Fra-1 immunoreactivity in the rat brain during normal postnatal development and after injury in adulthood

E. Pozas a,b,*,1, F. Aguado1c, I. Ferrer a,b

a Unitat de Neuropatologia, Servei d’Anatomia Patològica, Hospital Prínceps d’Espanya, Unitat de Neuropatologia Experimental, Universitat de Barcelona, Hospital de Llobregat, 08907 Barcelona, Spain
b Unitat de Neuropatologia, Servei d’Anatomia Patològica, Hospital Prínceps d’Espanya, Laboratori de Neurobiologia Cel.lular i Molecular, Universitat de Barcelona, Hospital de Llobregat, 08907 Barcelona, Spain
c Unitat de Neuropatologia, Servei d’Anatomia Patològica, Hospital Prínceps d’Espanya, Departament de Biologia Cel.lular i Anatomia Patològica, Universitat de Barcelona, Hospital de Llobregat, 08907 Barcelona, Spain

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Abstract

Fra-1 is a member of the Fos family whose functional role in the central nervous system is little understood. In the present study, Fra-1 immunoreactivity is examined in the rat brain during normal development and after different injuries in adulthood, by using Western blotting and immunohistochemistry. Western blots show a band at p35 which corresponds to the molecular weight of Fra-1. During postnatal development, Fra-1 immunoreactivity is observed in nerve fibers of all the main fiber tracts in the cerebrum, whereas Fra-1 immunoreactivity in adult rats is restricted to the hippocampus, mainly the molecular layer of the dentate gyrus and the mossy fiber layer. After administration of colchicine, an axonal transport inhibitor, Fra-1 immunoreactivity accumulates in the perikarya of many cerebral neurons, including those of the dentate gyrus, hippocampus, cerebral cortex, amygdala and thalamus. Fra-1 immunoreactivity is also found in the nuclei of reactive astrocytes, as revealed with double-labeling immunohistochemistry to Fra-1 and GFAP, following either intraperitoneal injection of kainic acid at convulsant doses, intrastratial injection of quinolinic acid, or intraventricular injection of colchicine. These results suggest a cytoplasmic role for Fra-1 in the neurons, whereas the localization of Fra-1 in the nuclei of reactive astrocytes suggests a participation of this transcription factor in the activation of the AP-1 sequence of selected genes in the early glial response after different brain lesions.

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1. Introduction

The protooncogenes of the fos and jun families encode the proteins Fos and Jun which constitute the core of the AP-1 transcription factors. All of these members contain a leucine zipper region and a basic region which are necessary for dimer formation and DNA binding activity, respectively. Dimerization is necessary for DNA binding to the specific consensus sequence in DNA. Variations in the composition of AP-1 affect the affinity of dimer formation. In addition, post-transcriptional changes of Fos and Jun affect their binding activity and transactivation potential (Sonnenberg et al., 1989; Ryseck and Bravo, 1991; Hadman et al., 1993).

In the central nervous system, members of the Fos and Jun family are implicated in different processes (Wilkinson et al., 1989; Sheng and Greenberg, 1990; Mellstrom et al., 1991; Morgan and Curran, 1991;...
Sprague–Dawley adult rats of 250–300 g received an intraperitoneal injection of KA (9 mg/kg body weight dissolved as 9 mg/ml in saline solution) (Sigma) or saline alone. Only rats exhibiting increased locomotor activity and rapid wet-dog shakes starting at about 30 min after injection of KA, followed by recurrent tonic-clonic convulsions during the first 5 h of observation, were selected. Another group of adult rats was stereotaxically injected, under tiobarbital (5 μg/g) (Sigma) anesthesia, in the left striatum with 1 μl of 34 nM of quinolinic acid (Sigma) or with saline alone at two different coordinates, as previously described (Ferrer et al., 1995). Finally, a third group of rats was injected in the lateral ventricle with 75 μg of colchicine (Sigma) in 10 μl of phosphate buffered saline (PBS) or with saline alone. Rats treated with KA and quinolinic acid were killed at 6, 12, 24 and 48 h, and 4 days after injection. Rats treated with colchicine were killed at 12, 24 and 48 hours after injection.

