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Research report

Focal brain injury induces multiple immediate early genes encoding zinc finger transcription factors

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Abstract

Focal brain injury is known to markedly induce the *fos* and *jun* families of immediate early genes (IEGs). This study employed *in situ* hybridization to examine the effects of focal brain injury, produced by the intracerebral injection of saline or ibotenic acid on the expression of IEGs encoding zinc finger transcription factors. Thirty minutes after the injections, NGFI-A, NGFI-B, NGFI-C, and *egr*-3 mRNAs were induced in dentate gyrus, hippocampal pyramidal cells, cerebral cortex, caudate-putamen and piriform cortex of the injured hemisphere. Nurr1 was induced in hippocampal pyramidal cells and dentate granule cells. After three hours the induction of NGFI-A, NGFI-B, NGFI-C and Nurr1 persisted in all brain regions except for the dentate granule cells. By six hours after injection mRNAs for most of the zinc finger genes had returned to control levels. However, the expression of *egr*-3 3 and 6 h after the injection was identical to that observed at 30 min after the injection and it was the only gene the expression of which persisted 6 h following the injections. Twenty-four hours after the injection, the expression of all five IEGs returned to control levels. In general, no gross differences in the IEG induction were observed between the animals injected with saline and ibotenic acid. Since these zinc finger genes were expressed in the same regions where *fos* and *jun* family members are induced by similar types of brain injury, it is suggested that these transcription factors may act in concert with Fos/Jun family members. These observations should be considered when studying the effect of invasive manipulations on IEG expression in the brain and add to the evidence for remote effects of focal brain injury.

Keywords: Hippocampus; Spreading depression; mRNA; NGFI-A; NGFI-B; NGFI-C; *egr*-3; Nurr1

1. Introduction

The *fos* and *jun* families of immediate early genes (IEGs) respond rapidly and transiently to various types of stimulation in the central nervous system. The Fos (Fos, FosB, Fra1 and Fra2) and Jun (Jun, JunB, JunD) family members form dimers which bind to specific DNA sequences and thereby regulate the expression of target genes [24]. Their expression in the brain has been used as markers of increased activity [30]. Expression of *fos* and *jun* genes can also be induced in the brain by various types of injury [6,7,8,32]. Dragunow et al. [6,7,8] demonstrated that focal brain injury caused by needle implantation and intracerebral saline injections leads to massive induction of the *fos/jun* genes in the injured hemisphere. The mechanism of IEG induction by penetrating brain injury may involve spreading depression and release of excitatory amino acids [32].

In contrast to the data describing the localization and induction of the *fos/jun* IEGs in the nervous system, relatively little is known about the IEGs encoding proteins containing zinc finger motifs. The zinc finger motif is a common DNA binding structure, and proteins containing zinc fingers are capable of binding to a variety of DNA sequences [16,27,29]. Some of

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these zinc finger genes are IEGs, i.e. their expression can be rapidly stimulated *in vitro* by for example growth factors and depolarizing agents without prior protein synthesis [4,18,20,26,33]. Furthermore, some of these genes have been shown to be expressed in the brain [4,10,18,20,26,33]. The best characterized IEG of this class is the nerve growth factor (NGF) inducible gene NGFI-A (also known as zif268, *egr*-1, *krox*-24 and TIS8). Originally cloned from NGF treated PC12 cells [23], it was found to be induced in the brain by diverse types of stimulation including stress [15,31], seizures [13,18] and focal brain injury [7]. NGFI-C was also cloned from NGF stimulated PC12 cells. NGFI-C is expressed in brain and is induced by metrazol induced seizures [3]. NGFI-B (also known as Nur77 and TIS1) was cloned from mouse 3T3 fibroblasts stimulated by growth factors [11]. It encodes an orphan receptor belonging to the steroid/thyroid receptor superfamily. Nurr1 (also known as RNR-1), isolated from a neonatal mouse brain cDNA library, also encodes an orphan receptor sharing 92% similarity with NGFI-B in its DNA binding domain [20], but unlike NGFI-B, Nurr1 is not induced in PC12 cells by NGF treatment. Nurr1 expression is greater in the brain than in any peripheral organ. Finally, the early growth response gene *egr*-3 was cloned from growth factor treated fibroblasts [26]. The Egr-3 protein activates transcription through a specific DNA sequence termed GSG (GCGGGGGCG), which is also recognized by NGFI-A and NGFI-C [2,3,26]. In contrast, NGFI-B recognizes a 6 base long sequence (AGGTCA), which is similiar to the estrogen and thyroid hormone receptor response element half-site [34,35]. Based on the strikingly similar DNA binding domain of Nurr1, it has been suggested to bind to a similiar, if not the same, DNA sequence [20].

