323

Subjects had their plasma osmolarities measured at the times listed (standard error of the mean for each osmolarity was less than 1 mmol/kg). They did not have access to food or water for the duration of the experiment. The relative immunostaining in the PVNm was scored as undetectable (0), trace (Tr), or up to maximal (3+).

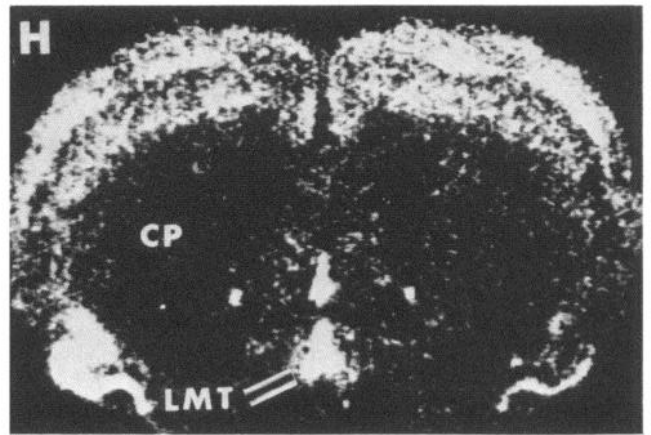
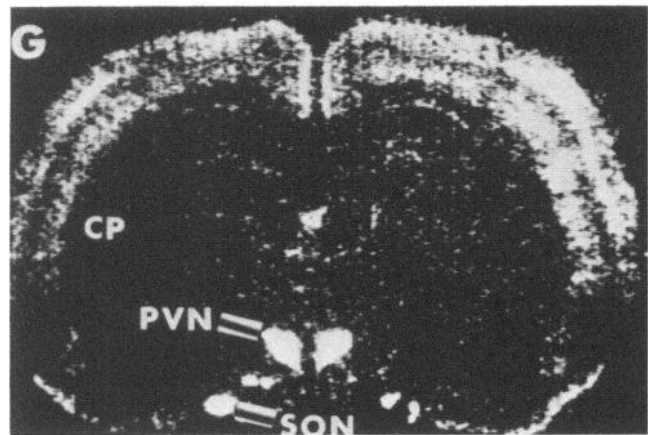
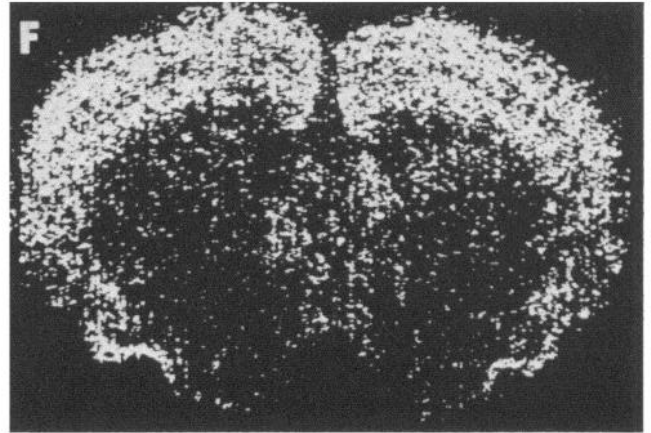
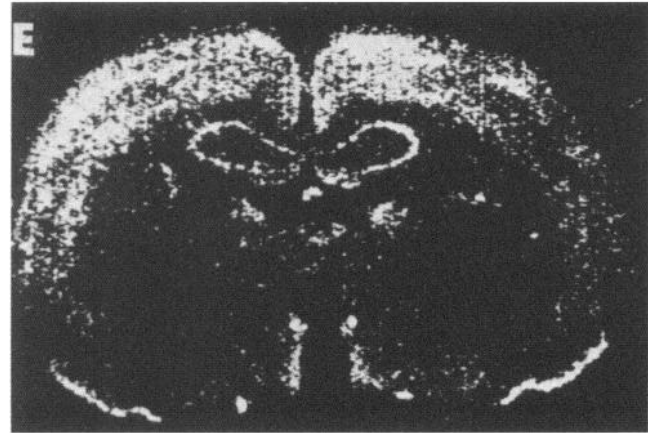
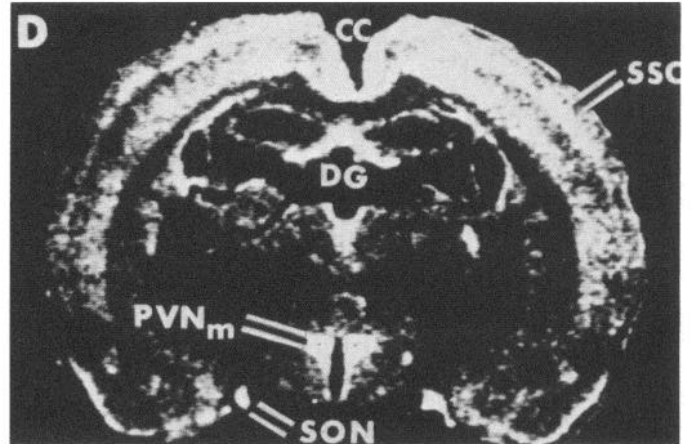
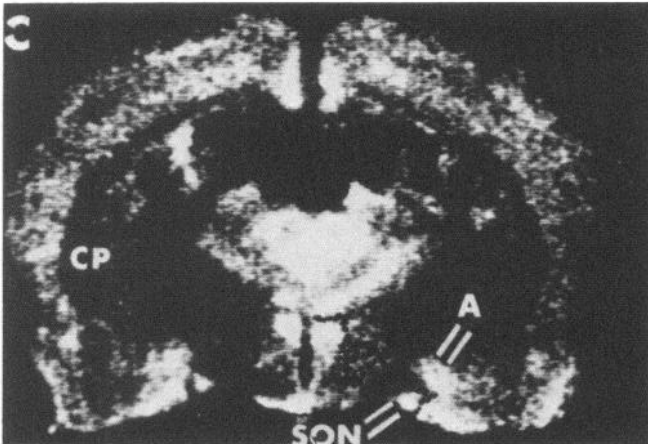
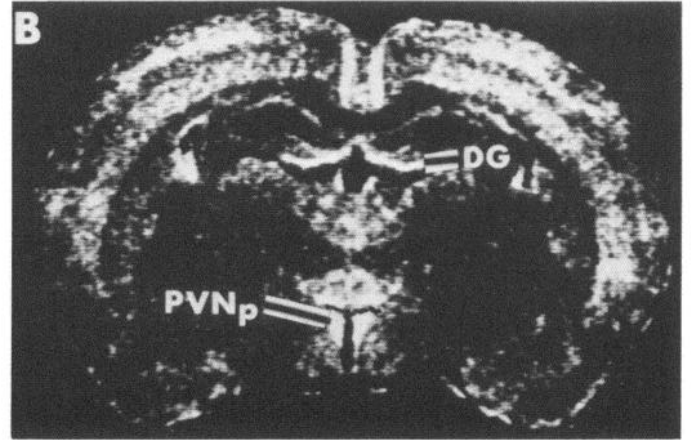


