ACTIVITY AND INJURY-DEPENDENT EXPRESSION OF INDUCIBLE TRANSCRIPTION FACTORS, GROWTH FACTORS AND APOPTOSIS-RELATED GENES WITHIN THE CENTRAL NERVOUS SYSTEM

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Abstract—This review primarily discusses work that has been performed in our laboratories and that of our direct collaborators and therefore does not represent an exhaustive review of the current literature. Our aim is to further discuss the role that gene expression plays in neuronal plasticity and pathology.

In the first part of this review we examine activity-dependent changes in the expression of inducible transcription factors (ITFs) and neurotrophins with long-term potentiation (LTP) and kindling. This work has identified particular ITFs (Krox-20 and Krox-24) and neurotrophin systems (particularly the brain-derived neurotrophic factor (BDNF)/tyrosine receptor kinase-B, Trk-B system) that may be involved in stabilizing long-lasting LTP (i.e. LTP3).

We also show that changes in the expression of other ITFs (Fos, Jun-D and Krox-20) and the BDNF/trkB neurotrophin system may play a central role in the development of hippocampal kindling, an animal model of human temporal lobe epilepsy.

In the next part of this review we examine changes in gene expression after neuronal injuries (ischemia, prolonged seizure activity and focal brain injury) and after nerve transection (axotomy). We identify apoptosis-related genes (p53, c-Jun, Bax) whose delayed expression selectively increases in degenerating neurons, further suggesting that some forms of neuronal death may involve apoptosis.

Moreover, since overexpression of the tumour-suppressor gene p53 induces apoptosis in a wide variety of dividing cell types we speculate that it may perform the same function in post-mitotic neurons following brain injuries.

Additionally, we show that neuronal injury is associated with rapid, transient, activity-dependent expression of neurotrophins (BDNF and activinA) in neurons, contrasting with a delayed and more persistent injury-induced expression of certain growth factors (IGF-1 and TGFβ) in glia. In this section we also describe results linking ITFs and neurotrophic factor expression. Firstly, we show that while BDNF and trkB are induced as immediate-early genes following injury, the injury-induced expression of activinA and trkC may be regulated by ITFs.

We also discuss whether loss of retrograde transport of neurotrophic factors such as nerve growth factor following nerve transection triggers the selective and prolonged expression of c-Jun in axotomized neurons and whether c-Jun is responsible for regeneration or degeneration of these axotomized neurons.

In the last section we further examine the role that gene expression may play in memory formation, epileptogenesis and neuronal degeneration, lastly speculating whether the expression of various growth factors after brain injury represents an endogenous neuroprotective response of the brain to injury.

Here we discuss our results which show that pharmacological enhancement of this response with exogenous application of IGF-1 or TGF-β reduces neuronal loss after brain injury. © 1999 Elsevier Science Ltd. All rights reserved

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ABBREVIATIONS

AD	Afterdischarge	ITF(s)	Inducible transcription factor(s)
ADX	Adrenalectomy	LSE	Limbic status epilepticus
ALS	Amyotrophic lateral sclerosis	LTD	Long-term depression
ATF-2	Activating transcription factor-2	LTP	Long-term potentiation
BDNF	Brain-derived neurotrophic factor	MK801	$(+)$-5-Methyl-10,11-dihydro-5*H*-di-
Cdk	Cyclin-dependent kinase		benzo[*a,d*]cycloheptene-5,1-imine maleate
CGRP	Calcitonin gene-related peptide	MPTP	*N*-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
CHS	Continuous hippocampal stimulation	MS	Multiple sclerosis
CHX	Cycloheximide	MSDB	Medial septal-diagonal band
CREB	Calcium/cyclic AMP-responsive element	NGF	Nerve growth factor
	binding protein	NMDA	*N*-Methyl-D-aspartate
CSF	Cerebrospinal fluid	NT-3	Neurotrophin-3
DRG	Dorsal root ganglion	NT-4/5	Neurotrophin-4/5
FFL	Fornix-fimbria lesion	PCD	Programmed cell death
Fra(s)	Fos-related antigen(s)	PD	Parkinson's disease
GH	Growth hormone	PNS	Peripheral nervous system
HD	Huntington's disease	Rb	Retinoblastoma
HI	Hypoxic-ischemic stroke	ROS	Reactive oxygen species
ICE	Interleukin-1β converting enzyme	SGP-2	Sulphated glycoprotein-2 (clusterin)
IEG(s)	Immediate-early gene(s)	TGF-β	Transforming growth factor-β
IGF-1	Insulin-like growth factor-1	trk	Tyrosine receptor kinase
IGFBP(s)	Insulin-like growth factor binding protein(s)	TUNEL	Terminal deoxynucleotidyl-transferase
ISNT	*In situ* nick translation		end-labelling

1. INTRODUCTION

In this review we will discuss the role of gene expression in neuronal functioning and discuss the specific role of various genes whose expression we have examined in detail. Over the past 20 years accumulating research suggests that alterations in the genetic program of neurons underlies the brain changes responsible for long-term potentiation (LTP), kindling, learning and memory, some forms of neuronal death especially in development (i.e. programmed cell death, PCD, which may or may not occur by apoptosis) and the response/recovery of the brain to injury. Most of this evidence comes from animal studies examining the effects of inhibitors of transcription and translation in animal models of learning and memory [i.e. LTP, Otani *et al.* (1989); Goelet *et al.* (1986); Nguyen *et al.* (1994); Huang *et al.* (1994)], models of epileptogenesis [i.e. kindling, Jonec and Wasterlain (1979)], brain injury [i.e. hypoxic-ischemic stroke (HI), Goto *et al.* (1990); Shigeno *et al.* (1990); Papas *et al.* (1992)] *in*

vitro neurodegeneration (Shah *et al.*, 1997; Martin *et al.*, 1988; Serghini *et al.*, 1994), axotomy (Lopez-Mascaraque and Price, 1997) and in neural development (Oppenheim *et al.*, 1990). Further evidence comes from research that has carefully characterized the expression of various genes in these models in detail. It has therefore become clear that gene expression is likely very important for both plastic and pathological processes within the nervous system. Understanding the nature of the changes in gene expression that are responsible for plastic and pathological events might allow us to produce treatments for diverse brain/nervous system diseases and disorders including dementia, Alzheimer's disease, Multiple sclerosis (MS), Parkinson's disease (PD), HI, Huntington's disease (HD) and amyolateral sclerosis (ALS). In this review we will attempt to provide a broad introduction to our field which involves examining the role of neuronal gene expression in brain functioning.

