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Regulation of ΔFosB and FosB-like Proteins by Electroconvulsive Seizure and Cocaine Treatments

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SUMMARY

Previous work has shown that c-Fos and several Fos-like proteins or Fras (Fos-related antigens) are induced acutely in brain in response to a wide variety of stimuli. In contrast, several stimuli induce apparently distinct Fos-like proteins, termed chronic Fras, after chronic administration. We show that ΔFosB, a truncated splice variant of FosB, responds like the other acute Fras: it is induced rapidly and transiently in cerebral cortex after acute electroconvulsive seizure (ECS) and in striatum after acute cocaine but does not accumulate after chronic ECS or cocaine treatment. Although the chronic Fras are immunochemically related to ΔFosB, they can be distinguished from ΔFosB based on their temporal properties in that they are induced after chronic ECS and cocaine treatments only. Moreover, the chronic Fras and ΔFosB can be distinguished by their migration patterns on one- and two-dimensional gel electrophoresis. The chronic Fras, therefore, appear to be novel FosB-related proteins. We also provide evidence that the chronic Fras heterodimerize primarily with Jun-D and Jun-B, as opposed to c-Jun. The possibility that AP-1 complexes containing the chronic Fras function as negative regulators of AP-1 mediated transcription is discussed.

The clinical efficacy of antidepressant and antipsychotic treatments requires their chronic administration. Similarly, addiction results in response to repeated exposure to a drug of abuse. One mechanism that could underlie such long-term changes in brain function elicited by these chronic treatments is the regulation of gene expression.

Among the most studied transcription factors are those encoded by immediate early genes, a prototypical example of which is c-Fos (1). c-Fos can be induced rapidly and transiently by many stimuli via cAMP, Ca^{+2}, and other second messenger systems (2–4). Several regulatory elements in the c-Fos promoter region are required for the regulation of its expression (5). c-Fos heterodimerizes with c-Jun to form the AP-1 complex, which binds with high affinity to the AP-1 site, with a consensus sequence of TGA(G/C)TCA. Crystalographic studies of the AP-1 complex have demonstrated that the carboxyl-terminal regions of the c-Fos/c-Jun heterodimer are flexibly joined to the DNA binding region (6), which may allow the carboxyl-terminal domains to interact with adjacent proteins such as TATA-binding proteins and other transcription factors. Binding of the AP-1 complex to an AP-1 site has been shown to both activate (7) and repress (8) transcription.

There are several additional members of the Fos family, including Fra-1 (Fos-related antigen-1), Fra-2, FosB, and ΔFosB (9). These proteins can also form heterodimers with Jun proteins, including c-Jun, Jun-B, and Jun-D. Different combinations of Fos and Jun proteins form different AP-1 complexes, which may exert different effects on promoters with AP-1 sites. The diversity of AP-1 complexes is even greater if the post-translational effects of phosphorylation are considered (10). Many neurally expressed genes contain AP-1 consensus sequences in their promoter regions, including NMDAR1 (11), proenkephalin (7), prodynorphin (12), tyrosine hydroxylase (13), D1 dopamine receptor (14), RIIβ subunit of protein kinase A (15), neurofilament-H (16), and neurotensin/neuromedin N (17). However, very few genes have yet to be shown to be regulated by AP-1 complexes *in vivo*.

The smallest known Fos family protein is ΔFosB, a trun-

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ABBREVIATIONS: ECS, electroconvulsive seizure; Fra, Fos-related antigen; AP-1, activator protein-1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

cated splice variant of FosB (18–20). ΔFosB lacks the FosB carboxyl-terminal domain, which constitutes the major transactivation domain required for regulation of transcription (21). Although the transcriptional activity of ΔFosB remains controversial (21–22), its transactivation activity appears to be very low (23). This suggests that ΔFosB is a negative regulator of AP-1 mediated transcription. Like other negative transcriptional regulators such as IkB and ICER (inducible cAMP early repressor) (24), ΔFosB may play an important role in negative feedback mechanisms. In the present study, we showed that ΔFosB is induced in the cerebral cortex and striatum by acute ECS and cocaine treatments, respectively. This represents the first demonstration that ΔFosB is induced in the brain, as has been reported previously in cultural cells (18–20).

Several novel Fras, induced by chronic ECS, cocaine, and other treatments (morphine, haloperidol, kainic acid, and 6-hydroxydopamine), have been identified (25–29). These so-called chronic Fras are induced gradually in a region-specific manner in response to repeated treatments and exhibit a half-life of ~7 days in brain. The chronic Fras may, therefore, mediate some of the long term changes in gene expression produced by repeated perturbation of the brain. We show here that the chronic Fras are immunochemically related to ΔFosB but can be distinguished from ΔFosB based on their temporal properties and migration on gel electrophoresis and would appear to be novel FosB-related proteins. We also provide evidence that the chronic Fras complex with Jun-D and Jun-B to form the chronic AP-1 complex. We propose that the chronic AP-1 complex containing the chronic Fras may serve as long-lasting negative regulators of AP-1-mediated transcription.

Materials and Methods

ECS treatment. Male Sprague-Dawley rats (initial weight, 140–260 g; Camm Research Institute, Wayne, NJ) were used for all experiments. ECS was administered, as before (30), via earclip electrodes (45 mA, 0.3 sec). Chronic and chronic/acute animals received a single ECS daily for 10 days. Control and acute animals received chronic sham treatments where electrodes were clipped onto the rats' ears but no current was applied. Prior sham treatments were used to reduce the effects of stress (31, 32). On day 11, acute and chronic/acute animals were given an acute ECS, and control and chronic animals were given sham treatments. For control, acute and chronic/acute treatments, rats were killed at varying times after their last treatment. For chronic treatment, rats were killed 18 hr after the last ECS treatment. Cerebral cortex was obtained by gross dissection.

Cocaine treatment. Male Sprague-Dawley rats (initial weight, 140–160 g) were used. Chronic cocaine treatment consisted of twice-daily intraperitoneal injections of cocaine hydrochloride (22.5 mg/kg; National Institute on Drug Abuse) for 8 days. For acute treatments, animals were pretreated twice daily with saline intraperitoneally for 4 days to desensitize the animals to any stress associated with the injections. Control animals received equal volumes of physiological saline (0.9% NaCl) intraperitoneally for the same drug treatment periods. Striatum was obtained by gross dissection.

Cell culture. The Rat-1A fibroblast is a cell line carrying the human *myc* gene under the transcriptional control of a retroviral long terminal repeat (33). Rat-1A cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Serum-deprived Rat-1A cells were stimulated with 20% dialyzed FBS for 6 hr, and then nuclear protein and total cellular RNA were harvested as reported previously (23).

RNA isolation. Total cell RNA was isolated from cortex or striatum by the guanidinium isothiocyanate/phenol procedure of Chomczynski and Sacchi (34). Tissue was homogenized in 3 volumes of GIT buffer (47.27 g of guanidinium isothiocyanate, 1.67 ml of 3 M sodium acetate, pH 6, 97 ml of RNase-free water, and 1.67 ml β-mercaptoethanol) with a Polytron. The cell lysate was then transferred to a sterile 30-ml Oak Ridge tube, and the following solutions were added: 0.1 volume of 2 M sodium acetate, pH 4, 1 volume of water-saturated phenol, and 0.2 volume of 49:1 chloroform/isoamyl alcohol. The contents in the tube were mixed by vortexing for 1 min and then centrifuged at 15,000 × *g* for 20 min. The aqueous layer was transferred to a fresh Oak Ridge tube, and 2 volumes of 100% ethanol were added. The tube was kept at −80° for 15 min and centrifuged as described above. The final pellet was resuspended in 100 μl of RNase-free water.