In a previous study, the distribution of these zinc finger IEGs was mapped in the rat brain after capsaicin induced stress. A clear increase of NGFI-A and NGFI-B mRNAs was found in the hypothalamic paraventricular nucleus (PVN), whereas no change in the expression of NGFI-C, Nurr1 and *egr*-3 was observed in any of the brain areas studied [15]. To investigate whether these genes can be induced by brain injury, the distribution and induction of these genes after neuronal damage caused by the intracerebral injections of saline and the neurotoxic glutamate analogue ibotenic acid was studied using *in situ* hybridization and Northern blotting.

2. Materials and methods

2.1. Animal treatments

Thirty-six adult male Sprague–Dawley rats divided into 9 groups of 4 animals each were used for in situ hybridization. Three additional animals were used for Northern blotting. For in situ hybridization the rats were injected under chloral hydrate anesthesia (250 mg/kg) either with 10 μl of normal saline or with 10 μl of ibotenic acid (1 μg/μl in normal saline) into the right hippocampus-striatum (approximate coordinates bregma −3.5 mm, lateral 2.5 mm, ventral 4–7 mm according to the atlas of Paxinos and Watson [28]). The animals were killed by decapitation 30 min ($n = 4$ for saline injected animals and $n = 4$ for ibotenic acid injected animals), 3 h ($n = 4$ and 4), 6 h ($n = 4$ and 4) or 24 h ($n = 4$ and 4) after the injection. Four intact animals that had no surgery performed were used as controls.

2.2. In situ hybridization

After decapitation the brains were removed and frozen on dry ice. Four to five brains were frozen in the same chuck. Each chuck was composed from brains representing each time point after either saline or ibotenic acid injection. Four chucks contained also brain obtained from control animal. Fourteen micrometer thick coronal sections were cut with a Microm HM 500 cryostat at Bregma levels −2.0 to −4.5 and mounted onto Fisherbrand Superfrost/Plus slides (Fisher Scientific, Pittsburgh, USA). The location of the injections was verified from the sections. The sections were stored at −20°C. In situ hybridization was performed as previously described [5]. Four sections cut from each chuck were hybridized with each oligonucleotide probe. The oligonucleotide probes complementary to the mRNAs encoding rat NGFI-A (nucleotides 1666–1711 [23]), mouse NGFI-B (nucleotides 182–227 [11]), rat NGFI-C (nucleotides 418–461 [3]), mouse *egr*-3 (nucleotides 189–234 [26]) and mouse Nurr1 (nucleotides 733–777 [20]) were labelled at the 3'-end with [^{35}S]dATP (DuPont-NEN Research Products, Boston, USA) using terminal deoxynucleotidyltransferase (Amersham Int., Buckinghamshire, UK). The sections were air dried at room temperature and subsequently hybridized at 42°C for 18 h with a mixture of 4×SSC, 50% formamide, 1×Denhardt's solution, 1% sarcosyl, 0.02 M phosphate buffer (pH 7.0), 10% dextran sulphate, 500 μg/ml heat-denaturated salmon sperm DNA, 200 mM dithiothreitol and 1×10^7 cpm/ml of the labelled probe. After hybridization, the sections were washed 4 times for 15 min each in 1×SSC at 55°C and thereafter left to cool for 1–3 h at room temperature. The sections were then dipped in distilled water and subsequently in 50, 60 and 90% ethanol and air dried at room temperature. The sections were covered with Amersham β-max autoradiography film (Amersham Int.). After exposing the films 4 weeks for NGFI-A, NGFI-C and *egr*-3 and 6 weeks for NGFI-B and Nurr1 they were developed using LX24 developer and AL4 fixative (Kodak, Rochester, USA).