**Figure 2.** *c-fos* mRNA *in situ* hybridization coronal brain autoradiographs from subjects administered (A) isotonic (IS) saline and (B) hypertonic (HS) saline 60 min previously. *PVN<sub>m</sub>*, magnocellular paraventricular nucleus of hypothalamus; *PVN<sub>p</sub>*, parvocellular PVN; *SON*, supraoptic nucleus; *HIp*, pyramidal neurons of hippocampus; *CC*, cingulate cortex; *PC*, piriform cortex; *CP*, caudate putamen; 3, third ventricle.

teinase K digestion, phenol/chloroform extractions, and ethanol precipitation, RNA was quantified by OD at 260 nm. Five micrograms of RNA was denatured in 50% formamide, loaded onto a 1.5% horizontal agarose gel, and electrophoresed in the presence of 2.2 M formaldehyde.

The RNA was transferred to a nylon membrane filter (Nytran, Schleicher and Schuell) by capillary blotting and hybridized at high stringency [1.5 × saline/sodium/phosphate/EDTA (SSPE), 50% formamide at 65°C] to the full-length complementary <sup>32</sup>P-RNA probe. After washing at 65°C





**Table 2. Optimal density ratios obtained from *c-fos* mRNA *in situ* hybridization autoradiographs of control subjects compared to subjects injected with isotonic (IS) and hypertonic (HS) saline**

Region	Control (n = 6)	5 min IS (n = 3)	20 min IS (n = 2)	1 hr IS (n = 7)	1 hr HS (n = 5)
LMT	1.06 ± 0.02	1.25 ± 0.06*	1.04 ± 0.02	1.06 ± 0.09	1.43 ± 0.14*!
PC	1.18 ± 0.21	2.11 ± 0.40*	1.38 ± 0.07*	1.30 ± 0.16*	1.34 ± 0.25*
CC	1.14 ± 0.06	1.36 ± 0.14*	1.25 ± 0.02*	1.19 ± 0.13	1.22 ± 0.11
LS	1.08 ± 0.05	1.22 ± 0.08*	1.14 ± 0.09	1.11 ± 0.07	1.10 ± 0.07
SCN	1.22 ± 0.03	1.34 ± 0.06*	1.29 ± 0.08	1.29 ± 0.11	1.30 ± 0.15
SSC	1.18 ± 0.13	1.35 ± 0.05*	1.31 ± 0.03*	1.22 ± 0.15	1.27 ± 0.15
PVA	1.11 ± 0.06	1.33 ± 0.05*	1.16 ± 0.04	1.17 ± 0.08	1.19 ± 0.09
AN	1.07 ± 0.04	1.19 ± 0.04*	1.10 ± 0.04	1.08 ± 0.06	1.08 ± 0.04
CP	1.06 ± 0.03	1.10 ± 0.02	1.05 ± 0.01	1.06 ± 0.04	1.03 ± 0.01
LH	1.05 ± 0.02	1.11 ± 0.03	1.05 ± 0.03	1.05 ± 0.02	1.08 ± 0.04
VL	1.05 ± 0.04	1.09 ± 0.02	1.06 ± 0.02	1.05 ± 0.04	1.03 ± 0.02

Controls were not injected. Experimental subjects either were injected intraperitoneally with isotonic (0.9%) saline (IS) and sacrificed at the indicated times or were injected with hypertonic (1.35%) saline (HS) and sacrificed 1 hr later. Optical densities (OD) of each structure were measured on x-ray films and divided by the OD of white matter to obtain OD ratios. The mean OD ratio ± SD for each structure for the animals in each group is listed. Differences ( $p < 0.05$ ) between saline-injected (IS and HS) and uninjected control animals (\*) and between the 1-hr isotonic and hypertonic saline-treated animals (!) were evaluated using the Student's *t* test.

for 1 hr each with  $2 \times$  SSC/0.1% SDS,  $0.5 \times$  SSC/0.1% SDS, and  $0.2 \times$  SSC/0.1% SDS, the membrane was exposed overnight to Kodak XAR film with an intensifying screen at  $-70^\circ\text{C}$ .