Preparation of riboprobe. A fragment (285 nucleotides) of the rat *fosB* gene was amplified by polymerase chain reaction with two primers (forward primer: 5′-GAAACGGATCAGCTCGAAGA-3′; backward primer: 5′-AAGAGAGAAGCCGTCAG GTT-3′). This fragment includes a 155-nucleotide sequence upstream of the 5′ alternative splicing site and a 130-nucleotide sequence downstream of the splicing site, which is absent in ΔFosB mRNA due to alternative splicing (see Ref. 18). The polymerase chain reaction product was cloned to the pCR-TA vector (Invitrogen) and sequenced with Sequenase 2.0 kit (United States Biochemical) to identify the orientation of the insert and confirm the *fosB* sequence. The pCR-TA derived plasmid was named pTA-fosB. A uniformly labeled riboprobe was prepared by T7 RNA polymerase transcription of *Hin*dIII-digested pTA-fosB. This probe consists of a 285-nucleotide region of the rat *fosB* sequence (Fig. 1A). Within this region, 130 nucleotides are deleted in ΔFosB. The probe was labeled with [α^{32}P]CTP (NEN) according to the procedures of Promega Biochemicals. The reagents for riboprobe synthesis were added to a microcentrifuge tube as follows: 4 μl of 5× transcription buffer (200 mM Tris·HCl, 30 mM $MgCl_2$, 10 mM spermidine, and 50 mM NaCl), 2 μl of 100 mM dithiothreitol, 0.5 μl of 40 units/μl RNasin, 3 μl of 10 mM ATP/GTP/CTP mixture, 1.5 μl of 0.5 mM UTP, 3 μl of 40 mCi/ml [^{32}P-α]CTP (from NEN), 5 μl of DNA template (1 μg), and 1 μl of 20 units/μl T7 RNA polymerase (final volume = 20 μl). The tube was incubated at 30° for 1 hr. Then, 1 μl of RQ DNase I (1 units/μl) was added and incubated at 37° for 15 min. The synthesized riboprobe was precipitated by 2.5 volumes of 100% ethanol and 0.5 volume of 7 M ammonium acetate, pH 6.0, and then resuspended in 200 μl of DEPC treated water. The radioactivity of the riboprobe was measured by liquid scintillation counting.

Ribonuclease protection assay. RNase T2 protection assay (35) was performed as follows. Total RNA (20 μg) and probe (2×10^5 cpm) were coprecipitated and resuspended in 30 μl of hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA). The RNA/probe mixture was then denatured at 85° for 2 min and hybridized at 42°. After 16 hr of hybridization, 300 μl of RNase T2 solution (50 mM sodium acetate, pH 4.4, 20 mM EDTA, 0.1 M NaCl, and 10 units/ml RNase T2) was added and incubated at 30° for 1 hr, followed by ethanol precipitation. The final RNA pellet was resuspended in 20 μl of loading buffer (98% formamide, 10 mM EDTA, 0.1% Bromphenol Blue, and 0.1% Xylene Cyanol). The RNA sample was then denatured at 90° for 2 min and electrophoresed in a 6% polyacrylamide gel at 200 V for 45–60 min with a Bio-Rad mini-gel apparatus.

Northern blotting. Northern blotting was performed as reported previously (36). Briefly, 20 μg of total RNA was electrophoresed in 1% agarose gels and then transferred onto nylon filters (ICN). The filters were UV cross-linked in the cross-link machine (Stratagene) before hybridization. The filters were prehybridized with salmon sperm DNA in hybridization buffer 5× SSPE (20 × SSPE = 17.5% NaCl, 2.8% NaH_2PO_4, 0.7% EDTA), 50% formamide, 10× Denhardt's

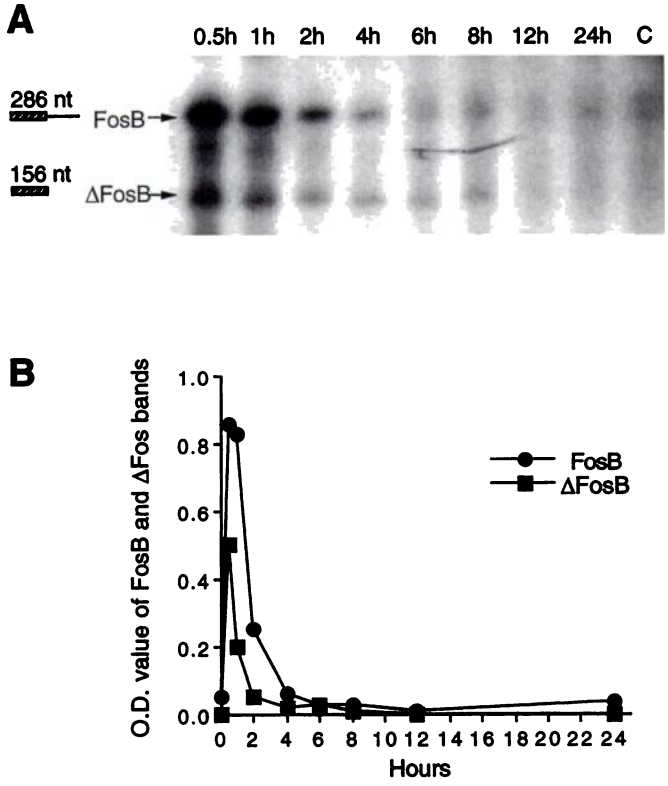

Fig. 1. Induction of ΔFosB mRNA by acute ECS treatment. A, Representative autoradiogram of an RNase T2 protection assay. *Left*, schematic structures of the protected fragments. *Shadowed box*, exonic sequence. *Line*, intronic sequence that is absent in ΔFosB mRNA. Twenty micrograms of total RNA from the cerebral cortex of each rat treated with a single acute ECS were hybridized with a FosB riboprobe and then subjected to RNase T2 digestion. The 286-nucleotide protected fragment (*top band*) corresponds to FosB mRNA, and the 156-nucleotide fragment (*bottom band*) corresponds to ΔFosB mRNA. The bands between the FosB and ΔFosB bands in acute 0.5- and 1-hr treatments may represent some degraded FosB products. B, Line graph indicates the optical densities obtained from autoradiograms of the RNase protection assay. Data represent three experiments for each time point.

solution (100 × Denhardt's = 2% Ficoll, 2% polyvinylpyrrolidone, 2% bovine serum albumin), 200 mg/ml denatured salmon sperm DNA, 0.1% SDS) for 5 hr and then hybridized with random prime-labeled Jun-D probe (kindly provided by Dr. Steve Hyman, Massachusetts General Hospital, Harvard University, Boston, Massachusetts) in 15 ml hybridization buffer (1–5, × 10^6 cpm/ml) at 42° overnight. The filters were washed (three times for 15 min in 0.2 × SSPE/0.1% SDS at 42°) and then blotted and exposed to X-ray film.