2.3. Northern blotting

Pieces of hippocampus and cortex obtained from intact rats and rats which had received bilateral intracerebral injections of saline 30 min before decapitation were homogenized with a polytron in a mixture of 6 M guanidium isothiocyanate, 8% 2-mercaptoethanol, 50 mM Tris-HCl and 10 mM EDTA. LiCl was added to the homogenate and nucleic acids were allowed to precipitate overnight at +4°C. The samples were then centrifuged at 12,000 rpm at +4°C for 90 min and resuspended in a solution consisting of 4 M urea, 2 M LiCl and 1 mM EDTA. After centrifugation at 12,000 rpm at +4°C for 90 min, the precipitate was incubated with DNAse (0.2 U/μl; Promega, Madison, USA) and RNAsin (1 U/μl; Promega) at +37°C for 60 min and subsequently in proteinase K (0.2 g/l; Sigma) at +37°C for 60 min. Total RNA was extracted with phenol-chloroform. The RNA was precipitated with ethanol, dissolved in 1 mM EDTA and the optical densities were measured at 260 nm. Five μg of RNA for NGFI-A, NGFI-B, NGFI-C and *egr*-3 and 10 μg for Nurr1 was electrophoresed into 1.5% agarose gel containing 1×MOPS and 7% paraformaldehyde and thereafter transferred to a nylon membrane (Nytran, Sleicher and Schuell, Keene, NH) for 24–48 h. The RNA

was immobilized to the membrane with UV light at 254 nm. The membrane was stained with 0.02% Methylene blue and the position of the 18S and 28S bands were marked. The membrane was prehybridized at 37°C for 1–3 h with the same mixture used for in situ hybridization, except that the concentration of salmon sperm DNA was 250 μg/ml. The oligonucleotide probes used above were labelled at the 3'-end with [^{32}P]dATP (DuPont-NEN Research Products) using terminal deoxynucleotidyltransferase (Amersham Int.) and about 1.5×10^8 of the labelled probe was added to the hybridization solution. To verify that equal amounts of each RNA sample were loaded into the gel, [^{32}P]dATP-labelled oligonucleotide probe against cyclophilin was added to the hybridization mixture. The membranes were hybridized at +37°C overnight and thereafter washed at +37°C for 3 h with increasing stringencies with the maximum stringency of 0.2×SSC and 0.1% SDS. The membranes were then covered with Kodak SB5 autoradiograhic film for 12 h to 7 days and the film developed with Kodak GBX developer and fixative.

3. Results

In the intact rat brain, NGFI-A, NGFI-B, NGFI-C and *egr*-3 mRNAs were expressed in the cerebral cortex, layers CA1-CA3 of the hippocampus and caudate-putamen (Fig. 1). Nurr1, in contrast, was expressed