**Plasma osmolarity.** Four rats were anesthetized, and PE50 catheters were inserted into the femoral artery with minimal blood loss. After obtaining a 100- $\mu\text{l}$  blood sample, 4 cc of HS was administered intraperitoneally. Blood samples (100  $\mu\text{l}$ ) were then obtained each hour for the next 8 hr and replaced with equal amounts of isotonic saline. The plasma osmolarities of these centrifuged blood samples were measured with a Wescor vapor pressure osmometer (Table 1).

Four additional rats were administered 4 cc of HS intraperitoneally. Two were allowed to begin drinking water 1 hr later, and two were allowed to begin drinking water 24 hr later. At 1, 2, 3, and 7 d after HS, subjects were anesthetized, 100- $\mu\text{l}$  blood samples were obtained via cardiac puncture, and plasma osmolarities were measured (see Table 3).

## Results

The plasma osmolarities of subjects administered intraperitoneal hypertonic saline and not allowed to drink were maximal at 1 hr and slowly decreased over the next 8 hr (Table 1). However, their plasma osmolarities were still quite elevated 8 hr after administration of the hypertonic saline (Table 1).

Differences in *c-fos* mRNA induction were detected between the hypertonic and isotonic saline-injected subjects and between the isotonic saline-injected subjects and the uninjected (U) control subjects (Fig. 1). Northern blots demonstrated a single species of *c-fos* mRNA in the hypothalamus. Hypertonic (H) and isotonic (I) saline induced *c-fos* mRNA in the hypothalamus compared to uninjected (U) controls at 30–180 min following saline administration (Fig. 1). However, injections of hypertonic saline induced *c-fos* mRNA to a greater extent (two- to threefold increases in optical density) than isotonic saline, the peak occurring 30–60 min following saline administration (Fig. 1).

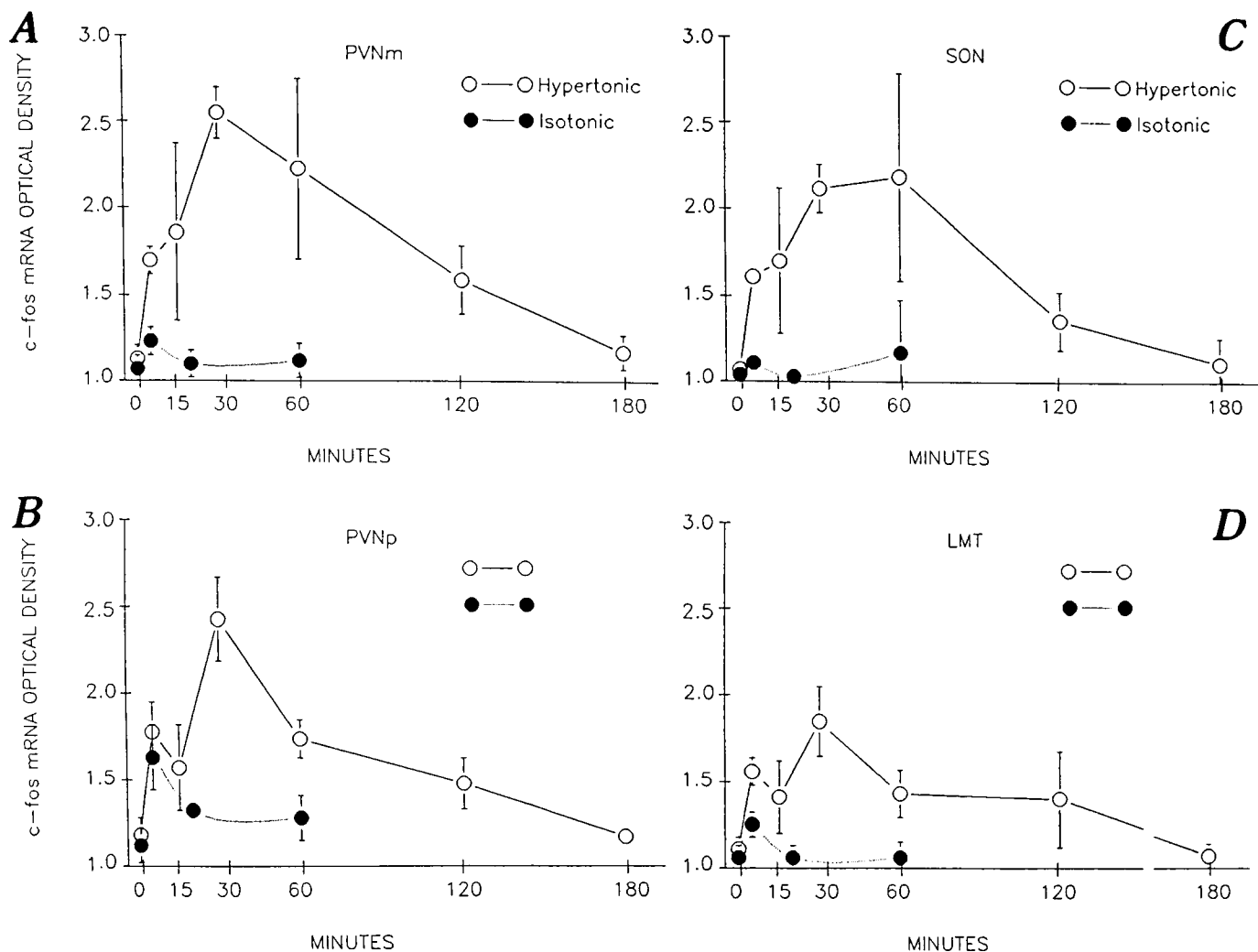
*In situ* hybridization showed that more *c-fos* mRNA was induced in the magnocellular paraventricular nucleus (PVNm),