2. A HIERARCHY OF GENE EXPRESSION EXISTS WITHIN THE CENTRAL NERVOUS SYSTEM WITH INDUCIBLE TRANSCRIPTION FACTOR EXPRESSION CENTRAL TO STIMULUS-RESPONSE COUPLING

Neurons are described as being plastic because they may respond to changes in their environment with either short or long-term changes in their phenotype. The earliest genetic response, or first wave of gene expression, usually involves increased transcription and translation of the family of immediate-early gene (IEG) transcription factors, whose prototypic members, Fos and Jun, are expressed in the nucleus of neurons (Dragunow et al., 1987). By definition, IEGs represent the first wave of gene expression in a cell in response to an environmental stimulus (be it a growth factor, neurotransmitter or other receptor stimulating substance) since their induction occurs in a protein synthesis-independent manner. That is, no other transcription factor proteins need to be transcribed/translated for the expression of the IEG to occur suggesting that the transcription factors that activate ITF induction are already (constitutively) expressed within the cell (see below). Not all IEGs are transcription factors however, and for reasons of clarity IEG transcription factors such as Fos and Jun are now generally referred to as inducible transcription factors [ITFs, as suggested by Gass and Herdegen (1995)]. Generally, these ITFs are not expressed by neurons in the basal state [although see Hughes et al. (1992)] in contrast to constitutively expressed transcription factors such as calcium/cyclic AMP responsive-element binding protein (CREB) and activating transcription factor-2 (ATF-2) whose activation via post-translational modification (usually phosphorylation) precedes and then initiates the increased transcription of these ITF genes.

Once initiated the expression of ITF genes usually occurs rapidly and transiently. For example, it has been shown that pharmacological activation of central cholinergic receptors with the muscarinic agonist pilocarpine will induce transcription of c-fos mRNA and detectable translation to Fos protein in both hippocampal and neocortical neurons within 30 min of the drug being injected intraperitoneally (see Fig. 1). However, after 8 hr of the drug being injected the expression of Fos had declined to below detectable levels (Hughes and Dragunow, 1993, 1994). It is thought that this induction profile (induced rapidly but only transiently) fulfils the necessary requirements of an efficient stimulus-response mechanism. Once they have been induced it is believed that these ITFs then go on to regulate a further, delayed, but usually more prolonged expression of effector genes (the second wave of gene expression) by interacting with the AP-1 site in the regulatory regions of these genes [see Hughes and Dragunow (1995a)]. The specific effector genes induced will depend upon the combinations of ITFs expressed within the neuron. For example, pilocarpine induces the ITFs Fos and Jun-B but not c-Jun in neurons (Hughes and Dragunow, 1994) similar to dopamine receptor antagonist drugs such as haloperidol (MacGibbon et al., 1995). In this way the combinatorial complexity of the ITF response allows specific regulation of effector gene expression.

Simplistically we can define at least four distinct phases in neuronal stimulus-transcriptional coupling.

1. Activation of a neurotransmitter or growth factor receptor leads to:
2. posttranslational modification (i.e. phosphorylation) of constitutively expressed transcription factors such as CREB (within seconds to minutes) which then
3. rapidly activate de novo transcription and translation of ITFs such as Fos and Jun (within minutes to hours).
4. The transient expression of these ITFs then regulates the more persistent (perhaps permanent) and delayed expression of ITF target genes.

The expression of these target genes which may encode structural proteins, enzymes, ion channels or neurotransmitters then results in long-lasting or permanent changes in morphology, structure and function of the nervous tissue. For a more detailed review of this topic see Hughes and Dragunow (1995a) and also refer to Table 1.
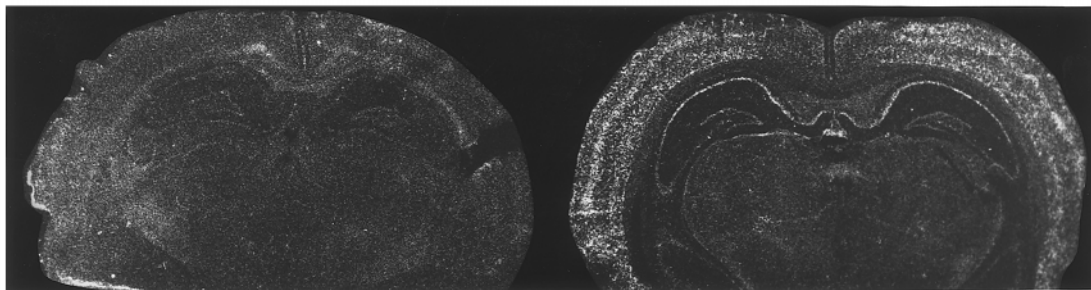
Fig. 1. Expression of c-fos mRNA in rat brain induced by systemic injection of the cholinergic agonist, pilocarpine (25 mg kg^{-1} i.p.) 30 min following drug injection. Left, c-fos mRNA expression in brain of vehicle injected rat; right, c-fos mRNA expression in brain of pilocarpine injected rat. Following injection of pilocarpine expression of c-fos mRNA is strongest in neocortical layers 2, 4, 5 and 6 and in the CA1 pyramidal cell layer of the hippocampus. c-fos mRNA was detected using a commercially available 40-mer antisense oligonucleotide probe directed against the c-fos gene [Oncogene Science Cat#353, see Hughes and Dragunow (1994)].