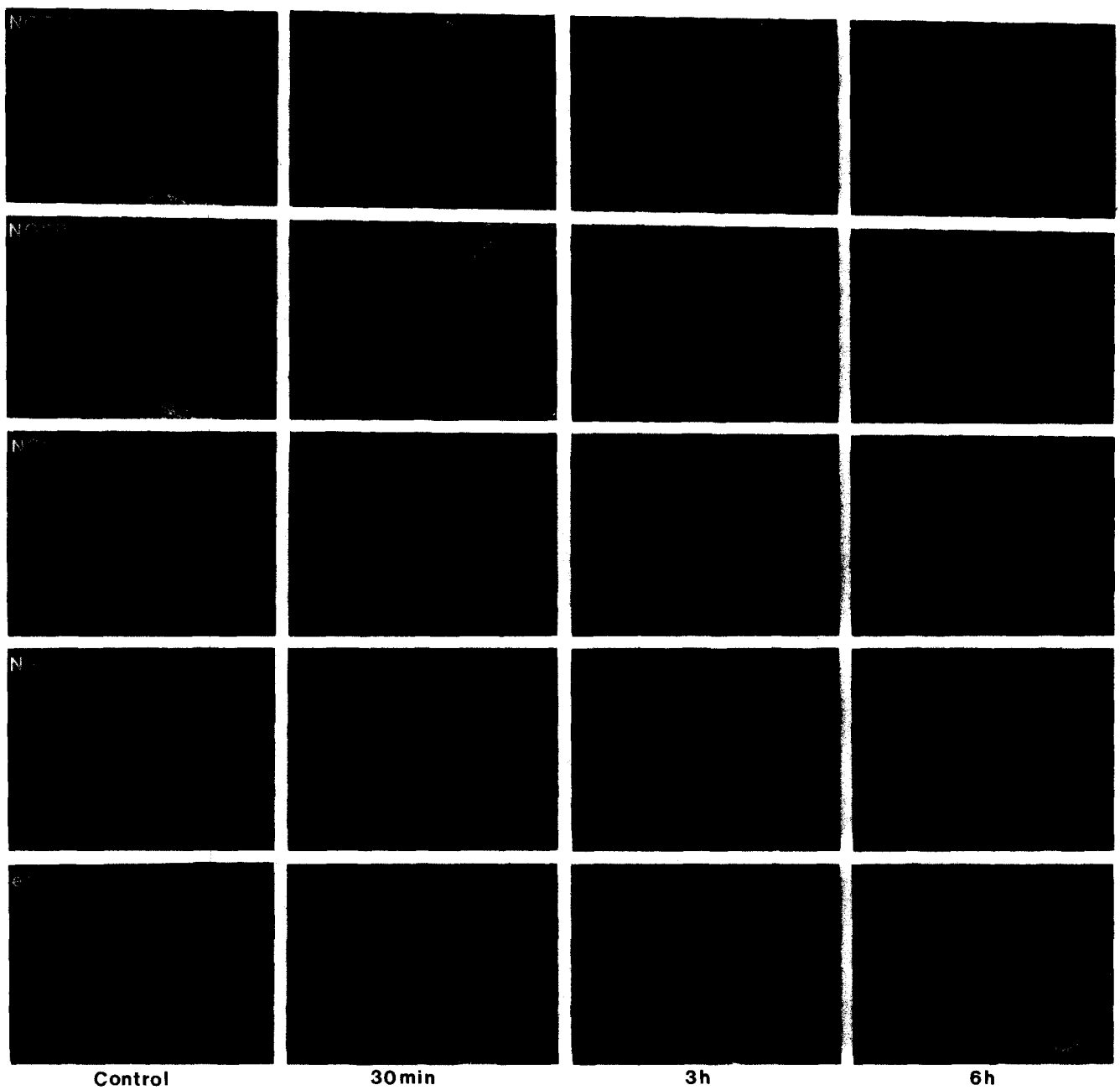

Fig. 1. Coronal sections of rat brain hybridized for NGFI-A, NGFI-B, NGFI-C, Nurr1 and *egr*-3 at indicated times after saline or ibotenic acid injection into the right hemisphere (the right side of each section in the figure). PVN, hypothalamic paraventricular nucleus; DG, dentate gyrus; CA1, CA1 pyramidal layer of hippocampus; M, meninges; CPu, caudate-putamen; Pir, piriform cortex; MHb, medial habenula; VI, layer VI of the cortex.

only in the medial habenula, layer 6 of the cortex and layers CA1-CA3 of the hippocampus. Thirty min after injection of saline into dorsal hippocampus, NGFI-A, NGFI-B, NGFI-C, *egr*-3 and Nurr1 mRNAs were substantially increased in several brain areas of the injured hemisphere. No significant changes in the IEG expression were observed in the contralateral hemisphere as compared with the expression in intact rat brain. The greatest increase for all five IEGs occurred in the dentate gyrus. NGFI-A, NGFI-B, NGFI-C and *egr*-3 expression also increased in cerebral cortex, caudate-putamen and piriform cortex. In hippocampus, NGFI-A and NGFI-B were slightly induced in CA1 pyramidal neurons, whereas the NGFI-C and Nurr1 mRNAs were modestly induced in CA1-CA3 pyramidal neurons. NGFI-A and NGFI-B were also induced in the PVN and in the meninges. Three hours after the injections, the levels of NGFI-A, NGFI-B and NGFI-C mRNA were still slightly elevated in hippocampal CA1-CA3 pyramidal cells, cerebral cortex, piriform cortex and caudate-putamen. Nurr1 expression remained elevated in hippocampal CA1, 2 and 3 pyramidal neurons. In the dentate gyrus the expression of NGFI-A, NGFI-B, NGFI-C and Nurr1 had returned to control levels by 3 h. Six hours after the injection, the expression of NGFI-A, NGFI-B, NGFI-C and Nurr1 in all brain regions was similar similar to that in the-intact animals. In contrast, *egr*-3 continued to be expressed in the cerebral cortex, piriform cortex, caudate-putamen and dentate granule cells for 6 h and returned to control levels 24 h after the injection (data not shown). The patterns of gene expression at each of the time points ($n = 4$ for each time) were virtually identical for all of the saline injected animals in the group. However, in one animal killed 24 h after saline injection there was increased NGFI-A and *egr*-3 expression in cortex and caudate-putamen and increased NGFI-C expression in cortex (data not shown).

