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LONG-TERM INDUCTION OF FOS-RELATED ANTIGEN-2 AFTER METHAMPHETAMINE-, METHYLENEDIOXYMETHAMPHETAMINE-, 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE- AND TRIMETHYLTIN-INDUCED BRAIN INJURY

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Abstract—A long-term induction of Fos-related antigens has been shown in neurons after brain injury, suggesting that Fos-related antigens are involved in enhancing the transcription of genes related to the process of regeneration and repair. In the present study, we report that levels of Fos-related antigen-2 are elevated in several models of chemically induced brain injury. Trimethyltin, which causes degeneration of neurons primarily in the hippocampus and other limbic regions, results in a five-fold induction of Fos-related antigen-2 immunoreactivity in neurons in the pyramidal and dentate layers of the hippocampus starting at seven days post-treatment and persisting for 60 days. Methamphetamine and methylenedioxymethamphetamine, agents which cause degeneration of dopaminergic nerve terminals in the striatum of the mouse, cause an increase in Fos-related antigen-2 immunoreactivity which begins at three days post-treatment and returns to basal levels by days 5 and 15, respectively. Treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine elevated levels of Fos-related antigen-2 in the mouse striatum at three days post-treatment. This abbreviated time-course of Fos-related antigen-2 induction is consistent with less severe insult (terminal damage) relative to trimethyltin (cell death), but induction occurs during the period of regeneration and repair in both models. Dexfenfluramine, a non-neurotoxic amphetamine, does not induce Fos-related antigen-2 expression. Decreasing core temperature of the mouse, which blocks amphetamine-induced neurotoxicity, also blocks Fos-related antigen-2 induction.

In summary, Fos-related antigen-2 is induced in models of both cell death and terminal degeneration, suggesting that this transcription factor may serve as a universal signal transduction molecule involved in the regulation of genes related to regeneration and repair in the CNS.

Key words: transcription factor, gene regulation, neuronal regeneration, AP-1, glial fibrillary acidic protein, terminal degeneration.

The various members of the activator protein-1 (AP-1) transcription factor family are induced in a specific temporal pattern with a short-term (hours) expression of some proteins and a long-term (days to weeks) expression of others. In the CNS, c-*fos* is rapidly induced in the hippocampus due to seizure activity, but returns to basal levels within 24 h.[19] Several Fos-related antigen (FRA)-immunoreactive proteins are also induced, but the temporal pattern of protein expression differs among the various FRAs.[16,30,36] After kainate administration, expression of Fos protein declines within 1–4 h; however, other FRA proteins persist. FRA proteins of 46,000 and 35,000 mol. wt, in particular, maintain an extended expression, and we have observed expression of the 35,000 mol. wt protein for several months after kainate treatment.[29] FRA transcription factors require a Jun protein to form a DNA binding complex.[10] These observations suggest that different FRA–Jun dimer combinations modulate genes in a distinct temporal sequence; acutely induced AP-1 proteins modulate a set of genes over the short term, while the persistently up-regulated AP-1 factors alter expression of a different set of genes. Chronic drug treatment or stimulation,[13] as well as damage to the brain, have been shown to cause long-term induction of FRA-immunoreactive proteins, suggesting that FRAs are regulating genes for adaptation to a persisting or permanent change in the biochemical environment.[28] Following brain injury, FRA proteins and other transcription factors with prolonged expression have been proposed to be involved in the regulation of genes related to repair and regeneration.[11,27]

The FRA-2 protein was originally identified as a 46,000 mol. wt protein induced in growth-stimulated chicken embryo fibroblasts.[22] Unlike Fos, FRA-2 exhibits delayed onset and prolonged kinetics,[22] suggesting protracted regulation of target genes. The role of FRA-2 in gene expression in the brain is not well understood; however, treatment with the excitotoxin, kainate, induces rapid and prolonged expression of FRA-2 mRNA in the rat hippocampus, suggesting that this factor is involved in repair of seizure-induced brain damage.[16] In this study, we examined FRA-2 expression in several brain injury models.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used. Male Sprague–Dawley rats (200–250 g) were used as subjects for experiments with trimethyltin

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Abbreviations: AP-1, activator protein-1; D-FEN, dexfenfluramine; FRA, Fos-related antigen; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein-2; MDMA, methylenedioxymethamphetamine; METH, methamphetamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TMT, trimethyltin.