Table 1. Characteristics and grouping of the various transcription factors mentioned in this review (ITF = inducible transcription factor)

TF gene	Characterization	Related genes
c-fos	ITF; Component of the AP-1 transcription factor complex. AP-1 binds at the ATGACTCA site	Fos-B, Fras
c-jun	ITF; with Fos forms AP-1	Jun-B, Jun-D
nur-77	Ligand activated ITF; orphan receptor. A member of the steroid/thyroid hormone superfamily. May therefore act as a ligand-activated transcription factor. Binds to AAAAGGTCA site	
CREB	Constitutively expressed TF. Activated by post-translational modification, that is, phosphorylation of Ser residue. Binds to CRE site, TGACGTCA	CREM
Krox-24	ITF; zinc-finger transcription factor, binds to GCGTGGGGCG sequence	Krox-20

3. ACTIVITY-INDUCIBLE GENE EXPRESSION

3.1. Long-Term Potentiation and Long-Term Depression (LTD)

Brief episodes of high-frequency (but non-seizure-inducing) electrical stimulation of the perforant path input to the dentate gyrus results in a persistent increase, or LTP in the synaptic efficacy of this monosynaptic excitatory pathway (Bliss and Lomo, 1973). Other pathways in the hippocampus also show LTP, which is an activity-dependent strengthening of synaptic efficacy that may be involved in memory storage in the brain (Bliss and Collingridge, 1993). At least three types of LTP that can be distinguished on the basis of varying decay rates (Jeffery *et al.*, 1990; Abraham and Otani, 1991; Abraham *et al.*, 1993); LTP1, which decays with an average decay constant of about several hours; LTP2, with a decay constant of about several days; and LTP3, with a decay constant of about several weeks (Jeffery *et al.*, 1990). These various types of LTP can be generated in dentate gyrus neurons by administering progressively greater numbers of bursts of pulses (10–50 bursts) as described with the 10 burst paradigm producing LTP2 and the 50 burst paradigm producing the more durable LTP3 in our experiments (Jeffery *et al.*, 1990). Furthermore, anesthetizing rats with sodium pentobarbital prevents LTP3 expression following 50-burst stimulation and, instead, generates LTP2 (Jeffery *et al.*, 1990). Based upon previous pharmacological studies we believe that LTP3, but not LTP1 or 2, requires *de novo* protein synthesis and altered gene expression (Otani *et al.*, 1989).

Over the past few years studies have focused upon identifying which genes are involved in LTP1, LTP2 or LTP3. Initial studies in anaesthetized rats however failed to observe any increased expression of the IEG Fos in dentate gyrus neurons with LTP (Douglas *et al.*, 1988; Dragunow *et al.*, 1989a) in contrast to the robust bilateral expression found after a kindling afterdischarge (AD; see Fig. 2 and Dragunow and Robertson, 1987a). In contrast however, unilateral Krox-24 expression (and less reliable expression of Jun-B and c-Jun expression) was found by others to be associated with LTP in anaesthetized rats (Wisden *et al.*, 1990; Schreiber *et al.*, 1991; Cole *et al.*, 1989) suggesting that perhaps Krox-24 (and perhaps c-Jun and Jun-B also) but not Fos was involved in some way with the production/maintenance of LTP. These early exper-

iments were performed in anaesthetized rats however and we now know that anaethetics interfere with LTP3 production (Jeffery *et al.*, 1990). We therefore performed subsequent LTP experiments in awake rats. These experiments in awake rats now clarify that stimulation producing LTP reliably increases rapid (within 30 min) unilateral expression of Fos-like immunoreactivity but not of c-fos mRNA (Dragunow *et al.*, 1989a; Jeffery *et al.*, 1990), c-Jun, Jun-B and Jun-D mRNA and proteins, Fos-related antigen (Fra) but not Fos or Fos-B proteins (Demmer *et al.*, 1993) Krox-24 mRNA and Krox-20 and Krox-24 proteins in dentate gyrus neurons (Richardson *et al.*, 1992) with Krox-20 showing prolonged expression after LTP (Williams *et al.*, 1995). These results suggest that some ITFs, especially Krox-20 and Krox-24 are related to LTP mechanisms. The expression of ITFs does not appear to correlate with the amount of LTP induced, that is, LTP initiation, but rather with the stabilization, that is, duration/maintenance, of the LTP. In particular Krox-20 and Krox-24 expression after LTP correlates better with LTP stabilization than the expression of Fos or Jun transcription factor family members (Richardson *et al.*, 1992; Abraham *et al.*, 1993; Williams *et al.*, 1995). Specifically, we have demonstrated that stimulation that generates LTP3, but not LTP1 or LTP2, will produce a strong induction of Krox-20 and Krox-24 in dentate gyrus granule cells via *N*-methyl-D-aspartate (NMDA) receptor activation (Abraham *et al.*, 1993). Stimulation that produces LTP2 will weakly induce Krox-24, but not Krox-20 (or Fos and Jun ITFs). While Fos and Jun family members are induced after LTP3 production (Demmer *et al.*, 1993) their induction does not correlate as well as the Krox transcription factors with LTP stabilization. The phenomenon of LTD may be just as important in memory processing as LTP. Like LTP, LTD may persist for a long period of time (up to 3 weeks) depending on the inducing stimulus (Abraham *et al.*, 1994). Because of its potential importance we also examined ITF expression with LTD. The major finding of these experiments was that ITF expression, in particular Krox-24 was as well correlated with the persistence of heterosynaptic LTD as with LTP (Abraham *et al.*, 1994).