supraoptic nuclei (SON), and lamina terminalis (LMT) at 5 and 15 min following administration of hypertonic (Figs. 3C; 4; 5A,C) compared to isotonic saline (Figs. 3B, 4). However, at 5 min the amount of *c-fos* mRNA in the parvocellular PVN (PVNp) was similar in hypertonic and isotonic saline-injected subjects (Figs. 3B,C; 4B). At 30, 60, and 120 min, more *c-fos* mRNA was induced in the PVNm, PVNp, SON, and LMT of the hypertonic (Figs. 2B; 3D,G,H; 4; 5B,D,F) compared to the isotonic (Figs. 2A; 3E,F) saline-injected subjects. At 180 min, there was no difference in the amount of *c-fos* mRNA in hypertonic compared to isotonic saline-injected subjects in PVNm, PVNp, SON, and LMT (Fig. 4).

Immunocytochemistry using the LA041 monoclonal antibody revealed few if any Fos immunoreactive cells in PVNm, PVNp (Fig. 6A), SON (Fig. 7A), and LMT (Fig. 6F) in uninjected controls and in subjects 5 min and 1 hr after being injected with isotonic saline (Fig. 7B). Following administration of hypertonic saline, some Fos immunoreactivity was detected by 30 min (Fig. 6B), and substantial Fos was expressed in PVNm (Fig. 6C), PVNp (Fig. 6C), SON (Fig. 7D), and LMT (not pictured) by 1 hr. This response reached a maximum at 2 hr (Figs. 6D,G; 7E) and disappeared by 4–8 hr. There was no immunostaining of PVNm, PVNp, SON, or LMT with the LA041 antibody at 8 (Table 1) or 24 hr after administration of hypertonic saline.

Using the R1B6 polyclonal antiserum and the polyclonal antiserum developed by Curran (Sonnenberg et al., 1989a,b), there was heavy immunostaining of PVNm (Table 3), PVNp, SON, and LMT for 1–24 hr in subjects given hypertonic saline and not allowed to drink. In subjects allowed to drink 1 hr after administration of hypertonic saline, R1B6 immunostaining was detectable in PVNm from 1 hr to 3 d later, but not at 4–7 d (Table 3). In subjects allowed to drink 24 hr after administration of hypertonic saline, R1B6 immunostaining was detectable in

←  
**Figure 3.** *c-fos* mRNA *in situ* hybridization coronal brain autoradiographs from an untouched control (A), from subjects administered isotonic saline 5 min (B) and 30 min previously (E and F); and from subjects administered hypertonic saline 5 min (C) and 30 min previously (D, G, H). PVNp, parvocellular PVN; PVN, paraventricular nucleus of hypothalamus; SON, supraoptic nucleus; LMT, lamina terminalis; CP, caudate putamen; A, amygdala; DG, dentate gyrus; SSC, somatosensory cortex; CC, cingulate cortex.



**Figure 4.** Expression of *c-fos* mRNA measured on x-ray film *in situ* hybridization autoradiographs for (A) the magnocellular paraventricular nucleus of hypothalamus (PVNm); (B) the parvocellular (PVNp) paraventricular nucleus; (C) the supraoptic nucleus (SON); and (D) the lamina terminalis (LMT) as a function of time after intraperitoneal administration of hypertonic (open circles) and isotonic (solid circles) saline.

the PVNm from 1 hr to 7 d later (Table 3). Similar findings were observed in PVNp, SON, and LMT.

*c-fos* mRNA was induced in many structures of subjects injected with isotonic saline (Figs. 2A; 3B,E,F; Table 2) compared to uninjected controls (Fig. 3A, Table 2). These structures included the PVNp (Figs. 2A, 3B), anterior hypothalamus (Fig. 3B), suprachiasmatic nucleus, cingulate cortex (Fig. 3B), neocortex (layers 2, 3, 5, 6) (Figs. 2A; 3B,E,F), septal nucleus (Fig. 3F), olfactory/piriform cortex (Figs. 2A; 3E,F), the paraventricular and intralaminar thalamic nucleus, anterior thalamic nucleus (Fig. 3B), dentate gyrus granule neurons (Fig. 3B), hippocampal pyramidal neurons (Fig. 2A), bed nucleus of the stria terminalis, and the cortical and medial amygdaloid nuclei. We interpret this pattern as being due to the stress of handling and injection. The *c-fos* mRNA stress response peaked at 5–10 min after isotonic saline administration and disappeared by 1 hr in all regions except piriform cortex (Table 2).