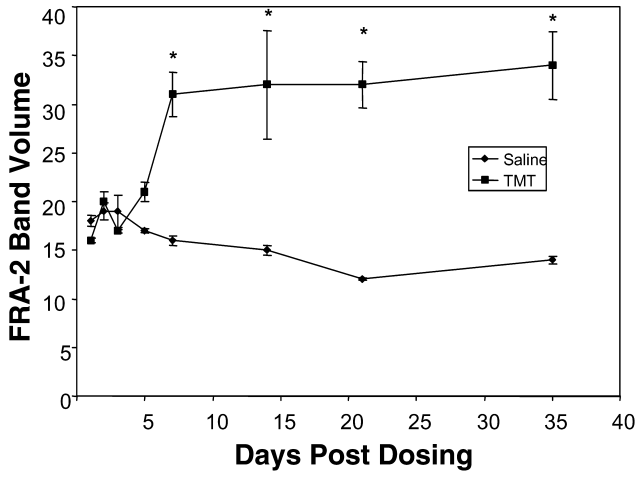

Fig. 1. Time-course of FRA-2 expression in the rat hippocampus after TMT treatment. Hippocampal protein extracts from rats treated with TMT (one to 35 days) and saline-injected were examined for FRA-2 expression using western blot analysis. FRA-2 expression is significantly increased four- to five-fold one week after TMT treatment and remained elevated at 35 days after treatment. $*P < 0.05$ ($n = 3$ for each group at each time-point). Each data point represents the mean ± S.E.M. for the densitometric values obtained.

(TMT). Rats were housed separately post-dosing to mitigate TMT-induced aggression associated with group housing.[8] TMT was administered at a single dose of 8 mg/kg (i.p.). Female C57Bl6/J mice were used as subjects for experiments with the known dopaminergic neurotoxicants, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), methamphetamine (METH) and methylenedioxymethamphetamine (MDMA).[24,25] Mice were housed in groups of six per cage, conditions that result in moderate post-METH increases in core temperature,[24] but that do not result in METH-induced malignant hyperthermia seen in METH-treated rats.[3,4] MPTP was administered at a single dose of 12.5 mg/kg (s.c.), which does not cause cell death. METH and MDMA were administered s.c. at doses of 10 and 20 mg/kg, respectively, every 2 h (beginning at 11.30) for a total of four doses. The non-neurotoxic amphetamine analog, dexfenfluramine (D-FEN), was used as a negative control and administered at a dose of 25 mg/kg for each of four doses administered on the same schedule as METH and MDMA. METH and MDMA dosages were chosen to give an approximately 50% decrease in dopamine levels;[24] this regimen is comparable to the effects seen with our MPTP model[22] and result in similar levels of gliosis.[24] The D-FEN regimen was chosen to give at least a 50% decrease in serotonin levels, the transmitter upon which it acts. The 4×50 mg/kg of D-FEN regimen does not increase glial fibrillary acidic protein (GFAP) levels, but produces greater and longer-lasting decreases in serotonin levels.

The mouse samples used in the present study were used in a previous examination of dopaminergic neurotoxicants.[18,24] Rats and mice were maintained on a 12-h/12-h light–dark cycle, with food and water available *ad libitum.* A lowered ambient temperature was achieved by placing mice in an Environmental Growth Chamber Model TC-1 (EGC, Chagrin Falls, OH, USA) at 15°C on a 12-h/12-h light–dark cycle. The animals were housed individually in order to prevent huddling and interference with the lowered ambient temperature.

Tissue preparation

The hippocampus and striatum were dissected free-hand and homogenized in hot 1% sodium dodecyl sulfate for subsequent analysis of FRA-2 by quantitative immunoblotting.

Fos-related antigen-2 analysis

Hippocampal and striatal homogenates were resolved on 10% sodium dodecyl sulfate gels and electrophoretically transferred to nitrocellulose. Blots were probed with rabbit anti-FRA-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection was achieved by enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK) and quantified by scanning the exposed films using a Personal Densitometer running ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA); care was taken to limit gel loads (total protein) from control and treated tissues to values that resulted in linear responses over the total range of exposure values.[23] Arbitrary volume units of exposure intensity were normalized to percentage of the corresponding control group. All band intensities fell within the linear range of film exposure values.

Statistical analysis

Data were analysed by ANOVA followed by least squares denominator test for mean comparisons. In all cases, experimental results were considered to be statistically significant when $P < 0.05$.