Gel shift assay. The gel shift assay was performed as described previously (25, 36). Brain tissue was homogenized with Dounce homogenizers in 20 volumes of the electrophoretic mobility shift assay buffer: 20 mM HEPES, pH 7.9, 0.4 M NaCl, 20% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 10 μg/ml leupeptin, 0.1 mM *p*-aminobenzamidine, 1 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol. The homogenates were incubated on ice for 30 min before centrifugation at 15,000 × *g* for 20 min at 4°. Aliquots of supernatants (20 μg of protein) were incubated at 20° for 20 min with 1 μg of poly(dI-dC)·poly(dI-dC), 40 μg of bovine serum albumin, 10 mM Tris·HCl, pH 7.9, 10 mM KCl, 1 mM EDTA, 4% glycerol, and 1 ng of the radioactively labeled AP-1 probe (5′-TCGACG**TGACTCA** GCGCGC-3′; the AP-1 site is in bold). This probe is from the promoter region of the human metallothionein II gene and is identical to that used previously (7, 25, 26). The samples were incubated for 20 min at 20° and electrophoresed at 150 V for 2 hr in a nondenaturing 6% acrylamide/0.16% *N,N′*-methylenebisacrylamide gel containing 25 mM Tris-borate buffer, pH 8.3, 1 mM EDTA, and 1.6% glycerol. The gels were dried and exposed to X-ray film. Levels of AP-1 binding were quantified by measuring the optical density of specific bands using an image-analysis system with the National Institutes of Health image software, version 1.52. Step-gradient gray scales were used to ensure that the densitometric measurements of autoradiograms were made within the linear range of detection. Supershift experiments were performed by incubating anti-c-Fos, anti-FosB, anti-Fra-1, anti-Fra-2, anti-Fra, anti-c-Jun, anti-Jun-B, anti-Jun-D and anti-Jun family antibodies (kindly provided by M. Gruda and R. Bravo, Bristol-Myers-Squibb) with the gel shift reaction components for 1 hr at 4° before the addition of the labeled probe.

Western blotting. One-dimensional Western blotting was performed as described previously (26). The tissue was homogenized in Dounce homogenizers in 10 volumes of electrophoretic mobility shift assay buffer. Fifty micrograms of protein of these cellular extracts were then subjected to SDS-polyacrylamide gel electrophoresis with a 10% acrylamide/0.27% *N,N′*-methylenebisacrylamide resolving gel overnight at 75 V and electrotransferred to nitrocellulose at 200 mA for 3 hr. The blots were blocked with four 15-min changes of 2% (for anti-Fra antibody, kindly provided by Dr. Michael Iadarola, NIDR, National Institutes of Health) or 0.5% [for anti-FosB(N) and anti-FosB(C) antibodies] nonfat dry milk powder in PBST buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and incubated overnight on a shaker at 4° in a 1:4000 dilution of anti-Fra antibody or a 1:1000 dilution of anti-FosB antibodies in blocking buffer with 0.05% sodium azide. All of the antibodies are affinity-purified polyclonal antibodies raised in rabbits. The anti-Fra antibody was raised against the M-peptide domain of c-Fos (KVEQLSPEEEEKRRIRRERNKMAAA, amino acids 129–153), which is a highly conserved region common to all known Fos-like proteins. The anti-FosB(N) and anti-FosB(C) antibodies were raised against TrpE/FosB fusion proteins corresponding, respectively, to the amino-terminal (amino acids 79–131) and carboxyl-terminal (amino acids 245–315) regions of FosB. The blots were washed four times for 15 min in blocking buffer and then incubated in a 1:4000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (Vector Labs) in blocking buffer for 2 hr. The blots were washed eight times for 15 min with PBST alone, developed with the enhanced chemiluminescence system of Amersham, and exposed to Hyperfilm-ECL (Amersham) for 5–60 sec. Levels of Fra immunoreactivity were quantified by measuring the optical density of specific bands using the above-mentioned image-analysis system, with step-gradient gray scales used to ensure that the densitometric measurements of autoradiograms were made within the linear range of detection. The specificity of the anti-Fra and anti-FosB antibodies has been confirmed in previous studies, wherein recognition of the immunoreactive bands was abolished by preabsorption of the antibodies with the peptide antigens (18, 26).

For two-dimensional Western blotting, samples (225 μg/sample) were separated by isoelectric focusing in tube gels for the first dimension according to a published procedure (26). The resulting tube gels were then layered across SDS-polyacrylamide slab gels (10% acrylamide/0.4% bis-acrylamide) and electrophoresed in the second dimension. Proteins in the resulting gels were transferred onto nitrocellulose, and immunolabeling was performed as described above for one-dimensional Western blotting.

Results

Regulation of ΔFosB mRNA by acute and chronic ECS treatments. To detect ΔFosB mRNA, we designed a riboprobe that can distinguish ΔFosB mRNA from FosB mRNA in an RNase protection assay (Fig. 1). This probe contains an exonic sequence and an intronic sequence of the

fosB gene. FosB mRNA is homologous to the probe in both exonic and intronic sequences and protected a 286 nucleotide fragment. The ΔFosB mRNA is homologous to the probe only in the exonic region and protected a 156-nucleotide fragment. These two protected fragments were readily separated in polyacrylamide gels.

By use of the RNase protection assay, we identified FosB and ΔFosB mRNAs in the cerebral cortex of rats treated with a single acute ECS. Both FosB and ΔFosB mRNAs were induced rapidly and transiently with equivalent time courses. FosB and ΔFosB mRNA levels were highest 30 min after the ECS and had returned to control within 12 hr. FosB mRNA was more abundant than ΔFosB mRNA during the course of this induction.

To test whether a chronic course of ECS affects FosB and ΔFosB mRNA expression in response to an acute ECS, we analyzed mRNA from cortex of rats treated chronically with ECS for 10 days before receiving an acute ECS on day 11 (Fig. 2). FosB and ΔFosB mRNAs were increased strongly 2 hr after the last ECS treatment, which suggests that there was no appreciable desensitization of this effect in response to chronic treatment. The FosB and ΔFosB mRNA levels returned to control 18 hr after the last ECS treatment, which is similar to that observed for an acute treatment.

To compare the induction of ΔFosB in brain by ECS treatments with a known ΔFosB induction system, aliquots of the acute and chronic ECS samples were analyzed along with Rat-1A cell samples (Fig. 3). These cells have been shown to express ΔFosB transcripts after serum stimulation (23). Equivalent migration positions of the protected fragments were observed between the ECS samples and the serum-stimulated Rat-1A cells, which confirms the accuracy of the RNase protection assay. Interestingly, levels of ΔFosB mRNA, relative to FosB mRNA, induced by ECS in the cortex were less abundant than that induced by serum stimulation in the Rat-1A cells.

Induction of ΔFosB mRNA by cocaine treatments. Acute cocaine administration has been shown to induce c-Fos and several Fos-like proteins in the striatum and nucleus accumbens, brain regions important for cocaine's behavioral effects (26, 36–40). However, induction of ΔFosB has not yet been reported. To test this possibility, we analyzed mRNA isolated from the striatum of rats treated with cocaine (Fig. 4). Induction of FosB and ΔFosB mRNAs was evident after a single acute treatment. However, both were induced to a much lesser extent after prior chronic treatment, which suggests that considerable desensitization had occurred. The ratios of FosB and ΔFosB induced by cocaine treatment in striatum and by ECS treatment in cortex were similar. However, FosB and ΔFosB were induced to a greater extent by ECS in cortex than by cocaine in striatum.