For morphological studies, the rats (n = 4 for each time-point) were anesthetized with diethyl ether and were perfused through the heart with saline followed by 4% paraformaldehyde in PBS. The brains were then removed from the skulls and immersed overnight in a similar fresh solution of fixative. The brains were cut into 40 μm thick slices on a freezing stage microtome. Sections were mounted on gelatin-coated slides and processed for paraffin embedding. Sections were stained with haematoxylin and eosin or processed for immunohistochemistry. For Western blot studies (n = 4 for each time-point), the rats were killed by decapitation. Immediately afterwards, the whole brain from rats for developmental studies, the hippocampus in those rats treated with KA or colchicine, and the striatum in those rats treated with quinolinic acid, were frozen in liquid nitrogen and stored at −80°C until use.

Animal welfare was conducted according to the Real Decreto 223:1988, which makes recommendations similar to those of the NIH report: Public Health Service Policy on the Human Care and Use of Laboratory Animals.

2.2. Immunohistochemistry

Immunohistochemistry was carried out following the avidin–biotin–peroxidase method (ABC kit, Vectorstain, Vector Laboratories). After blocking endogenous peroxidase with hydrogen peroxide and methanol, the sections were incubated with normal serum and then incubated at 4°C overnight with one of the primary rabbit antibodies. The antibodies to Fra-1 (sc-183, Santa Cruz Biotechnology), glial fibrillary acidic protein (GFAP) (Sigma), OX-42 and OX-6 (Serotec) were utilized at 1/500. After that, the sections were incubated for 1 h with biotinylated anti-rabbit IgG antibody (Vector) diluted 1/100, and finally with ABC at a dilution of 1/100 for 1 h at room temperature. The peroxidase reaction was visualized with 0.005% diaminobenzidine (Sigma) and 0.01% hydrogen peroxide. Samples of control and treated rats were processed in parallel to eliminate day-to-day variations in the immunostaining procedure. Double-labeling immunohistochemistry was conducted by incubating the sections following a two-step protocol. Briefly, the sections were first incubated with one of the primary antibodies, and...
the immunoreaction was visualized with diaminobenzidine and hydrogen peroxide. Subsequently, the sections were incubated with the second primary antibody, and the immunoreaction was visualized with 0.01% benzidine dihydrochloride, 0.025% sodium nitroferricyanide in 0.01% sodium phosphate buffer (pH 6.0), and 0.005% hydrogen peroxide. The first primary antibody was recognized by a brown homogeneous precipitate, whereas the second primary antibody was recognized by a dark blue granular precipitate. Specific immunoreaction controls in double-labeled sections included the omission of one or both antibodies and the inversion of the sequential order of these antibodies. The specificity of the immunoreaction for Fra-1 was tested by pre-incubating the antibody with the antigenic peptide (also from Santa Cruz Biotechnology).