Immunohistochemistry

Primary antibodies were used at the following concentrations: FRA-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit, 0.1 μg/ml), GFAP (Boehringer Mannheim Corporation, Indianapolis, IN, USA; mouse, 0.2 μg/ml) and microtubule-associated protein-2 (MAP-2; Boehringer Mannheim; rabbit, 0.1 μg/ml). Secondary antibodies (Vector, Burlingame, CA, USA; all used at 0.5 μg/ml) were rabbit immunoglobulin G coupled to horseradish peroxidase and mouse immunoglobulin G coupled to horseradish peroxidase.

Animals were perfused transcardially with a 50-ml syringe containing 1% paraformaldehyde (30 ml/100 g body weight) followed quickly by 4% paraformaldehyde (70 ml/100 g body weight). Brains were removed, stored in 4% paraformaldehyde overnight and cut as 30-μm coronal free-floating sections on a Vibratome. Selected sections were incubated with 3% H_2O_2 for 15 min to block endogenous peroxidase activity, permeabilized with 0.2% Triton X-100 containing 2% normal goat serum and incubated at 4°C with primary antiserum against FRA-2. Sections were washed three times for 5 min each in phosphate-buffered saline following each incubation step. Peroxidase-conjugated secondary antibodies were incubated with the sections for 2 h at room temperature. Following a phosphate-buffered saline wash, sections were incubated with tertiary antibody for 1 h. At room temperature, immunoreactivity was visualized by diaminobenzidine, which produced a brown color (single immunohistochemistry), or enhanced with nickel (double immunohistochemistry). In double-immunohistochemical procedures, sections were incubated with the second primary antibody overnight at 4°C, washed and treated with secondary and tertiary antibodies as described, and visualized with diaminobenzidine, producing a brown color.

In order to validate the specificity of the immunohistochemical reaction, primary antibodies were incubated with FRA-2 peptide before incubation with control sections (data not shown, but see Ref. 41).

RESULTS

Rats receiving 8 mg/kg (i.p.) TMT were killed one to 35 days after treatment. Levels of 46,000 mol. wt FRA-2 in the hippocampus significantly increased fivefold by seven days post-treatment and remained elevated for the full 35-day time-course (Figs 1, 4). To determine when FRA-2 levels return to baseline, an extended time-course was performed up to 90 days post-treatment. Levels of FRA-2 in the hippocampus remained significantly elevated for 60 days post-treatment (Fig. 2).

Immunohistochemical analysis revealed FRA-2-immunoreactive cells scattered throughout the CA1, CA3, hilar and dentate regions of the rat hippocampus after TMT treatment. Very few Nissl-stained cells were immunoreactive for FRA-2 in controls (Fig. 3A). Ten days after TMT treatment, several neurons show prominent FRA-2 expression in their nuclei (Fig. 3B). It is likely that these FRA-2-immunoreactive cells are neurons, because the nuclear immunostaining is co-localized with the Nissl stain (arrows; Fig. 3E) and double-immunohistochemical staining with MAP-2, a marker for neurons, showed co-localization with FRA-2 (Fig. 3C, D).

The expression of FRA-2 was examined in mice in the

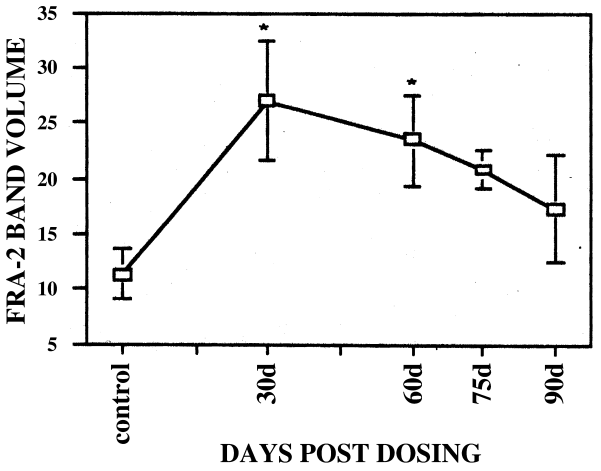

Fig. 2. Prolonged expression of FRA-2 after TMT-induced brain injury. Rats were saline-injected (controls) or treated with TMT and then killed at 30, 60, 75 and 90 days post-treatment. Total protein extracts were prepared from the hippocampi of these animals. Western blot analysis was used to examine FRA-2 expression. FRA-2 immunoreactivity increased nearly fourfold at 30 days and declined to approximately 2.5-fold at 75 and 90 days relative to control levels. ANOVA followed by Fisher's protected least squares denominator post hoc test found FRA-2 levels significantly different from control levels at 30 and 60 days post-treatment. $*P < 0.05$ ($n = 4$ for each group at each time-point). Each data point represents the mean ± S.E.M. for the densitometric values obtained.