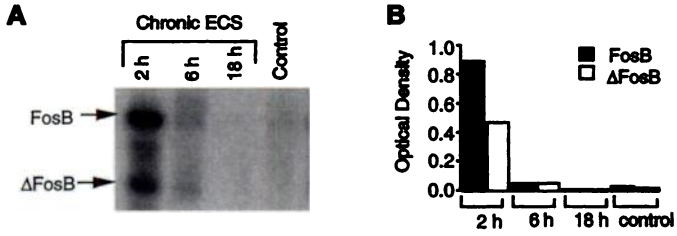

Fig. 2. Induction of ΔFosB mRNA by chronic and chronic plus acute ECS treatments. A, Twenty micrograms of total RNA from the cerebral cortex of each rat treated with ECS for 10 days were analyzed 2, 6, or 18 hr after the last ECS. The RNA samples were hybridized with a FosB riboprobe and then subjected to RNase T2 digestion. The 286-nucleotide protected fragment (*top band*) corresponds to FosB mRNA, and the 156-nucleotide fragment (*bottom band*) corresponds to ΔFosB mRNA. B, Bar graph indicates the optical densities obtained from autoradiograms of the RNase protection assay. Data represent four experiments for each time point.

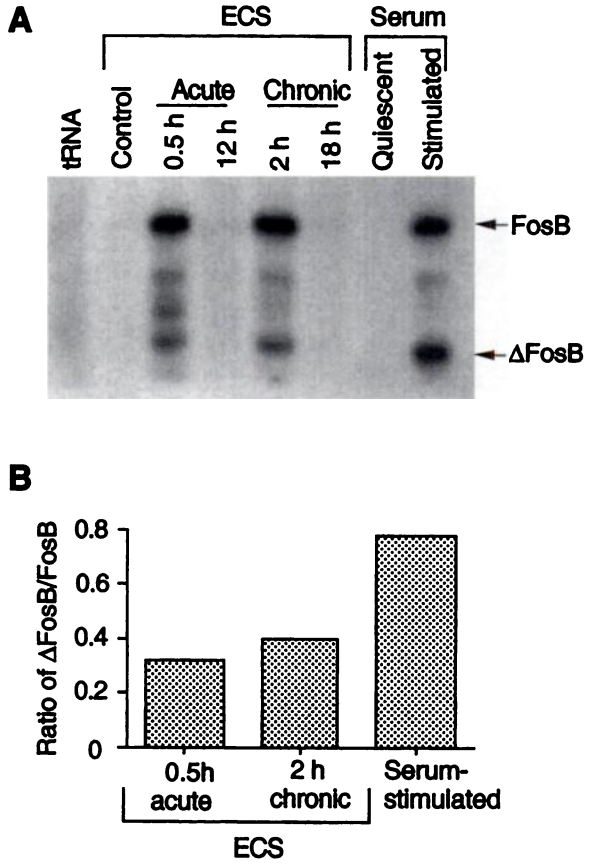

Fig. 3. ΔFosB mRNA induced by ECS in rat cerebral cortex is the same as that induced by serum stimulation in Rat-1A cells. A, Twenty micrograms of total RNA from the cerebral cortex of each rat treated with acute or chronic ECS and from Rat-1A cells were hybridized with a FosB riboprobe and then subjected to RNase T2 digestion. The 286-nucleotide protected fragment (*top band*) corresponds to FosB mRNA, and the 156-nucleotide fragment (*bottom band*) corresponds to ΔFosB mRNA. B, Bar graph represents the ratios of the optical densities of the ΔFosB versus the FosB band. Data represent three experiments for each determination.

Regulation of Fos/Fra expression by ECS and cocaine treatments. In previous studies (25, 26), we found that chronic ECS and cocaine treatments induced long-lasting chronic Fras of 35 and 37 kDa. The 35-kDa chronic Fra is similar in molecular weight to that reported for ΔFosB (18). However, the results described above demonstrated that ΔFosB mRNA was induced by acute treatments only, whereas appearance of the chronic Fras was observed after chronic treatments only (25, 26). Because patterns of protein induction can differ from those of RNA induction, we used Western blotting to study induction of ΔFosB and the chronic Fras at the protein level after acute and chronic ECS treatments. The anti-Fra antibody, which recognizes a highly conserved region of all known Fos-like proteins, reacted with c-Fos and several Fras induced by acute ECS treatment (Fig. 5). The bands in the 52–58-kDa range most likely correspond

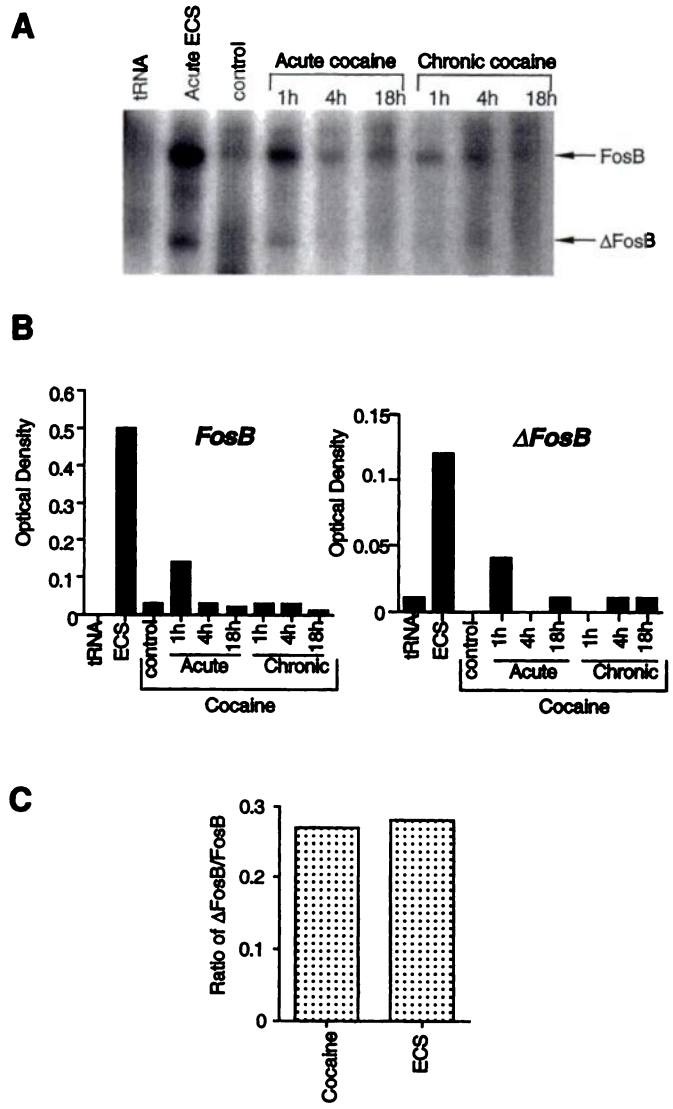

Fig. 4. Induction of ΔFosB mRNA by acute and chronic cocaine treatments. A, Twenty micrograms of total RNA from the striatum of each rat treated with a single acute cocaine injection or chronic cocaine twice daily for 10 days were hybridized with a FosB riboprobe and then subjected to RNase T2 digestion. The 286-nucleotide protected fragment (*top band*) corresponds to FosB mRNA, and the 156-nucleotide fragment (*bottom band*) corresponds to ΔFosB mRNA. B, Bar graph indicates the optical densities obtained from autoradiograms of the RNase protection assay. Data represent three experiments for each time point. C, Bar graph represents the ratios of the optical densities of the ΔFosB versus the FosB band. Data represent three experiments for each time point.