In addition to the ITFs we have investigated neurotrophin expression during LTP in awake rats. In these studies it is clear that brain-derived neurotrophic factor (BDNF) mRNA is increased unilater-
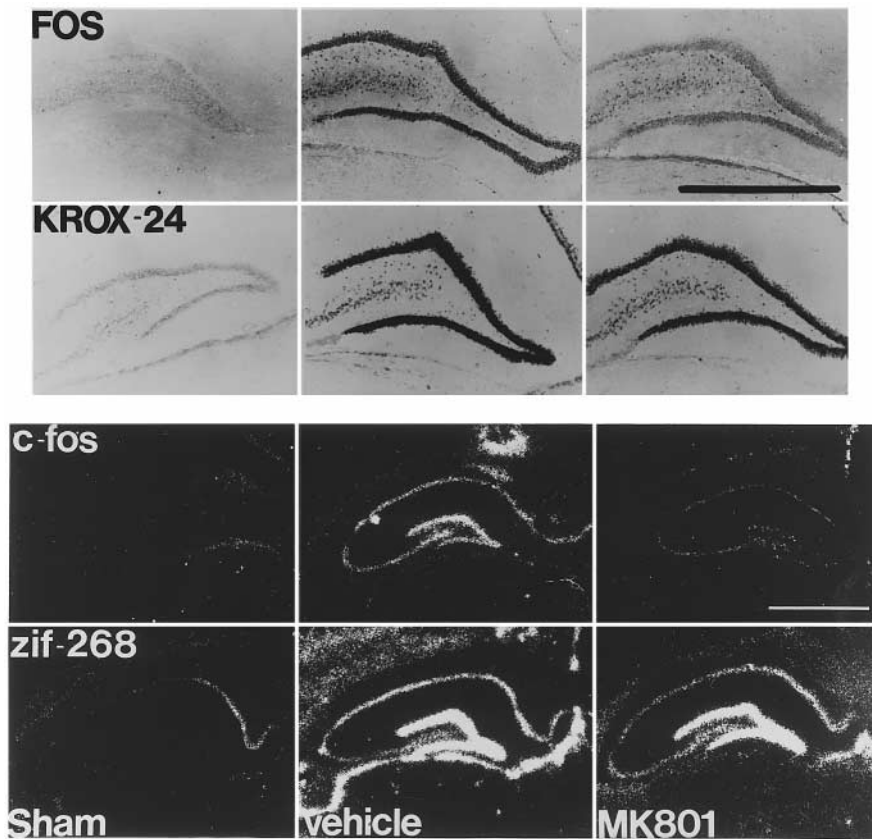
Fig. 2. Increased expression of the IEGs *c-fos* and *zif-268* (Krox-24) in dentate gyrus neurons 1 hr following a single kindling hippocampal afterdischarge and the effects of blockade of NMDA receptors with MK801. Top panel, expression of Fos and Krox-24 proteins by immunocytochemistry (see Hughes *et al.*, 1998) in Sham (column 1), 1 hr post AD rats (column 2) and in 1 hr post AD rats pretreated with MK801 (5 mg kg^{-1} i.p.) 30 min prior to electrical stimulation. Bar = 1000 μm. Lower panel, expression of *c-fos* and *zif268* (Krox-24) mRNA in Sham (column 1), 1 hr post AD rats (column 2) and in 1 hr post AD rats pretreated with MK801 (5 mg kg^{-1} i.p.) 30 min prior to electrical stimulation [see Hughes *et al.* (1998) for methods]. Bar = 1500 μm. Notice in both panels how MK801 differentially prevents the AD-induced expression of *c-fos* and *zif268* (Krox-24). Our results demonstrate clearly that MK801 significantly attenuates hippocampal kindling-induced expression of *c-fos* but not *zif268* (Krox-24) mRNA and protein in dentate gyrus neurons.

ally in dentate gyrus neurons with LTP of perforant-path dentate granule cell synapses. The expression of BDNF mRNA was first evident within 30 min of the 50 burst LTP3-inducing stimulus (but not with the 10 burst LTP2 producing stimulus), and remained elevated at 2 hr post-LTP induction (at maximal levels). By 4 hr however, BDNF mRNA levels had returned to baseline values (Dragunow *et al.*, 1993a). Further, BDNF mRNA expression could be blocked by pretreatment with the NMDA receptor antagonist, MK801, which also blocked LTP induction, and by sodium pentobarbital which shortens LTP3 persistence, suggesting that BDNF might participate in the NMDA-receptor mediated cascade of events that result in LTP3 stabilization. As yet it remains to be determined whether BDNF protein is also expressed during LTP induction. An additional report supporting our findings with BDNF demonstrates that both NGF and BDNF mRNA levels increase during LTP. In contrast to the increases seen for NGF and BDNF, these authors found decreased neurotrophin-3 (NT-

3) mRNA, and unaltered expression of neurotrophin-4/5 (NT-4/5) mRNA levels following LTP (Castren *et al.*, 1993). However, we have noticed that bilateral increases in both NGF and BDNF mRNA levels with LTP were seen in this study contrasting with the consistent unilateral expression of numerous ITF genes and now BDNF mRNA that we have observed in our LTP studies. The significance of this discrepancy remains unknown but may suggest the possibility of confounding seizure activity in their preparation (see Section 3.2).

Recently, we have also examined whether the expression of neurotrophin (trk) receptors is altered with 50-burst LTP (i.e. long lasting LTP3). While trkA mRNA (NGF receptor) expression remained unchanged (unpublished observations) the expression of both trkB (BDNF receptor) and trkC (NT-3 receptor) mRNAs were increased in dentate gyrus neurons (Dragunow *et al.*, 1997a). Increased trk mRNA expression was delayed with respect to ITF mRNA expression (which appears within 30 min) not appearing until 2 hr following stimu-

lation, and had declined to baseline levels by 4 hr post-tetanization. As with the ITFs and BDNF, the LTP-increased expression of trkB and trkC mRNAs was prevented by both MK801 and pentobarbitol. Further, 10-burst stimulation failed to increase the expression of trk mRNAs further supporting a role for neurotrophins and neurotrophin receptors in the maintenance of the durable form of LTP.

3.2. Kindling

Kindling is an animal model of seizure development or epileptogenesis, whereby periodic (i.e. once or twice daily) applications of an initially subconvulsive focal electrical stimulation eventually lead over a period of days or weeks to the formation of a generalized seizure. Focal electrical stimulation (usually to the amygdala or hippocampus, two brain areas which show strong kindling effects) results in a brief focal seizure called the AD. The AD is an absolute prerequisite for kindling to occur (Goddard, 1967; Goddard *et al.*, 1969; Racine, 1978). Like LTP, kindling appears to be a protein synthesis-dependent process (Cain, 1989; Dragunow *et al.*, 1989b; Jonec and Wasterlain, 1979; Perlin *et al.*, 1993) suggesting that the permanent chemical and/or neuronal circuitry changes [i.e. mossy fiber sprouting, Sutula *et al.* (1996)] that underlie kindling require the altered expression of existing proteins or the *de novo* synthesis of new 'kindling' proteins. Because of this we have examined kindling-induced changes in gene expression specifically associated with the kindling AD.