MPTP and substituted amphetamine models of dopaminergic nerve terminal degeneration.[24,25,37] Immunoblots using striatal protein three days after MPTP, METH and MDMA treatments revealed an increase in the 46,000 mol. wt FRA-2 protein (Fig. 4). METH and MDMA caused a threefold significant increase in striatal FRA-2 at three days post-treatment, which returned to control levels by days 5 and 15, respectively (Fig. 5): the cortex and cerebellum were not affected (data not shown). A 7°C decrease in ambient temperature abolished the METH- and MDMA-induced changes in FRA-2 (Fig. 6), but did not affect induction of FRA-2 by MPTP. D-FEN did not affect FRA-2 expression (data not shown). Immunohistochemical analysis showed low basal levels of FRA-2 expression throughout the matrix of the mouse striatum (Fig. 7A, B). Three days after METH treatment, FRA-2 immunoreactivity was increased in nuclei in cells throughout the striatum (Fig. 7C, D).

DISCUSSION

We have demonstrated that a protracted expression of neuronal FRA-2 is an element of the damage response that results from administration of several well-characterized neurotoxic agents. Both neuronal cell loss and nerve terminal degeneration in the absence of cell loss cause an increase in FRA-2 levels in neurons.

Much of our understanding of the mechanism of FRA-2's action is revealed by experiments producing chemically induced brain injury. TMT, which is used in fungicides, chemostabilizers and for various industrial purposes,[35] is a potent neurotoxicant for selective brain regions, particularly the hippocampal formation and piriform cortex, and these neurodegenerative changes are observable 16–24 h after administration.[1,2,6,8,17] Neuronal damage occurs in both dentate granule neurons and pyramidal neurons of the hippocampus;[1,7] however, dentate granule neurons recover from the initial injury whereas degeneration of pyramidal neurons, especially CA3 and CA4, progresses.[7] The neurodegenerative mechanism is still unknown, but TMT targets neurons that express stannin, a tin-binding protein.[39] TMT treatment has been shown to increase AP-1 transcription factors in the rat hippocampus.[41,42] Fos and Jun are induced within hours after TMT treatment and their expression returns to pretreatment levels within 24 h. However, the increased expression of FRA-2 is delayed and prolonged, suggesting a role for this protein in adaptation of surviving neurons to injury.

In contrast to TMT-induced neurodegeneration, METH, MDMA and MPTP cause loss of dopaminergic nerve terminals in the mouse neostriatum (as evidenced by decreased dopamine, tyrosine hydroxylase and silver degeneration staining) without the loss of neuronal perikarya in the substantia nigra.[18,24,37] Our demonstration that METH, MDMA and MPTP result in the induction of FRA-2 indicates that neurotoxic effects, which do not cause cell death, can result in elaboration of transcription factors. Since the terminal damage caused by the amphetamines, but not by MPTP, can be blocked by pharmacological agents, stress paradigms and environmental conditions that lower core temperature,[18] we were able to determine whether neuroprotection would be accompanied by an attenuated expression of FRA-2. Lowered ambient temperature completely blocked METH- and MDMA-, but not MPTP-induced expression of FRA-2, demonstrating a linkage between dopaminergic nerve terminal damage and the expression of this transcription factor. The lack of induction of FRA-2 following exposure to a non-neurotoxic amphetamine congener, D-FEN, is also consistent with this interpretation. FRA-2 is induced in surviving neurons following exposure to diverse types of neurotoxic agents that result in dissimilar types of neuronal damage (cell death vs terminal degeneration). This suggests that activation of FRA-2 signal transduction pathways may be common to neurons undergoing regeneration. The possibility exists, however, that FRA-2 plays a different role in each of these brain injury models, as with axotomy of retinal ganglion neurons.[33]