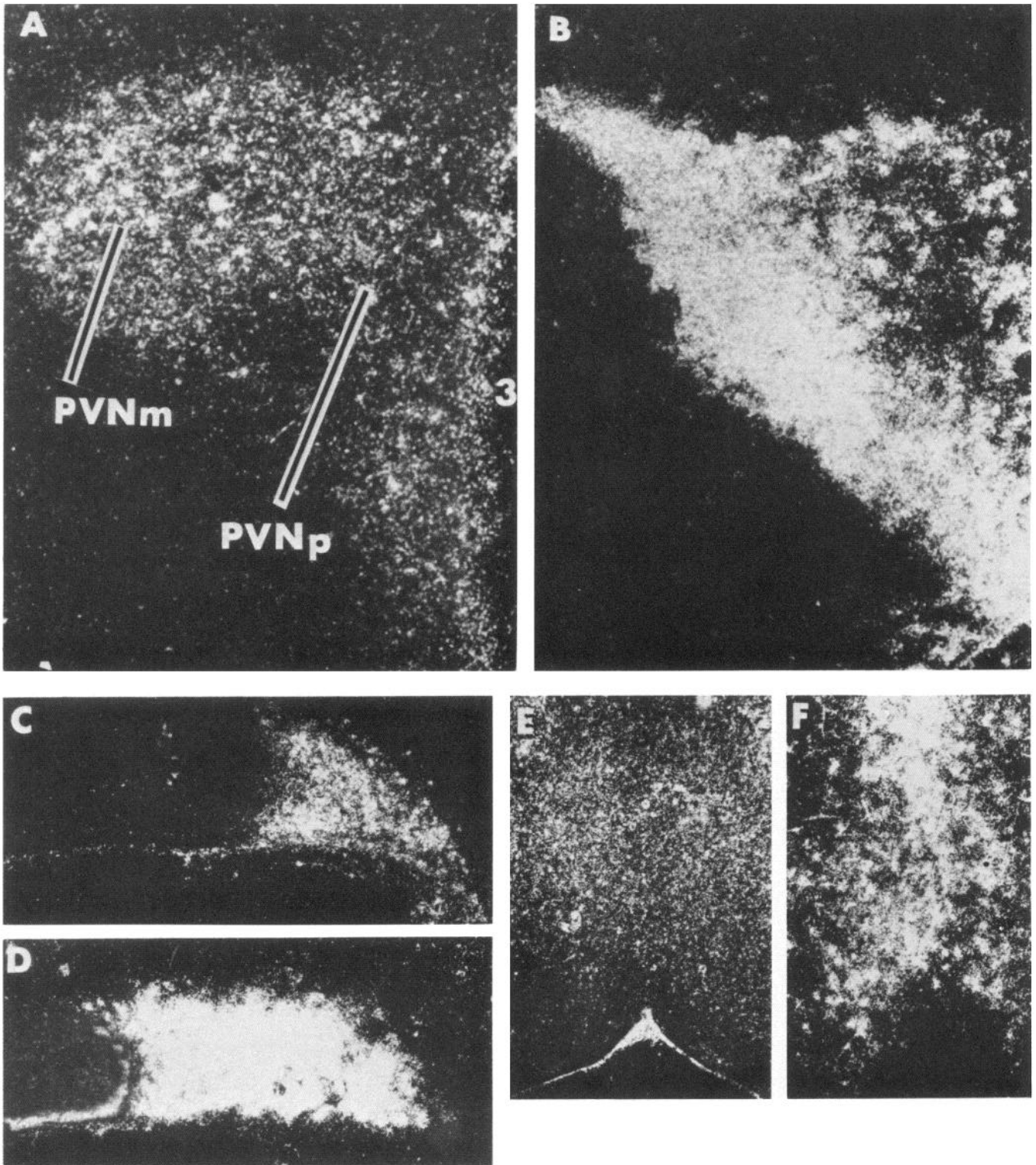
## Discussion

Intraperitoneal hypertonic saline induced a transient *c-fos* mRNA response that peaked around 30–60 min and returned to baseline

by 4–8 hr even though the osmotic stimulus continued for days. This is consistent with *in vitro* studies that show a transient *c-fos* mRNA response in spite of continued stimulation with growth factors, peptides, neurotransmitters, second messengers, and potassium depolarization (Kruijer et al., 1984; Curran and Morgan, 1985; Greenberg et al., 1986; Morgan and Curran, 1986; Sassone-Corsi et al., 1988; Areander et al., 1989; Bartel et al., 1989; Szekely et al., 1989). Moreover, other animal studies confirm a transient *c-fos* mRNA response following generalized seizures and other types of stimuli including hemorrhage (Morgan et al., 1987; Hoffman et al., 1989; Sonnenberg et al., 1989a,b).

Confusion about the duration of the Fos protein response has arisen because of the lack of specificity of antisera used. Fos-like immunoreactivity (FLI) has been observed for 1–3 d following water deprivation (Sagar et al., 1988), trigeminal nerve section (Sharp et al., 1989c), cortical injury (Dragunow and Robertson, 1988; Sharp et al., 1989a), and adrenalectomy (Jacobson et al., 1990). However, since the antibodies used in these studies recognize both Fos and FRA, it is likely that the FLI represented both Fos and FRA.