To study the effects of the kindling AD on gene expression rats are implanted unilaterally with a bipolar electrode into either the hippocampus or amygdala. At least 1 week following the surgery ADs are evoked by stimulating either brain region with a 2 sec biphasic electrical pulse (100 Hz, 2–500 μA). In the hippocampus the resulting AD consists of an initial burst of high frequency and amplitude spiking lasting from 10–30 sec followed by a period of post-ictal EEG silence which is terminated by a second wave of lower frequency spiking lasting from 10–30 sec. We have found that the hippocampal AD, in contrast to LTP stimulation, rapidly increases the bilateral expression of the ITF Fos in hippocampal dentate gyrus neurons (Dragunow and Robertson, 1987a). Fos expression was maximal within 30 min of the AD. Similarly, the expression of other ITFs (Fos B, Fras, c-Jun, Jun-B, Jun-D and Krox-24) increases rapidly (after 1 hr) but transiently (back to baseline levels within 24 hr) in dentate gyrus neurons following the AD [Dragunow *et al.* (1992a) and see Fig. 2]. In addition, a more selective and delayed (after 4 hr) expression of Fos, Jun-D and Krox-24 also occurs in hippocampal somatostatin and parvalbumin hilar interneurons (Dragunow *et al.*, 1992a). We found similar transient expression of ITFs (including Krox-20) within neurons of the amygdala after an amygdala AD (Dragunow *et al.*, 1988; Hughes *et al.*, 1994). In both paradigms ITF expression always returned to baseline levels after the transient, early and rapid rise in expression. It therefore seems unlikely that the long-lasting changes in gene expression that are responsible for the kindled state involve persistent ITF expression. Rather, since the ITFs can regulate the expression of other genes, it is likely that the expression of those genes [i.e. perhaps neuropeptideY, Marksteiner *et al.* (1990)], or perhaps genes even further downstream are responsible for the increased seizure sensitivity of the fully kindled brain. Candidate genes, that may have a role in mediating the synaptic remodelling that could be involved in kindling development (and perhaps LTP as described above) include the neurotrophins. Strong evidence suggests that NGF modulates the rate of kindling, since NGF antibodies attenuate kindling while NGF infusion into the brain will accelerate kindling progression (Adams *et al.*, 1997) while BDNF infusion induces long-lasting enhancement of synaptic transmission in the hippocampus (Kang and Schuman, 1995). Previous studies by other groups have shown that the expression of the neurotrophins increases during kindling (Ernfors *et al.*, 1991). Specifically, it has been shown that hippocampal kindling increases the expression of both NGF and BDNF but not NT-3 mRNA in dentate gyrus neurons. Further, we have also found transient increased levels of BDNF protein (see Fig. 3) and mRNA coding for the trkB and trkC neurotrophin receptors in dentate gyrus neurons following a hippocampal AD (Hughes *et al.*, 1998).

In addition to being dependent upon *de novo* protein-synthesis kindling is also dependent upon activation of the NMDA subtype of glutamate receptor since NMDA receptor antagonist drugs significantly impair the development of kindling without altering the generation of the AD (Gilbert, 1988; Gilbert and Mack, 1990; McNamara *et al.*, 1988; Morimoto *et al.*, 1991; Sato *et al.*, 1988; Vezzani *et al.*, 1988). The obvious question, therefore is whether the AD-induced expression of the above identified candidate 'kindling' genes is sensitive to NMDA receptor blockade. Unfortunately, until recently results have been inconclusive with no effect of NMDA receptor blockade on AD-induced neurotrophin expression (Ernfors *et al.*, 1991) and only partial blockade of Fos expression after the AD (Labiner *et al.*, 1989, 1993). Indeed we also showed conclusively that MK801 at a dose of 1 mg kg^{-1} i.p., 2 hr before amygdala kindling had no significant effect on the AD-induced expression of a number of ITFs (Hughes *et al.*, 1994). However, recent studies show that higher doses of MK801 may be required to totally prevent activation of the NMDA receptor. For example, it has been shown that while doses of MK801 of 1 mg kg^{-1} reduce NMDA-induced neurotoxicity by *ca* 70%, complete protection is not obtained until doses of 10 mg kg^{-1} MK801, are used (Lees, 1995). Indeed, we have found similar effects using MK801 at 1 vs 5 mg kg^{-1} i.p. to prevent focal hippocampal injury-induced expression of ITFs in dentate gyrus neurons, with 1 mg kg^{-1} only being partially effective, in contrast to almost complete blockade of ITF expression with the higher dose of 5 mg kg^{-1} i.p. [see Section 3.3 and Hughes *et al.* (1993a)]. For this reason we examined whether pretreatment (30 min) with MK801 at this dose would alter the AD-induced expression of any of the candidate
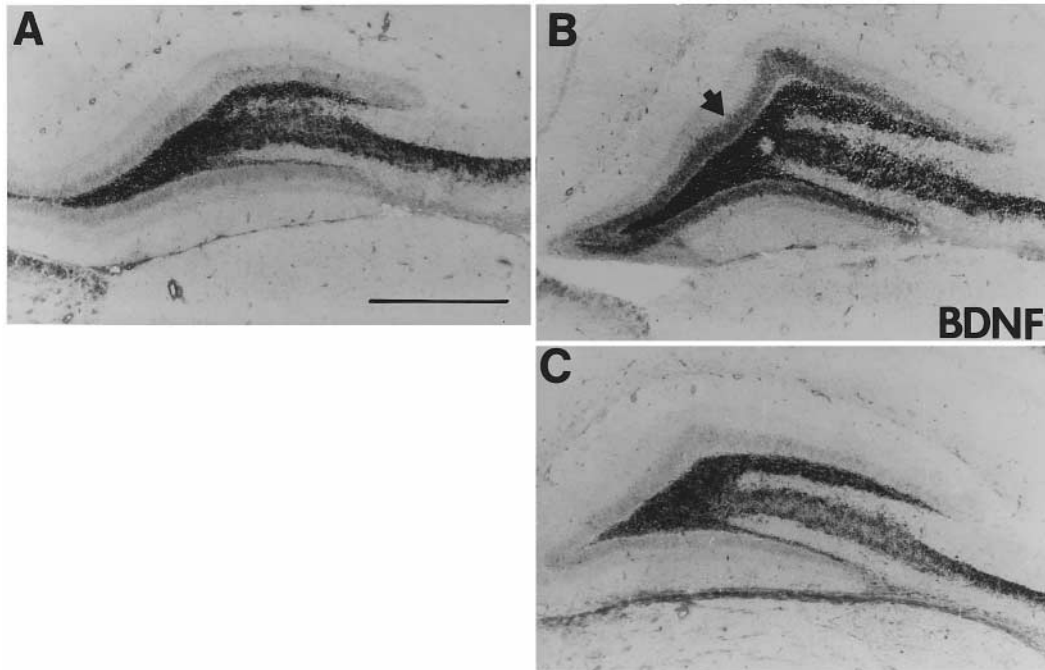
Fig. 3. Kindling AD-induced expression of BDNF protein in hippocampal dentate granule neurons at 4 hr is suppressed by MK801 pretreatment (5 mg kg^{-1} i.p. 30 min before stimulation) (A) BDNF protein as visualized by immunocytochemistry [see Hughes *et al.* (1998) for methods] was only basally expressed in the mossy fibre axons in sham operated rats. (B) 4 hr following a single hippocampal kindling AD, however, BDNF protein was substantially increased in dentate granule neurons (arrow). (C) The kindling-induced expression of BDNF protein was completely prevented by MK801 pretreatment. Bar = 750 μm.