Hippocampal ibotenic acid injections generally induced the IEGs in the same patterns and with the same time course as subjects injected with saline. There were two exceptions, however. In one animal there was marked bilateral induction of NGFI-A, NGFI-B, NGFI-C, Nurr1 and *egr*-3 (data not shown) in the dentate gyrus 30 min after ibotenic acid injection. In another animal, there was massive induction of NGFI-A and NGFI-B in the cortex and NGFI-C and *egr*-3 (data not shown) in the cerebral cortex, piriform cortex and caudate-putamen 6 h after the ibotenic acid injection. This cortical induction was significantly higher than the cortical expression in any of the other animals at any of the time points studied.

The specificity of the oligonucleotide probes was confirmed by Northern blot analysis. Total RNA ob-

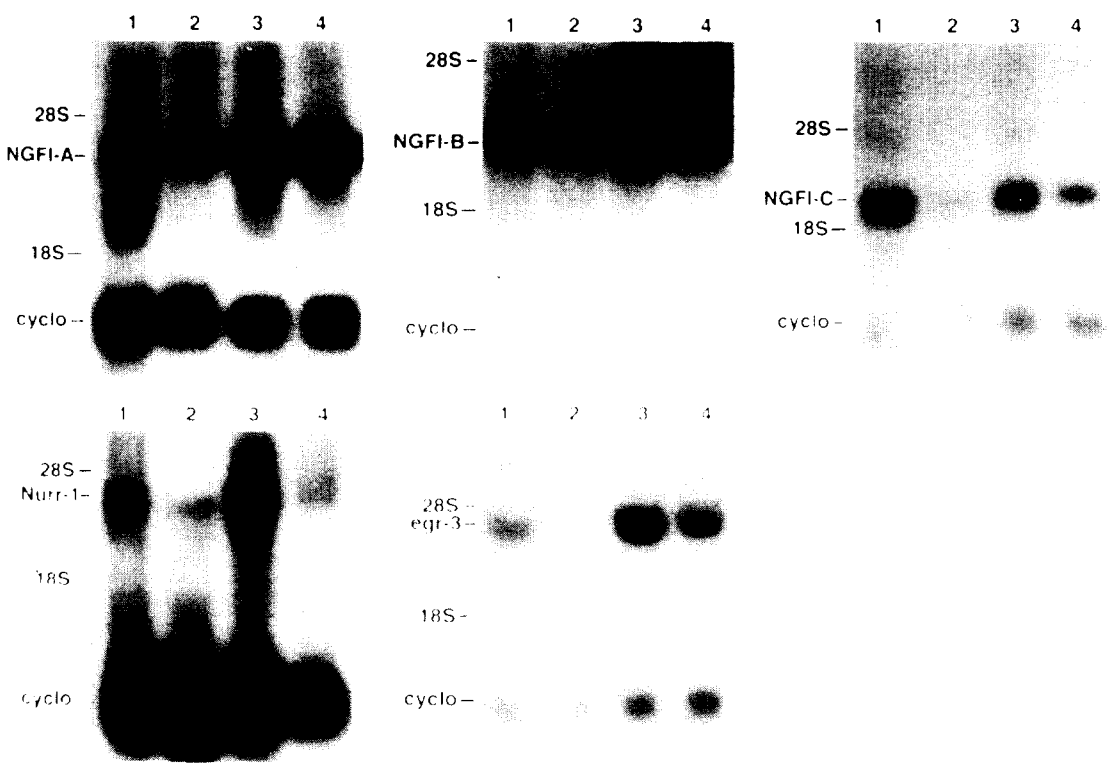

Fig. 2. Northern blots of NGFI-A, NGFI-B, NGFI-C, Nurr1 and *egr*-3 from untreated rat cortex (lane 1) and hippocampus (lane 2) and from cortex (lane 3) and hippocampus (lane 4) obtained from an animal which received an intracerebral injection of saline 30 min before sacrifice. Five (NGFI-A, NGFI-B, NGFI-C and *egr-3)* or 10 (Nurr1) μg of total RNA was loaded in each lane. Only a single band for each probe can be seen. Cyclo, cyclophilin.

tained from hippocampus and cortex of intact rats and of rats killed 30 min after being injected with physiologic saline were hybridized with the same probes which were used for in situ hybridization. Single bands of expected molecular size were seen in hippocampal and cortical samples obtained from control and saline injected rats (Fig. 2).