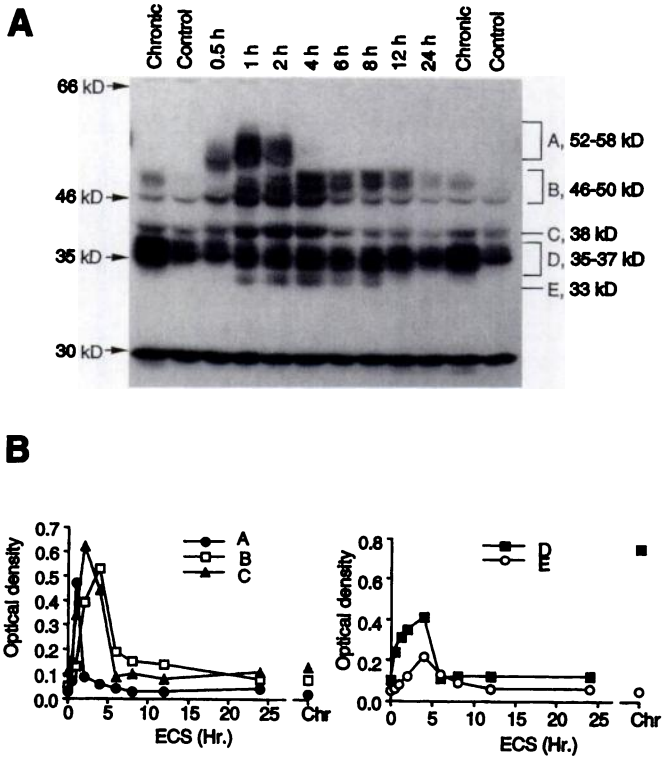

Fig. 5. Regulation of Fra expression by acute and chronic ECS treatments. A, Time course of induction of Fra immunoreactivity after acute ECS (0–24 hr) and chronic ECS (18 hr after the last of 10 daily treatments) using the anti-Fra antibody (see Materials and Methods). Five groups of Fra bands are designated as A to E, corresponding, respectively, to c-Fos, FosB/Fra-2, Fra-1, chronic Fras, and ΔFosB. Some of the bands in group A may represent phosphorylated forms of c-Fos, and those in group B may represent phosphorylated forms of FosB and Fra-2. B, Line graphs indicate the averaged optical density of the bands in each group at different time points. Data represent four experiments for each determination.

to c-Fos and its phosphorylated isoforms. Most of the bands in the 46–50-kDa range most likely correspond to FosB and its phosphorylated forms based on similar banding patterns obtained with specific anti-FosB antibodies (see below). Other bands in the 46–50-kDa range, and the prominent 38-kDa band, may correspond to Fra-2 and Fra-1, respectively, based on the reported molecular weights of these proteins, but this identification remains tentative. The 33-kDa band most likely represents ΔFosB, as is described below. The sequence of the induction waves of these "acute" Fras was: c-Fos > Fra-1 > Fra-2/FosB and ΔFosB.

The 35- and 37-kDa bands, which correspond to the chronic Fras, were induced significantly only after chronic ECS treatment, although basal levels of the proteins were detected in the control and acute samples (Fig. 5). The average optical density of these bands after chronic treatment was ~8 times that observed in control. The small and transient increase, induced by acute treatments, in the 35-kDa band apparent in Fig. 5 was not observed in all animals studied and appeared to reside in proteins distinct from the chronic Fras (Fig. 6).

We used two anti-FosB antibodies to further analyze the induced Fras. The anti-FosB(N) antibody (directed against the amino terminus of FosB) can recognize both FosB and ΔFosB proteins; the anti-FosB(C) antibody (directed against the carboxyl terminus of FosB) can recognize FosB only. Fig. 6 shows that the 33-kDa band was detected by the anti-FosB(N) antibody but not the anti-FosB(C) antibody. Moreover, direct comparison with serum-stimulated Rat-1A cells showed that the 33-kDa band induced by acute ECS migrated at the same position as the ΔFosB protein in the Rat-1A cells. These results confirm the 33-kDa protein as ΔFosB.

The 35- and 37-kDa chronic Fras both showed the same immunoreactivity to the anti-FosB(N) and anti-FosB(C) antibodies as ΔFosB (Fig. 6A), reacting with the former but not the latter. These findings indicate that the chronic Fras are ΔFosB-like proteins, consistent with our previous observations (26). In contrast, note that both antibodies revealed a