LA041 is probably specific for authentic Fos, since it was

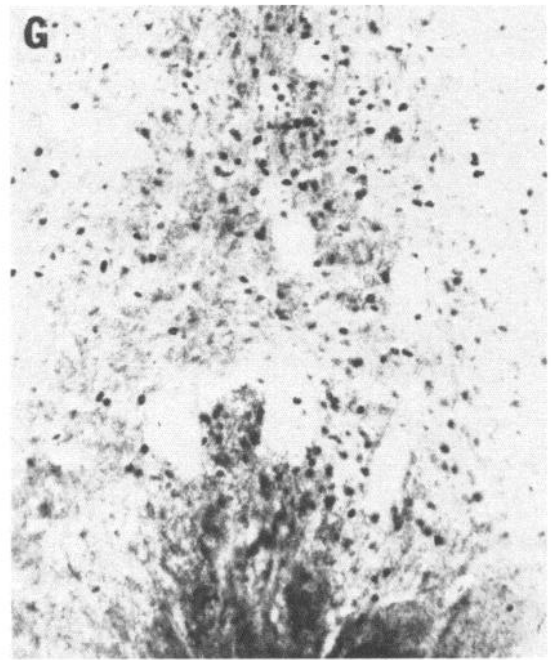
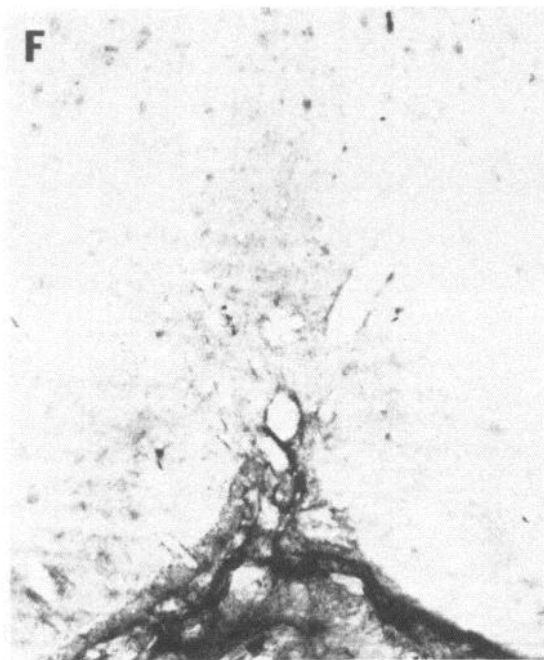
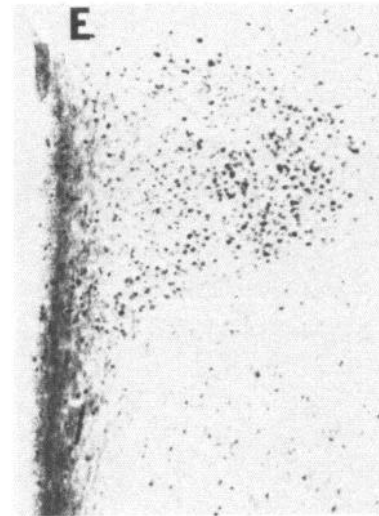
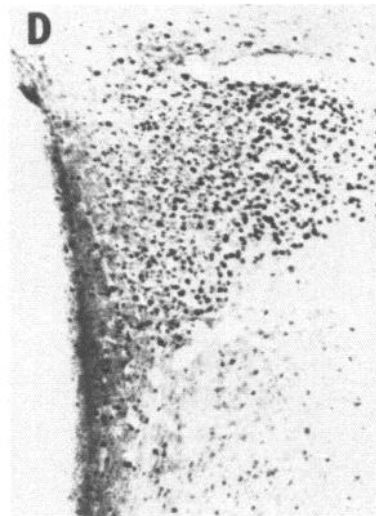
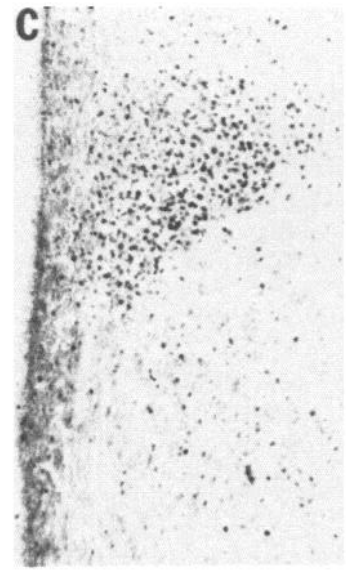
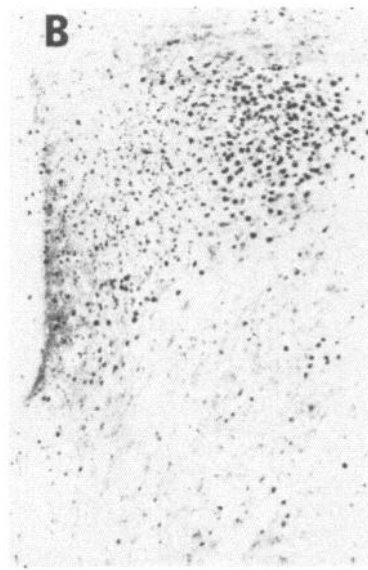
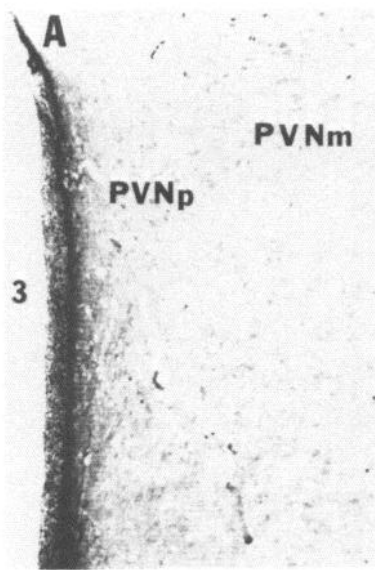


**Figure 5.** <sup>35</sup>S-labeled *c-fos* mRNA in the *PVNm* and *PVNp* of subjects administered hypertonic saline 15 min (*A*) and 60 min (*B*) previously, in the SON of subjects administered hypertonic saline 15 min (*C*) and 60 min (*D*) previously, and in the LMT of subjects administered hypertonic saline 5 min (*E*) and 60 min (*F*) previously.