2.3. Gel electrophoresis and Western blotting

The whole brains or dissected regions were homogenized with Hepes buffer containing 1 mM DTT, 0.5 mM phenylmethylsulfonil fluoride and 1 mg/ml of pepstatin, aprotinin and leupeptin (Sigma). The protein content was determined by the method of Bradford (1976). Sodium dodecyl-sulphate-polyacrilamide gel electrophoresis (10% and 12% SDS-PAGE) was carried out as described by Laemmli (1970) using a mini-protein system (Bio-Rad) with low range molecular weight standards (Bio-Rad). Equal amounts of protein (between 15 and 30 μg in different assays) were loaded in each lane with loading buffer containing 0.125 M Tris (pH 6.8), 20% glycerol, 10% mercaptoethanol and 0.002% bromophenol blue. The samples were heated at 95°C for 10 min prior to gel loading. Protein analyzed on gel electrophoresis was transferred to nitro-cellulose membranes (Amersham) using an electrophoretic transfer system (Trans-blot Semy-dry Transfer cell, Bio-Rad) at 40 mA for 1 h. The membranes were then washed with Tris–buffered saline solution (pH 7.4) and 0.1% Tween-20 (TTBS), and then blocked with TTBS containing 5% skimmed milk, for 30–60 min. Following this, the membranes were incubated at 4°C overnight with the same polyclonal antibody to Fra-1 used for immunohistochemistry at a dilution of 1/500, 1/1000 for tubulin (Sigma) after stripping of membranes, in TTBS containing 5% skimmed milk. After washing, the membranes were incubated with anti-rabbit IgG labeled with HRP (Amersham) diluted 1/1000 for 1 h at room temperature, washed again, and developed with the chemiluminescence ECL system (Amersham) followed by apposition of the membranes with autoradiographic films (Hyperfilm, ECL, Amersham). Control of protein content in each lane was evaluated by staining selected gels with Coomassie blue and membranes with Ponceau (Sigma), and with incubated the membranes whit antibodies for tubulin, mentioned above. The specificity of the immunoreaction was tested by pre-incubating the antibody Fra-1 with the antigenic peptide.

3. Results

3.1. Fra-1 in the developing and adult brain

The Fra-1 polyclonal antibody recognized a unique band at p35 in Western blots. This band was abolished after preincubation with the antigenic peptide. The intensity of the band was strong in rats aged 3 days and decreased with age, although a transient increase was observed in rats aged 21 days (Fig. 1(A)). The control of protein content was evaluated with antibodies to tubulin after stripping the same membranes (Fig. 1(B)).

In the developing forebrain, Fra-1 immunoreactivity was exclusively observed in nerve fibers, whereas no immunostaining was detected in the nuclei of neurons. At P3, intense immunoreactivity was present in nerve fibers of the main fiber tracts including fimbria/fornix, corpus callosum, anterior commissure, hippocampal mossy fibers, and molecular layer of the hippocampus, as well as nerve fibers in the neocortex, thalamus, caudate/putamen and, to a lesser degree, hypothalamus (Fig. 2). Fra-1 immunoreactivity decreased in all these regions excepting the hippocampus, mainly the molecular layer of the dentate gyrus and the mossy fiber layer, from day 21 onwards.

Fig. 1. Western blotting to Fra-1 (A) and tubulin (B), from whole rat brain homogenates at postnatal (P) days 3, 7, 12, 21, and adulthood. A shows a unique band at about 35 kDa whose intensity decreases with age, with a transient increase at postnatal day 21. No variations in the intensity of the bands through time is observed in B. Total protein per lane 15 μg.
Fig. 2. Fra-1 immunoreactivity in the developing rat brain at postnatal day 3 (P3) (A, B) and P7 (C, D). At P3, Fra-1 immunoreactivity is observed in the corpus callosum (CC), anterior commissure (AC), and fascicles of the striatum (ST), as well as in the mossy fibers layer (mf), hilus of the dentate gyrus (hi), stratum radiatum (rd) and molecular layer (mol) of the hippocampus. At P7, Fra-1 immunoreactivity is present in the molecular layer of the hippocampus (mol-h), molecular layer of the dentate gyrus (mol-dg), mossy fiber layer and stratum radiatum. S, septum; CA1, CA3: areas of hippocampus; DG: dentate gyrus; v: lateral ventricle. (A), bar = 500 μm; (B, C), bar = 200 μm; (D), bar = 100 μm.