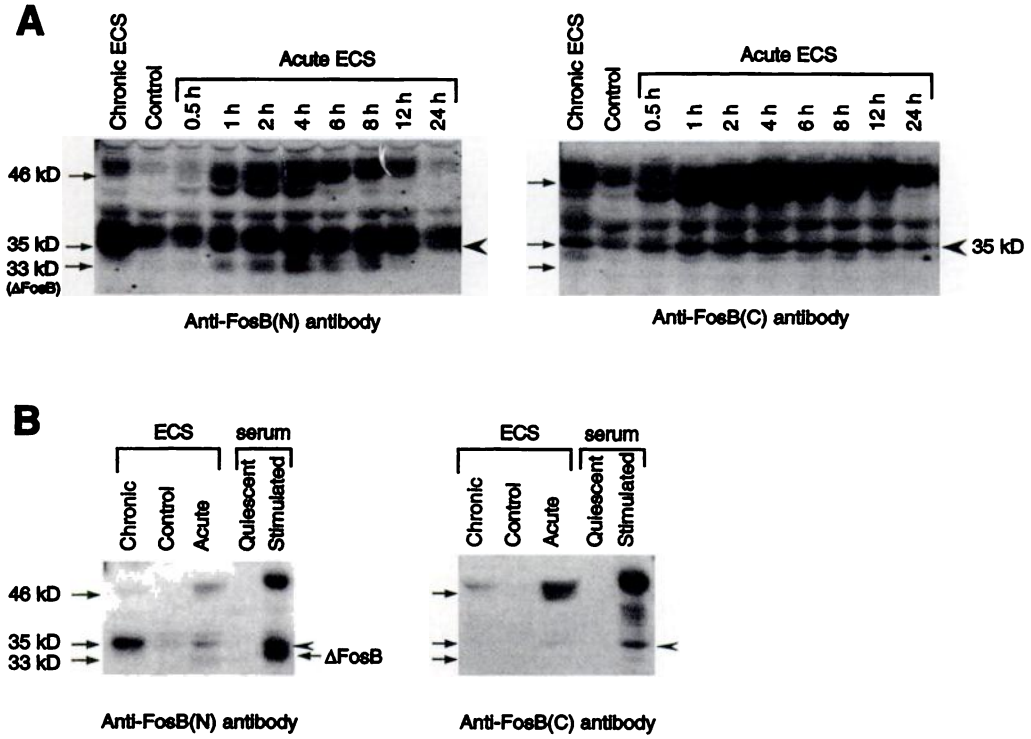

Fig. 6. Induction of ΔFosB and ΔFosB-like immunoreactivity by acute and chronic ECS treatments. A, The 33-kDa ΔFosB band (just below the 35-kDa band) in acute 1–8 hr ECS treatment samples reacted with anti-FosB(N) terminus antibody but not with anti-FosB(C) terminus antibody. The 35- and 37-kDa bands in the chronic ECS treatment sample showed the same immunoreaction pattern as the 33-kDa band induced by acute treatment in that they are recognized by anti-FosB(N) but not by anti-FosB(C) antibodies. A 35-kDa band in the acute treatment samples was recognized by the anti-FosB(C) antibody (*arrowhead*). However, this band is not related to the chronic Fras, based on the observation that it was induced consistently by acute, but not by chronic, ECS. This band probably accounts for the increased immunoreactivity in the 35-kDa range, recognized by the anti-FosB(N) antibody, in the acute ECS treatment samples. B, The 33-kDa band induced by acute ECS treatment migrated at the same position as the ΔFosB band induced by serum-stimulation as recognized by the anti-FosB(N) antibody (*left autoradiogram*). These bands were not recognized by the anti-FosB(C) antibody (*right autoradiogram*). The anti-FosB(N) antibody also recognized a ~35-kDa band (*arrowhead*) in the serum stimulated cells. However, this band is not ΔFosB because it was recognized by the anti-FosB(C) antibody as well. All of the bands in the serum-stimulated Rat-1A sample migrated slightly slower than those in other samples due to a higher concentration of SDS in the Rat-1A sample. Results are representative of three experiments.

small induction of proteins in the 35-kDa band after an acute ECS. Interestingly, a 35-kDa protein induced by serum stimulation in the Rat-1A cells also reacted with both anti-FosB(C) and FosB(N) antibodies. This 35-kDa protein could represent a degradation product of FosB but is clearly immunochemically distinct from ΔFosB and the chronic Fras, which react with the anti-FosB(N) antibody only. This immunoreactivity presumably accounts for the small and transient increase, after acute ECS, in Fra levels in the 35-kDa range evident in Fig. 5.

Based on one-dimensional Western blots, the chronic Fras migrate at different molecular weights compared with ΔFosB (Fig. 4). However, such a difference could conceivably be due to post-translational modifications such as phosphorylation. Therefore, we used two-dimensional Western blotting to further compare the patterns of Fos-related proteins induced by acute and chronic ECS treatments. Fig. 7 shows that two of these chronic Fras migrated to positions that are far from that of ΔFosB in the two-dimensional blots. A third chronic Fra migrates just above ΔFosB but with a similar pI.

To test the possibility that ΔFosB is induced by cocaine treatment, we analyzed Fra proteins in striatum of rats treated with cocaine acutely and chronically. The results with cocaine parallel those with ECS. The one-dimensional Western blot shown in Fig. 8 illustrates that the ΔFosB protein with a molecular weight of 33 kDa was induced only by acute, not by chronic, cocaine treatment. The 35- and 37-kDa chronic Fras, in contrast, were significantly induced by chronic treatment only.

Chronic Fras form AP-1 complexes with Jun-D and Jun-B. Chronic ECS and cocaine treatments have been shown to induce a long-lasting AP-1 complex in cortex and striatum, respectively, which is supershifted by anti-Fra antibody (25, 26, 36). To further characterize the Fos-related components in the chronic AP-1 complex, supershift assays with anti-c-Fos, anti-FosB, anti-Fra-1, anti-Fra-2, and anti-Fra antibodies were performed. The long-lasting AP-1 complex induced by chronic cocaine treatment was supershifted by anti-Fra antibody and disrupted specifically by anti-FosB antibody but not affected by the other antibodies (Fig. 9A), even though these other antibodies can disrupt AP-1 complexes containing purified c-Fos, Fra-1, or Fra-2.[2] Disruption, instead of supershifting, of the AP-1 complex by anti-FosB antibody may be due to the fact that the binding of the antibody destabilizes the AP-1 complex. Similar results with these various antibodies were obtained with cortical samples after chronic ECS treatment (data not shown). These findings demonstrate that the long-lasting AP-1 complex con-

[2] M. Gruda, personal communication.

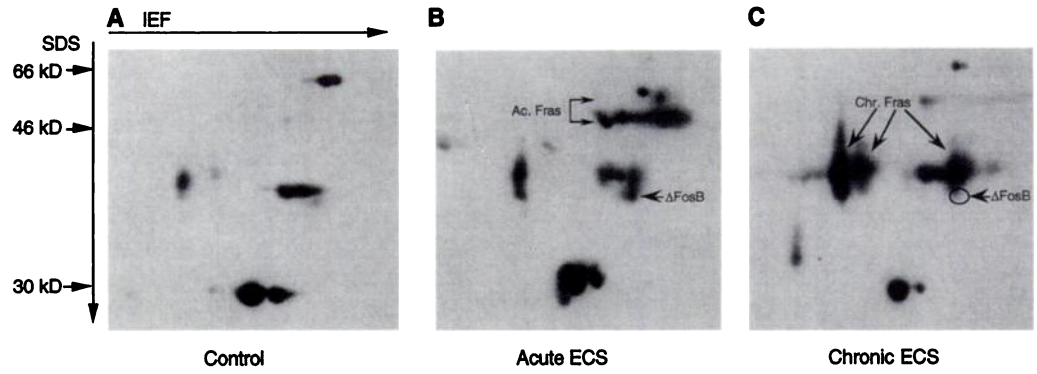

Fig. 7. Two-dimensional immunoblots of Fra immunoreactivity in cerebral cortex of ECS-treated rats. The first dimension was separated by isoelectric focusing (*IEF*), with the basic end to the *left of each panel*. The second dimension was performed by SDS-polyacrylamide gel electrophoresis. Fra immunoreactivity in control (A), acute ECS (B), and chronic ECS (C) samples, using the anti-Fra antibody (see Materials and Methods), are shown. ΔFosB in the acute ECS sample was identified, based on its induction pattern (induced only by acute treatment) and its molecular weight (33-kDa). The chronic Fras (*Chr. Fras*) were identified, also based on their induction pattern (induced or upregulated by chronic treatment only) and their molecular weights (35 and 37 kDa) as described previously (26).

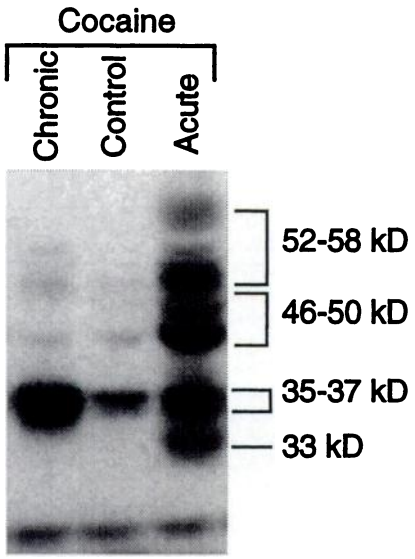

Fig. 8. Induction of ΔFosB and chronic Fras by cocaine treatments. A 33-kDa band, corresponding to ΔFosB, was induced by a single acute cocaine injection. The 35- and 37-kDa bands, corresponding to the chronic Fras, were induced by chronic cocaine treatment. The 52–58-kDa bands induced by acute cocaine may represent c-Fos and its phosphorylated isoforms, and the 46–50-kDa bands presumably represent FosB, Fra-2, and their isoforms.

tains FosB-related proteins. Because FosB and ΔFosB are present only in the acute state, the FosB-related proteins in the AP-1 complex induced by the chronic treatments are likely to be the chronic Fras.