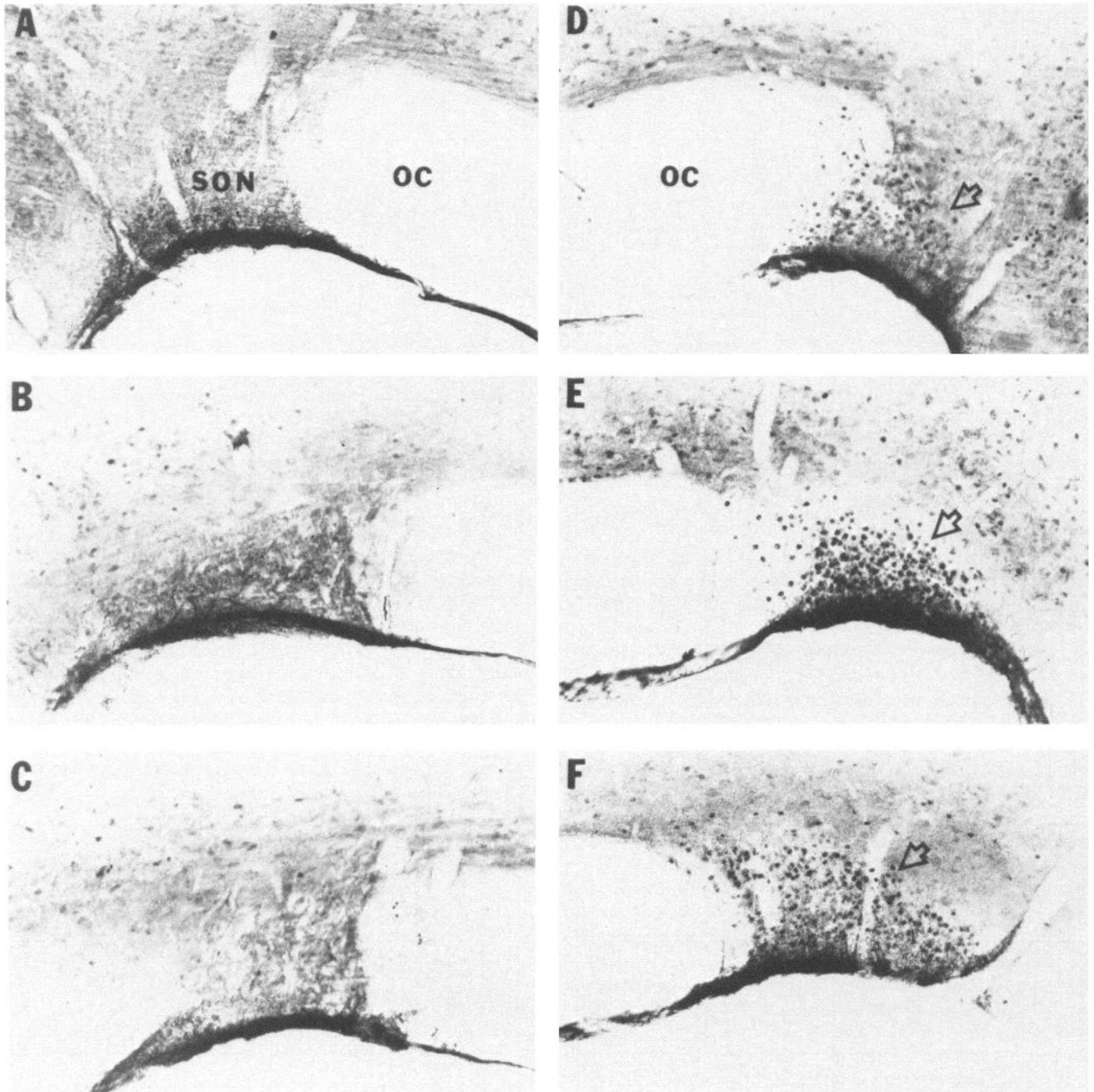
raised to a peptide on the N-terminal end of Fos and it produces one band on Western blots (DeTogni et al., 1988). Using the LA041 monoclonal antibody, Fos disappears by 4–8 hr following cortical injury (Sharp et al., 1990) and osmotic stimuli (present study). These data suggest that the temporal expression of

Fos protein parallels the *c-fos* mRNA response. That is, the Fos protein is induced and disappears within 4–8 hr of the osmotic stimulation. This agrees with Sonnenberg et al. (1989a,b), who found that Fos protein is induced and disappears over a 4–5 hr period following administration of most convulsants.