4. Discussion

Intracerebral injections of saline or ibotenic acid markedly induced the five zinc finger IEGs examined in this study. The mRNAs encoded by these genes were induced in cerebral cortex, piriform cortex, caudate-putamen and hippocampus of the injured hemisphere, with the greatest increases occurring in the dentate gyrus. No changes in the IEG mRNA levels were observed in the contralateral hemisphere. This indicates that the IEG induction was caused by the injury and not by the stress caused by animal handling, anesthesia etc. Furthermore, these results demonstrate that focal brain injury, unlike stress [15], is capable of inducing all of these zinc finger genes in the brain. The results of the present study extend previous findings by Dragunow et al. [7] demonstrating the inducibility of NGFI-A and certain members of the *fos / jun* family in the brain after focal brain injury.

With the exception of Nurr1, the localization of these zinc finger IEGs in normal rat brain was strikingly similar: low to moderate mRNA levels were observed in the hippocampus, cerebral cortex and caudate-putamen. The induction of NGFI-A, NGFI-B, NGFI-C and *egr*-3 caused by focal injury occurred in these same brain areas. This suggests that the protein products of these genes may act in concert with each other and with the members of the Fos and Jun family. However, there were some significant differences in the time course of induction and localization of these IEG mRNAs. First, the localization of Nurr1 in intact brain was clearly different from the other genes. Furthermore, the induction of Nurr1 appeared to be specific to hippocampus in contrast to the hippocampal and cortico-striatal induction of NGFI-A, NGFI-B, NGFI-C and *egr*-3. Second, the rapid and massive induction of NGFI-A, NGFI-B, NGFI-C and Nurr1 in the dentate gyrus disappeared by 3 h after the injection, whereas *egr*-3 mRNA continued to be expressed for 6 h in dentate. *egr*-3 was also induced for 6 h in cerebral cortex, piriform cortex and caudate-putamen, at a time when NGFI-A, NGFI-B, NGFI-C and Nurr1 mRNAs had returned to basal levels. This sequential expression of genes encoding transcription factors may in turn lead to a sequential expression or repression of target genes at different time points. Thus, Egr-3 may serve as a late response transcription factor affecting the transcription of late response target genes. Interestingly, the binding sequence of Egr-3 is the same as that of NGFI-A and NGFI-C. Therefore, one role of Egr-3 may be to regulate the same target genes as NGFI-A and NGFI-C, except that Egr-3 would be affecting the same target genes at later times when expression of NGFI-A and NGFI-C had returned to control levels. The finding that the induction of Nurr1 is restricted to the hippocampus suggests that there are specific zinc finger genes that might have hippocampal specific target genes.

A significant induction of NGFI-A and NGFI-B, but not of NGFI-C, *egr*-3 and Nurr1, was also seen in the PVN. This may reflect the stress produced by the surgery and intracranial injections in these animals, and is consistent with our previous studies demonstrating that stress induces NGFI-A and NGFI-B, but not NGFI-C, Nurr1 or *egr*-3, in the PVN [15]. NGFI-A and NGFI-B mRNA levels were also increased in the meninges, which might be due to the effects of stress, release of factors from the damaged neuronal and glial cells, from bleeding into the subarachnoid space, or other unknown factors.