3.2. Fra-1 immunoreactivity after injury in the adult brain

3.2.1. Colchicine administration
At 12 h post-injection, Fra-1 immunoreactivity increased in the mossy fibers of the hippocampus. Fra-1 immunoreactivity was also observed in the perikarya of granule cells of the dentate gyrus, lateral habenula, amygdala, layers IV–V of the neocortex, and ventral and medial thalamus (Fig. 3(A–E)). At 24 h after injection, Fra-1 immunoreactivity increased in these regions and appeared in scattered cells throughout the hippocampus. At this time, Fra-1 immunoreactivity was also increased in the cytoplasm of neurons in the superficial layers of the neocortex located at the site of the injection (Fig. 3(F–H)), whereas the immunostaining of the mossy fibers showed varicosities suggesting degeneration of these fibers secondary to the death of selective granule cells in the dentate gyrus. At 48 h, Fra-1 immunoreactivity decreased in all these regions, but a number of strong Fra-1-immunoreactive cell nuclei appeared in the hippocampus and thalamus (Fig. 3(I)). Double-labeling immunohistochemistry disclosed that these cells were astrocytes (see later).

3.2.2. Quinolinic acid
At 6–12 h after quinolinic acid injection, Fra-1 positive cell nuclei were observed in the white matter around the lesioned striatum (Fig. 4(A, B)). At 24 h, Fra-1 immunoreactivity was detected in a number of cell nuclei in the lesioned striatum, neighbouring white matter and superficial layers of the neocortex (Fig. 4(C, D)). Lack of immunoreactivity was observed at 4 days post-injection. In addition to cell nuclei, neurons of layers IV and V of the neocortex at the site of the injection showed Fra-1 immunoreactivity in their cytoplasm.

3.2.3. Kainic acid
At 12 and 24 h post-injection, Fra-1 immunoreactivity was observed in the cell nuclei of glial cells in the corpus callosum and hippocampal commissure. In these regions, some cells also showed cytoplasmic immunoreactivity. In addition to the cells in the white matter, Fra-1 immunoreactivity was found in certain cell nuclei in the hippocampus, entorhinal cortex, lateral thalamus and upper layers of the neocortex (Fig. 4(E)). At 48 h after KA administration, Fra-1 immunoreactivity was still present in cell nuclei of the hippocampus, entorhinal cortex and thalamus (Fig. 4(F)). At 4–7 days, Fra-1 immunoreactivity was restricted to a few cell nuclei in the entorhinal cortex.

3.2.4. Double-labeling immunohistochemistry
Double-labeling immunohistochemistry showed that Fra-1-immunoreactive nuclei colocalized GFAP im-
munostaining in all the paradigms of brain injury, thus indicating that the vast majority of Fra-1-immunoreactive glial cells were astrocytes (Fig. 5(A, B)). Very occasionally, microglial cells showed Fra-1 immunoreactivity, as revealed with double-labeling immunohistochemistry with Fra-1 and OX-42 antibodies (data not shown).

3.2.5. Western blotting

Western blots of the lesioned hippocampus of kainic acid-treated rats showed a band at p35, the intensity of which increased at 48 h following KA administration (Fig. 6(A)). Similarly, Western blots of the lesioned striatum in quinolinic-injected rats showed a band at p35 whose intensity increased at 48 h when compared
Fig. 4. Fra-1 immunoreactivity in the rat brain at 12 h (A, B) and 24 h (C, D) following intrastriatal injection of quinolinic acid, and at 24 h (E) and 48 h (F) following intraperitoneal KA injection. Fra-1 immunoreactivity is observed in the nuclei of probable glial cells in the white matter (A, B) and striatum (C, D) surrounding the area of quinolinic acid injection, as well as in the nuclei of reactive glial cells in the CA1 area (E) and entorhinal cortex (F) following KA injection. (A, C), bar in (C) = 50 μm; (B, D, E, F), bar in (F) = 25 μm.

with controls (Fig. 6(B)). Finally, Western blots of the hippocampus of control and colchicine-treated rats showed a band at p35, the intensity of which augmented in colchicine-treated rats at 48 h post-injection. In addition, a strong band just below the band of 35 kDa appeared in colchicine-treated samples (Fig. 6(C)). Both bands disappeared by incubating the antibody Fra-1 with the antigenic peptide, thus suggesting that both bands were Fra-1 (data not shown).