'kindling' genes mentioned above. This recent study clearly shows that MK801 differentially regulates the expression of these genes (Hughes *et al.*, 1998). MK801 pretreatment produced a very strong inhibition of the AD-induced expression of Fos, Jun-D and Krox-20 in dentate gyrus neurons but had a much smaller effect on Jun-B and c-Jun expression. In contrast, MK801 did not significantly inhibit Krox-24 expression in granule cells or the delayed expression of Fos, Jun-D or Krox-24 in hilar interneurons (see Fig. 2). Significantly, MK801 abolished the delayed (at 4 hr) expression of BDNF protein in dentate gyrus neurons (Fig. 3), and the expression of mRNA coding for the BDNF receptor (trkB mRNA), but not trkC mRNA which is also expressed in dentate gyrus neurons following the AD (Hughes *et al.*, 1998). These results agree with those of Labiner *et al.*, showing that MK801 attenuates AD-induced Fos expression (Labiner *et al.*, 1989, 1993) but contrast with the earlier findings of Ernfors *et al.* showing no effect of MK801 on AD-induced expression of BDNF mRNA in granule cells (Ernfors *et al.*, 1991). The lack of effect of MK801 in the paper by Ernfors *et al.*, may be due to insufficient concentrations of MK801 being reached in the brain at the time of kindling since they used a short injection-test interval (10 min vs our 30 min) and previous studies have shown that MK801 only has antiepileptic effects evident after 30 min (Williamson and Lothman, 1989). This coupled with the low doses used (0.33 and 1 mg kg^{-1})

which may not entirely block NMDA receptors for the reasons mentioned, may explain the lack of effect of MK801 seen in their studies.

3.3. Neurotransmitter/Drug-Induced Gene Expression

Physiological activation of neurotransmitter receptors or drug-induced activation/antagonism of neurotransmitter receptors can induce changes in neuronal gene expression. The short and longer-term changes in gene expression induced by central nervous system (CNS) active compounds may underly both therapeutic and adverse effects associated with drugs use (i.e. drug tolerance, sensitization, dependence). Since we have previously reviewed this area [see Hughes and Dragunow (1995a)] and more recently other reviews and papers in this area have been published (Nestler, 1997; Nestler and Aghajanian, 1997; Hiroi and Graybiel, 1996; MacGibbon *et al.*, 1994, 1995) we shall not discuss it any further here.

4. INJURY-INDUCIBLE GENE EXPRESSION

4.1. Hypoxia/Ischemia

Much of our work on injury-related gene expression has been performed using two well characterized and reproducible models of perinatal (21 day Wistar rat) HI injury (Sirimanne *et al.*, 1994; Beilharz *et al.*, 1995a) modified from the original

Levine model (Levine, 1960). Modifications have been performed to increase consistency of neuronal loss and animal survival (Sirimanne et al., 1994). With these models 21 day old rats undergo permanent, unilateral right carotid artery ligation. They are subsequently transferred to an infant incubator at 34°C (relative humidity of $85 \pm 5\%$) for a 2 hr recovery period before being exposed to hypoxia of 8% oxygen in nitrogen for either 15 (moderate model) or 60–90 min (severe model). Rats exposed to 15 min of HI suffer no mortality and 90% develop selective neuronal loss in the frontoparietal cortex (pyramidal neurons of layers 4 and 5 in middle cerebral artery territory) and hippocampal CA1 region of the ipsilateral hemisphere over several days post-HI insult. The selective, delayed, neuronal death in this model may occur by apoptosis since neurons undergoing selective neuronal death have an apoptotic morphology and show non-random (oligonucleosomal, 180bp ladder) DNA fragmentation, using terminal deoxynucleotidyl-transferase end labelling (TUNEL) and gel electrophoresis of extracted DNA which peaks at 3–5 days post-HI (Beilharz et al., 1995a). Activated microglia identified using lectin histochemistry are associated with areas undergoing delayed neuronal death, 3–5 -days post-HI. In contrast, rats exposed to 60–90 -min of hypoxia suffer 5% mortality and consistently develop widespread severe cortical necrosis with further neuronal loss in striatum, thalamus, hippocampus and dentate gyrus of the ipsilateral hemisphere (Sirimanne et al., 1994). While DNA fragmentation is also seen in this severe model it is random at the early time-points after HI with electrophoresis of extracted DNA producing a smear with no clear laddering effects. At later time-points, however, that is, at 3 days post-HI some DNA laddering is seen perhaps suggesting a role for delayed apoptosis in this severe model of HI [Beilharz et al. (1995a) and see Fig. 4].