**Figure 7.** Coronal sections of the supraoptic nucleus (SON) stained immunocytochemically for Fos using the LA041 monoclonal antibody are pictured for an untouched control (*A*), 1 hr after administration of isotonic saline (*B*), and 5 min (*C*), 1 hr (*D*), 2 hr (*E*), and 3 hr (*F*) after administration of hypertonic saline.

Hypertonic saline induced FLI, detected using the R1B6 and Curran polyclonal antibodies, for up to 7 d. Since hypertonic saline induced authentic Fos, detected using LA041, for only 4–8 hr, the FLI present between 8 hr and 7 d likely represented FRA. Similarly, Sonnenberg et al., (1989a,b) showed that, though

Fos disappears by 4 hr following a seizure, 46- and 35-kDa FRA are expressed for at least 8 hr. Our observations suggest that Fos regulates transcriptional events related to the initial stimulus, since *c-fos* mRNA and Fos protein appear only transiently. FRA, however, continue to be expressed and regulate transcrip-

**Figure 6.** Coronal sections of the paraventricular nucleus stained immunocytochemically for Fos using the LA041 monoclonal antibody are pictured for an untouched control (*A*) and for subjects administered hypertonic saline 30 min (*B*), 1 hr (*C*), 2 hr (*D*), and 3 hr (*E*) previously. LA041 immunostaining in LMT 2 hr following administration of hypertonic saline (*G*) is also compared to LMT staining in an untouched control (*F*).

**Table 3. Polyclonal R1B6 immunostaining of PVNm neurons, representing FRA at times 1–7 d, and the plasma osmolarity (mmol/kg) in three groups of adult rats at various times following intraperitoneal administration of hypertonic saline**

	Time after HS administration									
	0 h	1 h	2 h	3 h	4 h	1 d	2 d	3 d	4 d	7 d
HS—no drinking										
Immunostaining	0	3+	3+	3+	3+	3+				
HS—drink 1 hr later										
Immunostaining	0	3+			3+	2+	1+	1	0	0
Plasma osmolarity	291					313	290	291		
HS—drink 24 hr later										
Immunostaining	0	3+			3+	3+	2+	2+	1+	1
Plasma osmolarity	290					318	291	290		290

In the first group, the subjects were given hypertonic saline intraperitoneally and not allowed to drink water for 24 hr. In the second group, the subjects were allowed to begin drinking water ad libitum 1 hr after intraperitoneal administration of the hypertonic saline. In the third group, the subjects were allowed to begin drinking water ad libitum 24 hr after intraperitoneal administration of the hypertonic saline. The immunostaining was scored as undetectable (0) to maximal (3+).

tional events for long time periods that equal or exceed the duration of the stimulus.

Hypertonic saline produces both an osmotic and stress stimulus. To control for the latter, isotonic saline was given intraperitoneally since handling and the discomfort of injection should produce stress without an osmotic effect. Since hypertonic saline significantly increased *c-fos* mRNA in PVNm, PVNp, SON, and LMT at 30–120 min compared to isotonic injected subjects, we propose that the osmotic stimulus induced *c-fos* in those structures.

The PVNm, SON, LMT, and subfornical organ (SFO) are known to participate in osmotic regulation. SFO neurons, thought to be the primary transducers of osmotic stimuli in the brain (Johnson, 1985), increase their firing rate in response to hypertonic solutions and project to the LMT. Neurons in and near the LMT respond to the osmotic stimuli (Johnson, 1985). They are thought to be the integrative center for volume and osmotic information, to project to the magnocellular hypothalamic nuclei (Chaudhry et al., 1989; Tanaka, 1989), and to regulate oxytocin and vasopressin secretion. The magnocellular neurons of the PVN and SON contain oxytocin and vasopressin and send their axons into the posterior pituitary gland to secrete those hormones into the systemic circulation. The magnocellular neurons increase their firing rate in response to the systemic administration of hypertonic solutions (Brimble and Dyball, 1977). It is reasonable, therefore, that the osmotic stimulus induced the *c-fos* and *fra* immediate early genes in all of these regions. Whether or not osmotic and/or stressful stimuli induce Fos and FRA in SFO neurons is still under study.

Stress (or arousal) likely accounts for the induction of *c-fos* in subjects injected with isotonic saline compared to uninjected controls. Stress induces *c-fos* in cortex, amygdala, thalamus, hypothalamus, hippocampus, septum, PVNp, and other regions (Table 2). Stress (Ceccatelli et al., 1989) and adrenalectomy (Jacobson et al., 1990) have been shown to induce *c-fos* in corticotropin-releasing factor (CRF)-containing PVNp neurons. PVNp, however, appears to respond to both stress and osmotic stimulation (Fig. 6). PVNp has CRF-containing neurons that are activated during periods of stress, and it has neurons in which vasopressin is induced following osmotic stimulation (Bondy et al., 1989). Further studies are needed to determine

whether hormones and/or specific neural circuits mediate stress induction of *c-fos* in PVNp and other brain regions.

The significance of the osmotic and stress induction of the *c-fos* and related genes is uncertain. The induction of *c-fos* could lead to long-term changes in expression of hormone and/or neurotransmitter genes (Sonnenberg et al., 1989c). Alternatively, Fos and FRA might regulate transcription from cytoskeletal genes that produce the morphological changes in PVN and SON neurons induced by osmotic stimuli (Kalimo, 1975; Tweedle and Hatton, 1976). In any event, it is plausible that *c-fos* and other immediate early genes participate in the mechanism by which neurons make long-term biochemical adjustments to short-term changes in neural activity.

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