To test which Jun protein forms the AP-1 complex with the chronic Fras, supershift experiments with anti-Jun antibodies were performed. The AP-1 complex induced by chronic cocaine in the striatum was disrupted partially by anti-Jun-D and anti-Jun-B antibodies and completely by anti-Jun Family antibody (Fig. 9B). In contrast, anti-c-Jun failed to disrupt the chronic AP-1 complex even though it can disrupt AP-1 complexes containing purified c-Jun.[2] These findings suggest that the long-lasting AP-1 complex induced by chronic cocaine treatment contains appreciable levels of Jun-D and Jun-B, but not of c-Jun. Interestingly, anti-Jun-B antibody disrupted the acute AP-1 complex to a greater extent than the chronic AP-1 complex, whereas anti-Jun-D antibody disrupted the chronic AP-1 complex only (Fig. 9, B and C). Anti-c-Jun antibody also failed to affect the acute AP-1 complex (Fig. 9C), consistent with the observation that c-Jun is not induced in the striatum by acute cocaine administration (44).

Analysis of Jun-D expression showed that Jun-D mRNA is constitutively expressed in cortex and striatum and is not affected by acute or chronic ECS or cocaine treatment in these brain regions (Fig. 10). In contrast, previous studies found that levels of c-Jun and Jun-B mRNA, while induced acutely, are reduced in cerebral cortex after chronic ECS treatment (30) and in nucleus accumbens after chronic cocaine treatment (36).

Discussion

Induction of ΔFosB expression *in vivo* by acute ECS and cocaine treatments. A major finding of this study is that FosB and ΔFosB mRNAs are rapidly and transiently induced in brain by acute ECS and cocaine treatments. This is the first evidence for ΔFosB induction *in vivo*. The rapid and transient induction pattern of ΔFosB suggests that this protein may be involved in stimulus-transcription coupling in the nervous system as has been proposed for other Fos- and Jun-like proteins (1). Induction of FosB mRNA by pentylenetetrazol seizure (41), noxious stimulations (42), and cocaine (36, 39) has been reported previously. Because the size of FosB mRNA (4145 nucleotides) is similar to that of ΔFosB (4005 nucleotides) (18), the FosB mRNA detected by Northern blotting in these studies probably included ΔFosB mRNA. Thus, ΔFosB may behave as a typical acute Fra in the nervous system, being induced rapidly and transiently in specific brain regions in response to diverse stimuli.

The role that ΔFosB plays in brain function remains unknown. Structural and functional studies of ΔFosB have shown that there is no apparent transactivation domain in the ΔFosB molecule that could mediate regulation of transcription (20, 23). The TATA binding protein (TBP) binding motif and the proline-rich sequence of FosB, which constitute the major transactivation domains of the protein (21), are deleted in ΔFosB because of the frame shift mutation caused by alternative splicing (18). Therefore, ΔFosB is very likely to function as a negative regulator of transcription. ΔFosB can inhibit repression of the c-fos promoter by FosB or c-Fos (18).

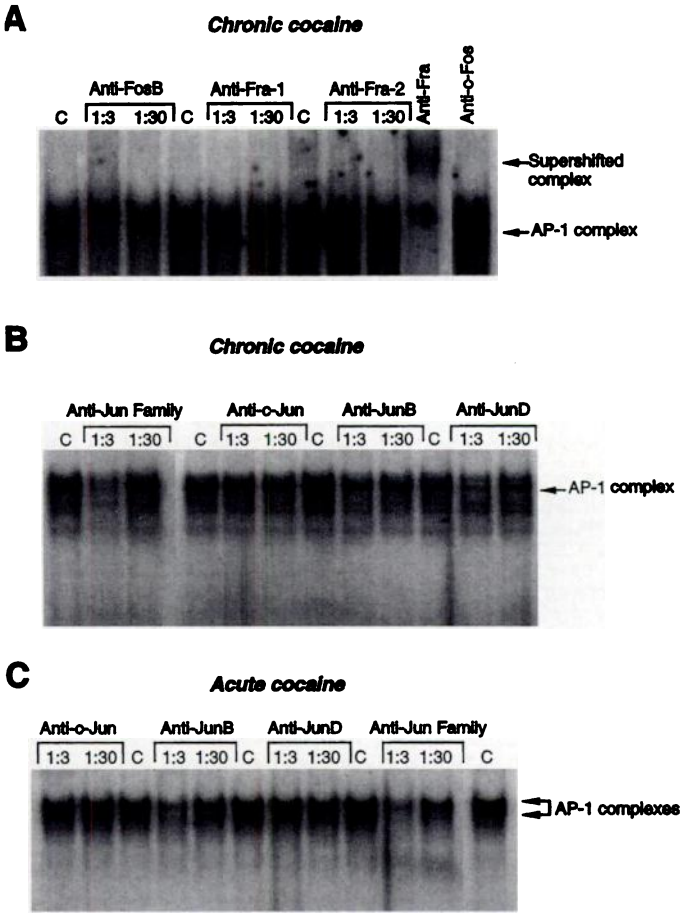

Fig. 9. Disruption of the chronic and acute AP-1 complexes by anti-Fos and anti-Jun antibodies. Nuclear extracts from striatum of rats treated with cocaine chronically or acutely were incubated with ^{32}P-labeled AP-1 oligonucleotide. Antibodies were added to supershift or disrupt AP-1 complexes with the corresponding components. Two dilutions of each antibody were used. For control, no antibody was added. A, Anti-c-Fos, anti-FosB, anti-Fra-1, anti-Fra-2, and anti-Fra antibodies were added to supershift or disrupt the chronic AP-1 complex induced by cocaine chronically. The chronic AP-1 complex was supershifted by anti-Fra antibody and disrupted by anti-FosB antibody. B, Anti-c-Jun, anti-JunB, anti-JunD, and anti-Jun family antibodies were added to disrupt the chronic AP-1 complex induced by cocaine chronically. The chronic AP-1 complex was disrupted partially by anti-Jun-D and anti-Jun-B antibodies and completely by anti-Jun family antibody. C, Anti-c-Jun, anti-Jun-B, anti-Jun-D, and anti-Jun family antibodies were added to disrupt the AP-1 complex induced by cocaine acutely. The acute AP-1 complex was disrupted by anti-Jun-B and anti-Jun family antibodies.