Thus these two models provide an opportunity to investigate gene expression associated with delayed, selective neuronal death which may occur by apoptosis (moderate model), or with early necrosis and perhaps delayed apoptosis (severe model). Our first experiment investigated the expression of the ITF Fos, after severe HI. Severe HI lead to a time-dependent induction of immunoreactive Fos expression in neurons but not glia or ependyma in the non-ligated hemisphere. Early induction in the non-ligated hemisphere was due to increased neuronal activity since it was prevented by anticonvulsant drugs and was only seen in animals that had seizures post-HI (Gunn et al., 1990). In the ligated hemisphere Fos protein was induced in glial-like cells in the corpus callosum, fornix-fimbria and internal capsule and in ependymal cells lining the lateral ventricle starting from 2 hr after HI but subsiding by 3 days. In contrast, no neuronal Fos expression was seen on the ligated side following HI. In later studies, ITF expression was investigated on the ligated side in the moderate model and we clearly found increased neuronal ITF expression in areas undergoing delayed death, but not necrosis, following HI (Dragunow et al., 1994). Moderate HI was associated with rapid (within hours) but transient ex-

pression of Fos, Fos-B, Jun-B, Jun-D, and c-Jun proteins and nur-77 mRNA (see Fig. 4) but not Krox-20 protein in neurons on the ligated side related to increased neuronal activity and selective, delayed (24–48 hr) expression of c-Jun and nur-77 mRNA (and to a lesser extent, Fos/Fras and Fos-B) in neurons that underwent delayed neuronal death (Fig. 4). Krox-24 in contrast showed an initial induction followed by a long-lasting suppression of its expression in regions undergoing cell loss in the ligated hemisphere. In support of our earlier results, severe HI, resulted in seizures and rapid neuronal loss and infarction (necrotic cell death) on the ligated side after HI, and was associated with early induction of all ITFs examined on the non-ligated side of the brain. In contrast, no neuronal ITF expression was seen on the ligated side. However we did find that expression of Fos, c-Jun, Jun-B, Jun-D and Krox-24, but not Krox-20 or Fos-B, was induced in non-nerve cells on the damaged side of the brain after both moderate and severe HI. Note that this ITF expression pattern in non-nerve cells after HI (i.e. all examined expressed not including Fos-B or Krox-20) is the same as the ITF pattern expressed in non-nerve cells after focal hippocampal injury [see below and Dragunow and Hughes (1993)]. Thus, these studies show that moderate HI induces rapid but transient (1–24 hr) ITF expression in neurons and glia in damaged regions on the ligated side with strong selective and prolonged (24–48 hr) expression of one ITF protein in particular, that is, c-Jun occurring in neurons undergoing delayed neuronal death. This suggests that c-Jun may play an important role in delayed neuronal death (Dragunow et al., 1993b) and this will be discussed in more depth later in this review. In addition whether or not nur-77, which has been associated with apoptosis in the immune system, and which is also selectively expressed in addition to c-Jun in neurons undergoing neuronal apoptosis, may play a role in neuronal apoptosis will also be discussed.