Unlike other AP-1 transcription factors, which are only transiently expressed, such as Fos, several FRAs have been shown to have prolonged expression after mechanical injury in the brain,[34] repeated drug treatment[13,31,32] and excitotoxicity.[16,30] While the FRA associated with chronic or repeated treatments was identified as delta FosB,[21,32] not all the FRAs induced after brain injury have been characterized. Kainate-induced neurodegeneration in the rat hippocampus results in a prolonged expression of FRA-2 and a 35,000 mol. wt FRA;[16,30] neurons of the dentate gyrus express high levels of the 35,000 mol. wt FRA lasting up to three months after a single injection of kainate.[29,30] Neurons of the dentate gyrus survive the kainate-induced neurotoxicity and sprout axon collaterals to form new synapses to adapt to the injured environment.[26] Granule neurons of the olfactory bulb, which exhibit neurogenesis throughout most of the lifetime of the rat, express the highest basal levels of the 35,000 mol. wt FRA relative to other brain regions.[29] FRA-2 is expressed in neurons throughout the hippocampus that survive TMT neurotoxicity. FRA-2 and the 35,000 mol. wt FRA are expressed from weeks to months after a single injury event, whereas chronic FRAs require constant stimulation or their levels decline by 50% within a week.[13] The prolonged expression of FRA proteins associated with injury suggests that