4. Discussion

The present results show that Fra-1 immunoreactivity is present in the main fiber tracts in the developing rat brain, whereas immunoreactivity is mainly restricted to the mossy fibers of the hippocampus in the adult brain. These observations suggest that Fra-1 is synthesized in neurons and then transported to nerve fibers. To prove this hypothesis, colchicine, a drug that binds to tubulin blocking axonal transport (Dahlstrom, 1968; Kreutzberg, 1969; Grafstein et al., 1970), was injected in adult rats. In addition, colchicine acts as a neurotoxin for certain neuronal populations, including granule cells of the dentate gyrus (Hansson, 1972; Goldschmidt and Steward, 1982). Administration of colchicine produces an accumulation of Fra-1 in the perikarya of neuronal cells and in degenerated mossy fibers. By using an antibody to Pan-Fos it has been possible to detect the presence of members of the Fos family in the cytoplasm of neurons during development and after KA injection (Alcantara and Greenough, 1993; Bing et al., 1996). However, the present results obtained with an antibody specific for Fra-1 suggest that the Fos-related immunoreactivity described in neuronal cell bodies and fibers is related to Fra-1. It is known that Fra-1 is capable of binding with DNA and dimerizing with many transcription factors, including members of the AP-1 and non AP-1 family (Cohen and Curran, 1988; Cohen et al., 1989; Mechta et al., 1997). In addition, Fra-1 may inhibit AP-1 complexes and antagonize the transcriptional effect of tumor promotion by different agents (Yoshioka et al., 1995). However, Fra-1 immunoreactivity is not detected in neuronal cell nuclei in both developing and adult brains. Furthermore, Fra-1 immunoreactivity is not
detected in the nuclei of neurons after brain injury. Therefore, it is difficult to explain how Fra-1 can work as a transcription factor in neurons. Yet the presence of Fra-1 in nerve fibers and its localization during development and after colchicine administration suggest a participation of Fra-1 in other roles than those restricted to the nucleus. The fact that Fra-1 is the only member of the Fos and Jun families that resists the proteolytic action of the cytoplasmatic enzyme calpains (Carillo et al., 1994) may be an important property to elicit some cytoplasmic function. The detection of a few unrelated transcription factors in synaptosomal preparations from the rodent brain (Kaltschmidt et al., 1993; Guerrini et al., 1995) has suggested a novel synapse-to-nucleus signalling system (Kaltschmidt et al., 1994). Whether Fra-1 should be included in this category remains unknown. Information about the presence or absence of Fra-1 at the presynaptic terminal is crucial to support this proposal.

Following colchicine, kainic acid or quinolinic acid administration to adult rats, strong Fra-1 immunoreactivity is observed in the nuclei of reactive astrocytes. Fra-1 immunoreactivity in glial cells is restricted to within and around the lesioned areas in these models after 24 h to 4 days, thus correlating with the early reactive astrocytic response to brain damage. It is worth mentioning that c-Fos is detected in glial cells in basal conditions and after different treatments in vivo and in vitro (Masood et al., 1993; Pechan et al., 1993; Bennett and Schwartz, 1994; Pennypacker et al., 1996). Jun B has also been detected in cultured microglial cells (Priller et al., 1995), whereas c-Jun is induced in glial cells of the developing brain and in glial cells of the gerbil brain following ischemic tolerant states (Mack et al., 1994; Kato et al., 1995; Ferrer et al., 1996). These observations suggest a role for AP-1 sequence in the glial response after brain lesions. Fra-1 in the nuclei of astrocytes may indicate a functional role for this transcription factor in stimulating different genes containing the AP-1 sequence in their promoter. In this line, it is noteworthy that GFAP and NGF, two genes that are expressed in astrocytes and are activated by different injuries, contain the AP-1 sequence (D’Nello and Heinrich, 1991; Sarid, 1991; Braselmam et al., 1992; Ma-
sood et al., 1993; Pennypacker et al., 1994; Yu et al., 1995). Therefore, it is possible that Fra-1 may be involved in the expression of different genes that participate in the astroglial response following brain damage.

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