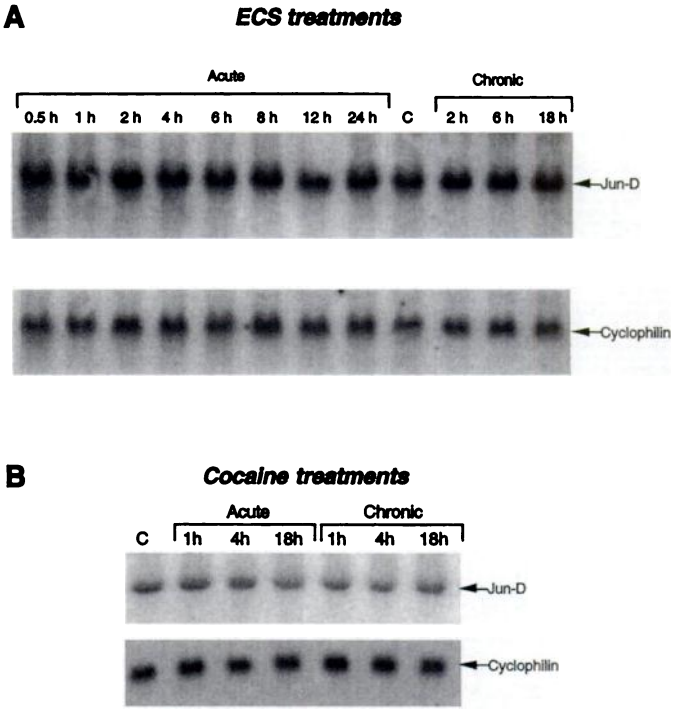

Fig. 10. Lack of induction of Jun-D mRNA by acute and chronic ECS and cocaine treatments. The autoradiograms show the expression of Jun-D mRNA in cortex of rats treated with ECS acutely or chronically (A) or in striatum of rats treated with cocaine acutely or chronically (B). Twenty micrograms of total RNA from each sample were used for the Northern blot analysis. The same RNA blot was first hybridized with the Jun-D probe and then the cyclophilin probe, which was used as an internal control. Results are representative of three experiments.

The rapid and transient induction of ΔFosB by ECS and by cocaine may be one of the acute negative feedback mechanisms elicited in response to these stimuli.

The pattern of ΔFosB induction by acute ECS and cocaine is similar, which suggests that similar mechanisms may be involved. Several regulatory elements in the promoter region of the *fosB* gene have been identified, including a serum response element and an AP-1 binding site (43). The serum response element responds to protein kinase C-dependent and MAP kinase-dependent signals. Since the time (30 min) for maximal induction of ΔFosB mRNA is shorter than the time (60 min) for the maximal expression of c-Fos and related proteins, the AP-1 site is probably not required for mediating ΔFosB induction.

Regulation of ΔFosB and the chronic Fras by chronic ECS and cocaine treatments. ΔFosB mRNA was still induced significantly by an ECS treatment that followed a course of chronic ECS. In contrast, the ΔFosB mRNA response was largely desensitized in the case of cocaine. In both cases, however, chronic ECS or cocaine treatments did not lead to a sustained induction of ΔFosB mRNA. Chronic ECS and cocaine treatments also did not result in sustained induction of ΔFosB protein. Instead these treatments induced several Fos-related proteins, termed chronic Fras, as reported previously (25, 26). Our data clearly demonstrate that these chronic Fras are different from the ΔFosB induced acutely. First, ΔFosB and the chronic Fras exhibit different temporal properties. The 33-kDa ΔFosB band, which comigrates with ΔFosB induced in serum-stimulated Rat-1A cells, was induced rapidly by an acute ECS or cocaine treatment and returned to control levels within 12 hr but did not accumulate after chronic treatments. In contrast, the chronic Fras were not induced significantly by an acute treatment but rather accumulated after chronic treatments. Second, the 33-kDa ΔFosB and the 35–37-kDa chronic Fra bands migrate differently on one- and two-dimensional gel electrophoresis. Thus, the chronic Fras appear to be novel FosB-related proteins. However, the question remains as to whether the chronic Fras represent the products of novel Fos-like genes, novel splice variants of the FosB gene, or novel post-translational modifications of FosB or related proteins. This question will be answered on further characterization of the chronic Fras, which is under way. Nevertheless, there is evidence that the chronic Fras are not a phosphorylation

variant of ΔFosB. Two of three chronic Fra bands induced migrate more than 1 pH unit more basic than ΔFosB. The third chronic Fra band exhibits a similar pI as ΔFosB. These positions would be highly unusual for phosphorylated isoforms of ΔFosB, which would be more acidic.

Characterization of these chronic Fras with anti-FosB carboxyl- and amino-terminal antibodies showed that the chronic Fras are immunochemically related to ΔFosB. Because the carboxyl terminus of c-Fos and FosB contains the major transactivation domain of the protein (21), the lack of a FosB-like carboxyl-terminal antigen in the chronic Fras suggests that these chronic Fras may have little or no transactivation activity. One interesting possibility, then, is that ΔFosB and the chronic Fras subserve similar functions. However, the fact that different proteins may serve as transcriptional inhibitors in the acute- versus chronic-treated states raises the interesting possibility that the expression of different genes are regulated under these two conditions.

The chronic Fras also exhibited strong immunoreactivity to the anti-Fra antibody, which is raised against the conserved DNA-binding and leucine zipper domains (M-peptide) of Fos family proteins. This suggests that the chronic Fras contain similar DNA binding and leucine zipper domains. Thus, like other Fos-related proteins, chronic Fras presumably form heterodimers with Jun proteins, with the heterodimer binding to DNA with an AP-1 consensus sequence. This is supported by the previous observation that chronic ECS and cocaine treatments induce long-lasting AP-1 complexes containing the chronic Fras (25, 26). By use of supershift assays, we provide evidence in the current study that the chronic Fras form heterodimers with Jun-D and Jun-B, with Jun-D apparently more specific for the chronic Fras. This interpretation is supported by preliminary immunoprecipitation experiments, which demonstrated that the 35 and 37-kDa chronic Fras can be coprecipitated by anti-Jun-D and anti-Jun family antibodies.[3] This confirms that the chronic Fras are components of the long-lasting AP-1 complex, and that they interact directly with Jun-D. Interestingly, Jun-D is expressed constitutively and not altered after acute and chronic treatments. However, it does not form appreciable levels of AP-1 complexes with acute Fras, such as c-Fos, FosB, and Fra-1, that are present in the acute-treated state. The specific interaction between Jun-D and the chronic Fras may be due to the fact that Jun-D has a higher affinity for the chronic Fras than c-Jun and Jun-B and/or that Jun-D is the major Jun protein available for the chronic Fras in the chronic state, because c-Jun and Jun-B are at least partially down-regulated by chronic treatments (30, 36). As stated above, it is likely that the chronic Fras are weak transactivators because they lack the carboxyl-terminal antigen containing the major transactivation domain of FosB. Since Jun-D is reported to be a weak transactivator relative to c-Jun and Jun-B (45), it is conceivable that the AP-1 complex formed by Jun-D and the chronic Fras is an even weaker transactivator.

Together, the findings of the present study support the hypothesis that the chronic AP-1 complex, formed by the chronic Fras and Jun-D, serves as a negative regulator of AP-1 mediated transcription in response to chronic treatments. We have shown previously that the chronic AP-1 complex exhibits different DNA binding properties compared to the acute AP-1 complex (25). Analysis of the specific genes with AP-1 sites in their promoters that are regulated by the chronic AP-1 complex is necessary for definition of the functions of the chronic Fras. Delineation of the molecular identity of the chronic Fras will clearly facilitate this process. In this manner, the chronic Fras may be involved in modifying intracellular signal transduction pathways, or cellular structural features, which contribute to the long term adaptive changes that occur in brain in response to repeated stimulations.

[3] N. Hiroi and E. J. Nestler, unpublished observations.

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