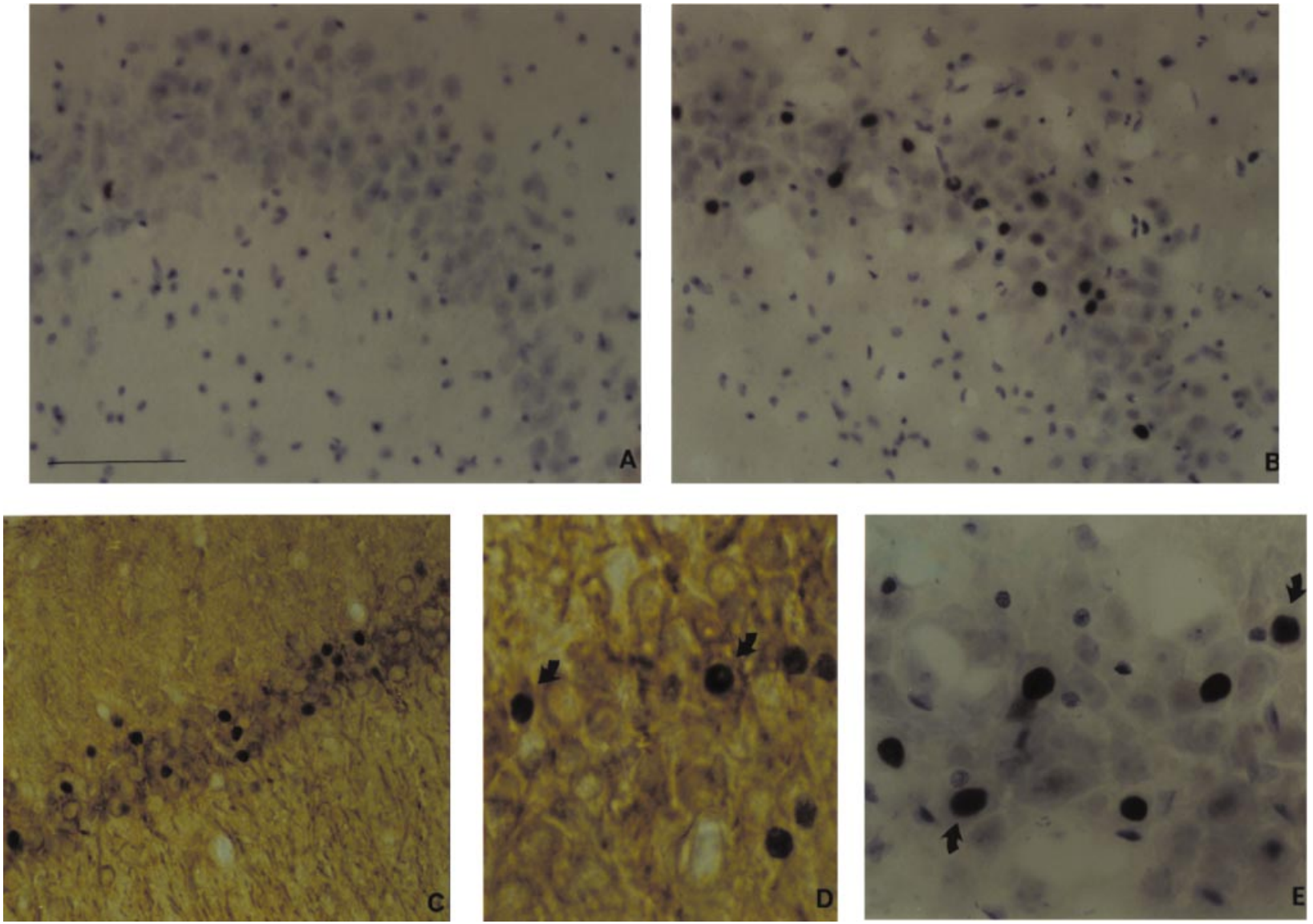

Fig. 3. Immunohistochemical localization of FRA-2 in the rat hippocampus after TMT treatment. Brains from rats saline-injected or 10 days after TMT treatment were sectioned and sections through the hippocampus were immunostained for FRA-2 (black), then counterstained with Nissl (blue) or double-immunostained for MAP-2 (brown). Hippocampal sections from saline-injected rats revealed few FRA-immunoreactive cells in the Nissl-stained neuronal layer of CA3 (A). TMT treatment resulted in numerous cells in CA3 containing FRA-2-positive nuclei (B). FRA-2 immunoreactivity is co-localized with the Nissl stain (arrows). Sections containing the hippocampus from TMT-treated rats exhibit double immunoreactivity for FRA-2 and MAP-2 in the neuronal layer of CA1 (C, D). At high magnification, the FRA-immunoreactive nuclei are present in MAP-2-positive neurons (arrows; D). Scale bar in A = 100 μm.

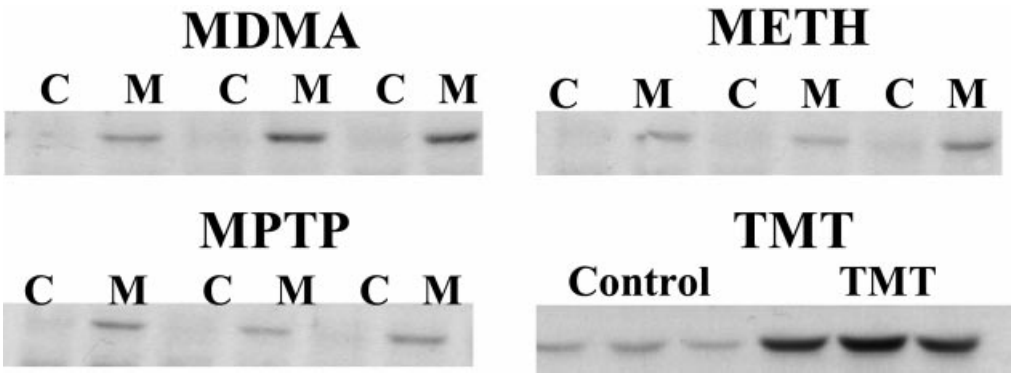

Fig. 4. Induction of FRA-2 after treatment with neurodegenerative drugs. Mice were treated with METH, MDMA and MPTP, and were killed three days after initial treatment. Striatal protein was analysed for FRA-2 immunoreactivity in treated (M) and saline-injected mice (C), and is increased following drug treatment. TMT and saline were administered to rats and after 35 days. Hippocampal protein extracts were analysed for FRA-2 immunoreactivity using western blot analysis, which revealed an induction in FRA-2 protein following TMT treatment. Each lane represents a different rat.