The target genes of the transcription factors being studied are largely unknown. One set of genes might be members of the NGF family of neurotrophic factors. Ballarín et al. [1] showed that focal brain injury produced by inserting a needle into the brain and intracerebral saline injection induce NGF and brain derived growth factor (BDNF) in dentate granule cells and piriform cortex 1 h after the injury. This increase is preceded by a massive induction of c-*fos* in the same area suggesting a role of c-*fos* in stimulating the transcription of NGF and BDNF. Indeed, the first intron of the NGF gene contains an active AP-1 site recognized by Fos/Jun dimers, through which Fos is capable of stimulating the transcription of NGF [12]. In the present study we demonstrate a substantial increase of zinc finger containing transcription factors in dentate granule cells and piriform cortex 30 min after intracerebral injection. These zinc finger transcription factors could also participate in the induction of NGF and BDNF. It is not known whether the NGF and BDNF genes contain the cis-acting sequences recognized by the zinc finger transcription factors. However, even the absence of such sequences does not necessarily indicate that these transcription factors are not capable of regulating the transcription of these genes, since other mechanisms including protein-protein interactions between different transcription factors, may be involved. This type of interaction has been demonstrated between Fos/Jun proteins and the glucocorticoid receptor. The glucocorticoid receptor is a zinc finger containing transcription factor belonging to the steroid/thyroid receptor superfamily [9], which is capable of interacting with the Fos-Jun dimers through the

zinc finger domain [17]. The localization of the members of the Fos/Jun families and the zinc finger IEGs in the same brain regions after focal brain injury opens up the possibility of the interactions between Fos/Jun family members and the zinc finger transcription factors in regulating NGF and/or BDNF transcription.

The key event in triggering the expression of these IEGs appears to be the trauma associated with insertion of the needle. In a subsequent series of experiments we have observed a similar induction after needle implantation only into the brain (Bontiempi et al. unpublished observations). These implants were performed with the guidance of a stereotaxic apparatus, which resulted in a more consistent localization of the lesion and time course of the IEG expression. Thus, the slight variations seen between the animals in the present study might be due to differences in location of injection and severity of injury. The widespread expression of IEGs may seriously interfere with studies that involve invasive manipulations of the brain, e.g. intracerebral and intracerebroventricular injections and electrode implantation. Lee and Bondy [21] have demonstrated that even craniotomy and meningeal disruption alone results in widespread induction of NGFI-A in ipsilateral cortex. Therefore, the results obtained after invasive manipulations of the brain have to be carefully compared with sham-operated controls or, alternatively, chronically implanted animals may have to be used.

Several types of brain insults including penetrating brain injury induce spreading depression in the brain. Spreading depression is initiated by an intense high frequency burst followed by a silent period after which the neurons gradually recover. In rats, the suppression of electrical activity spreads across the cortex within minutes. Spreading depression is characterized by disruption in ion homeostasis, recurrant waves of neuronal depolarization and endogenous release of neurotransmitters which further act on their specific receptors [19]. The resultant release of excitatory amino acids appears to play a central role in IEG expression, since N-methyl-D-aspartate (NMDA) receptor antagonists, such as MK-801, abolish spreading depression [22,25] and also abolish the cortical injury-caused induction of c-*fos* [6,14,32]. These previous c-*fos* results would suggest that prior administration of MK-801 might also block induction of these zinc finger IEGs, if the mechanism of induction is in fact related to the spreading depression.

We did not observe any difference in cortical or hippocampal IEG expression after intracerebral saline and ibotenic acid injections. This is consistent with the hypothesis that both types of injections initiated spreading depression which was responsible for the IEG induction. This would also account for IEG induction throughout dorsal and ventral hippocampus and cortex, in regions far away from the single injection sites. This also suggests that the increased neuronal death caused by the ibotenic acid injection does not significantly increase the induction caused by focal penetrating brain injury. However, two of the ibotenic injected animals did have more massive or prolonged IEG induction. This may be due to the variation in the injection site or suggest that ibotenic acid might have some additive effects on IEG induction in some subjects.

The present report demonstrates that trauma induces multiple IEGs encoding zinc finger proteins in the brain. All five IEGs were induced predominantly in the hippocampus, though there were some differences among the five genes in localization in other brain regions and in the time course of the induction. This IEG induction produced by mechanical trauma must be taken into account when studying the effect of acute invasive manipulations on IEG expression. These results extend the *cis*-acting DNA elements affected by focal brain injury from the widely studied AP-1 binding sequence to the less extensively characterized sequences recognized by the zinc finger transcription factors and thus they may widen the spectrum of target genes involved. The data might also suggest that there are zinc finger IEGs which are specifically induced in the hippocampus (Nurr1), and therefore perhaps hippocampal specific target genes.

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