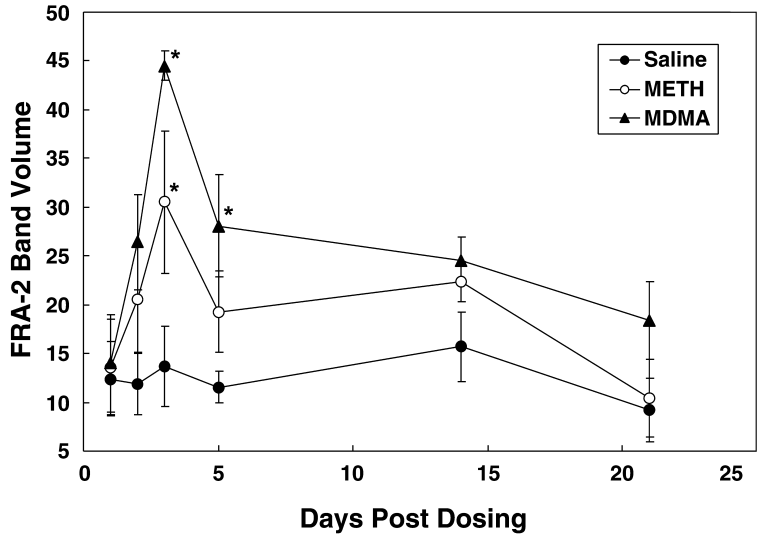

Fig. 5. Time-course of FRA-2 induction after METH and MDMA treatment. Mice were treated with METH, MDMA or saline, and were killed at one, two, three, five, 15 and 20 days after initial treatment. Striatal protein was analysed by immunoblot for FRA-2 immunoreactivity. FRA-2 levels were significantly increased at three and five days after MDMA treatment, and three days after METH treatment. $*P < 0.05$, for both treatments. Each data point represents the mean ± S.E.M. for densitometric values obtained from each of three mouse striatal homogenates.

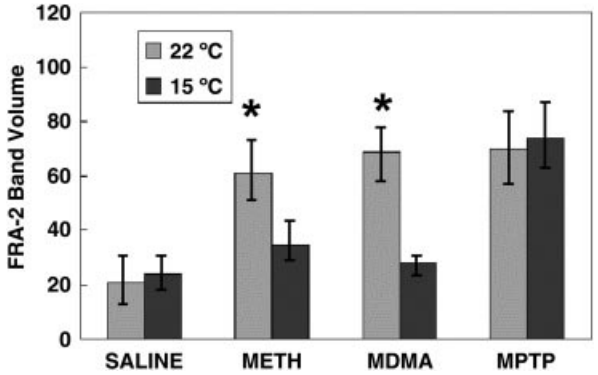

Fig. 6. Lowering ambient temperature blocks METH- and MDMA- but not MPTP-induced expression of FRA-2. Mice were treated with METH, MDMA and MPTP or saline, and exposed to either 15 or 22°C (ambient room temperature) for three days. Striatal protein was analysed for FRA-2 immunoreactivity by immunoblot. Each data point represents the mean ± S.E.M. for densitometric values obtained from each of three mouse striatal homogenates. $*P < 0.05$.

long-term activation of genes is needed to complete regeneration and repair of the injured CNS.

FRA-2 is induced within days after neuronal or terminal degeneration in two different brain regions. At this time, the stimulus that increases FRA-2 is unknown. FRA-2 expression is temporally correlated with the induction of reactive gliosis, as assessed by enhanced expression of GFAP.[5,18,24] When GFAP levels decline, FRA-2 expression returns to basal levels, raising the possibility that reactive astrocytes are responsible for enhanced FRA-2 levels in surviving neurons. Activated astrocytes release a number of substances, such as neurotrophic factors and cytokines, that could increase the expression of FRA-2.[9,38] An astrocytic response precedes neuroplastic changes in the CNS after injury, indicating that neurotrophic molecules originating from reactive astrocytes may be inducing expression of FRA-2. Alternatively, deafferentation with the subsequent loss of neurotransmitter stimulation may induce FRA-2 expression. The striatal neurons and the dentate granule neurons lose dopaminergic and glutaminergic innervation, respectively. Lack of afferent

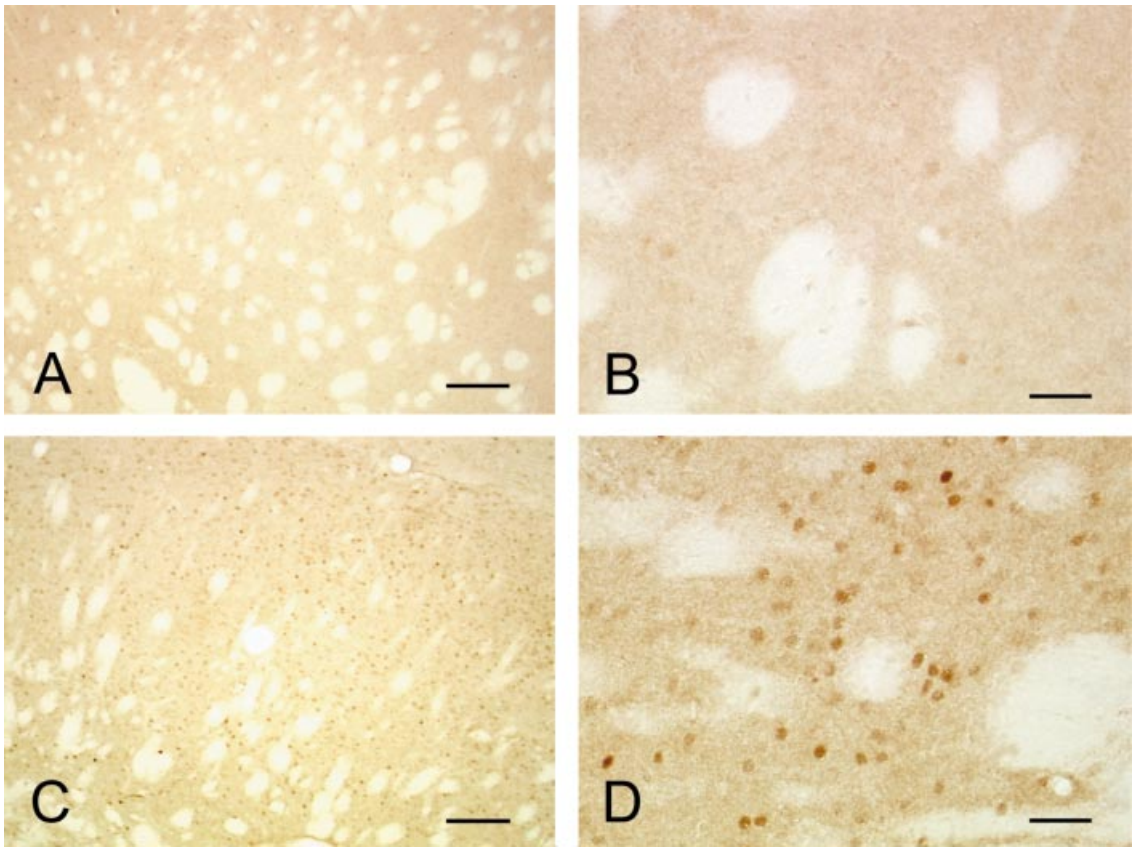

Fig. 7. Immunohistochemical localization of FRA-2 in mouse striatum after METH treatment. Mice were treated with saline (A, B) or METH (C, D). Three days following treatment, brains were removed and sectioned for FRA-2 immunohistochemistry. The striatum from saline-injected mice contained few FRA-immunoreactive cells. However, METH-treated mice showed numerous cells throughout the striatum containing FRA-2-positive nuclei. Scale bars = 132 μm (A, C), 33 μm (B, D).

stimulation may activate FRA-2 expression to regulate genes to restructure the neuronal cytoarchitecture. One or both of these mechanisms may play roles in the expression of FRA-2.

The temporal expression of FRA-2 overlaps the neuroplastic events that follow injury to the brain, and dopaminergic terminal sprouting has been reported following MPTP treatment[12] with a similar time-course as FRA-2 expression. Several genes related to regeneration and repair are transcriptionally up-regulated or potentially up-regulated by AP-1 transcription factors, suggesting an influence of AP-1 on neuroplastic gene expression.[11,14] Neuronal damage caused by TMT is associated with reactive synaptogenesis, as evidenced by synapsin-1 returning to control levels.[5] FRA-2 is a component of AP-1 DNA binding activity in the hippocampus at eight days after TMT treatment.[41] These long-term FRA-containing AP-1-binding complexes may target neuroplastic genes containing functional AP-1 regulatory sites in their promoter region, such as growth-associated protein-43.[20] Matrix metalloproteinase-1 gene contains two AP-1 sites, with one involved in basal transcription and the other in phorbol ester-induced transcription.[40] AP-1 regulation has also been reported for matrix metalloproteinase-9.[15] Synapsin-1, a synaptic vesicle-associated protein present in all nerve terminals, declines initially after TMT treatment in the rat hippocampus, but expression rebounds three months later,[5] and its promoter has an AP-1-like site. These results describe changes in gene expression caused by acute induction of FRA-2; however, the AP-1 transcription factors that regulate long-term changes in gene expression related to neuroplasticity are not well characterized.

CONCLUSION

FRA-2 expression is associated with neurodegeneration, axotomy and terminal degeneration. However, the limited evidence suggests that reactive astrocytes are producing neurotrophins that are inducing FRA-2 expression in neurons surviving injury to undergo neuroplastic changes in response to the injured environment. This transcription factor may be a universal signal transduction molecule for neurons to adapt to injury by activating genes related to repair and regeneration. However, more studies are necessary to elucidate the function of FRA-2 and its target genes.

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