In contrast, while severe HI also induced rapid, transient ITF expression within glia on the ligated hemisphere, severe HI only induced ITF expression in neurons in the non-ligated hemisphere. Neuronal expression was due to increased neuronal activity, that is, post-HI seizure activity. In addition to these results with ITFs we find that the expression and phosphorylation state of the constitutively expressed TF (CREB) is altered with HI. Constitutively expressed CREB exists in both an active (phosphorylated, pCREB) and an inactive (dephosphorylated, CREB) form in neurons (Walton et al., 1996a). Using the moderate HI model, in areas of selective neuronal death we found a loss in the expression of both CREB and pCREB associated with apoptosis, for example in CA1 pyramidal neurons at 48–72 hr. This may simply be due to cell loss but could involve selective down regulation of expression since other ITFs (i.e. c-Jun) are continuing to be expressed at high levels in these same regions at similar times. In contrast, however, we found a biphasic increase in pCREB in dentate gyrus neurons that are selectively resistant to moderate HI and in neocortical neurons on the ligated side, the majority of which survive the insult with the first phase
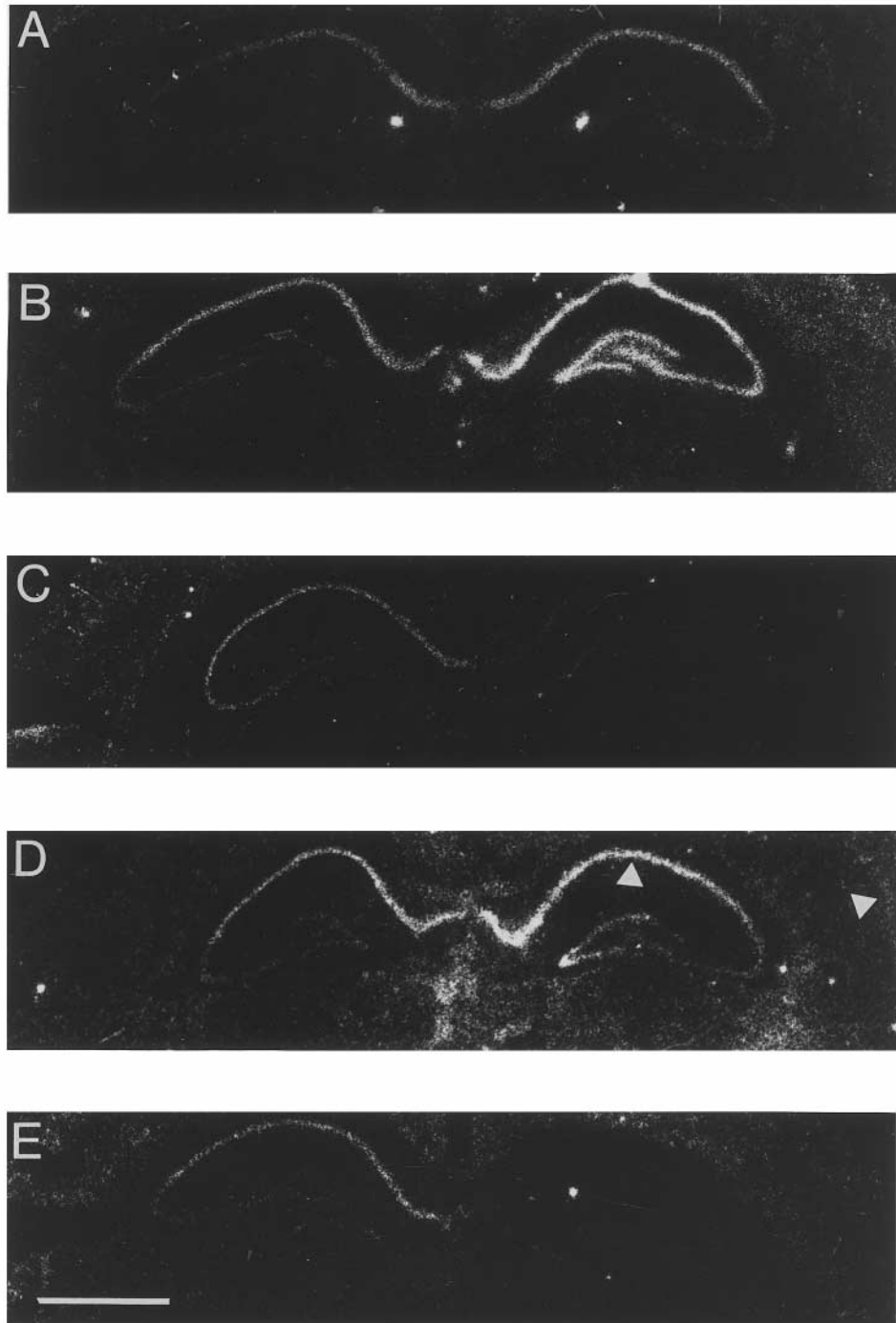
Fig. 4. Increased expression of *nur-77* mRNA after moderate (15 min) hypoxic-ischemic injury. Autoradiograms showing *nur-77* mRNA expression on the control (left) and injured (right) sides of the brain at (A) 0, (B) 3, (C) 12, (D) 48 and (E) 168 hr following the HI insult. Note the early seizure-induced increase of *nur-77* mRNA 3 hr after HI (compare A with B) and the delayed, injury-associated selective increase of *nur-77* mRNA in the CA1 pyramidal cell layer and layer 3–5 of the neocortex 48 hr following HI (D; solid arrow heads). Bar = 1750 μm.

of induction peaking at 6 hr and the delayed phase peaking at 48 hr following HI (Walton *et al.*, 1996a). This might support some neuroprotective function for pCREB in neurons after HI. It is also timely at this point to mention that we have found that the expression of the Redox activator of Fos and Jun, that is, Ref protein, which is found consti-tutively expressed in the nucleus of neurons and astrocytes within the nervous system (Dragunow, 1995) is also lost in CA1 regions following moderate

HI at time-points when strong coincident expression of c-Jun protein and nur-77 mRNA is clearly evident (Walton *et al.*, 1997a) perhaps suggesting selective down-regulation of expression.

It has been known for some time now that the injured brain produces neuronal growth promoting factors in a time-dependent manner in response to injury. This 'neurotrophic' activity peaks between 3 and 10 days following injury and is higher in the neonatal compared to the adult brain (Nieto-Sampedro *et al.*, 1982). It has been suggested that the production of these factor(s) might represent an endogenous neuroprotective response of the brain to limit further injury. To determine the identity of these factor(s) we have examined the expression of numerous growth factors after HI using our neonatal brain injury models.

While the neurotrophins (NGF, BDNF and NT-3) were obviously leading candidates, it became immediately clear in our studies that their expression was unlikely to be responsible for the delayed (3–10 days) increase in neurotrophic activity seen after brain injury. In our severe model of HI, the expression of the neurotrophin BDNF (and to a lesser extent NGF-β and NT-3) increased at early (3–24 hr) but not late (3–5 days) time-points in nerve cells of the non-ligated (uninjured) but not ligated (injured) hemisphere (Dragunow *et al.*, 1994; Beilharz *et al.*, unpublished observations) suggesting regulation by increased neuronal activity (seizures) but not neuronal injury.

In contrast to the activity-induced expression of neurotrophins in nerve cells on the non-ligated hemisphere, we found robust *and* delayed (3–5 days) injury-associated expression of insulin-like growth factors and various IGF-binding proteins (IGFBPs) in predominantly non-nerve cells of the ligated hemisphere following severe HI (Gluckman *et al.*, 1992; Beilharz *et al.*, 1995b; Klempt *et al.*, 1992a; Beilharz *et al.*, 1993). Using immunocytochemistry and *in situ* hybridization to detect IGF-I mRNA and protein we found increased expression of IGF-I mRNA in microglia and increased IGF-I immunoreactivity associated with astrocytes and microglia in the ligated hemisphere (lateral cortex, hippocampus, striatum, thalamus and pyriform cortex) 3–5 days following severe HI (Gluckman *et al.*, 1992). In contrast, no change was seen in the expression of IGF-I on the ligated hemisphere at earlier time-points (1–5 hr) or in the non-ligated hemisphere. The induced IGF-I mRNA was largely of the Ea form with only a slight induction of the Eb form. The Ea form is classically considered to represent the autocrine/paracrine form of IGF-I and is non-growth hormone (GH) responsive, while the Eb form represents the main GH-responsive endocrine form of IGF-I (LeRoith and Roberts, 1991).

We also examined whether expression of the IGF-I specific binding proteins (IGFBP-1 to IGFBP-6) was altered by HI. The IGF binding proteins are thought to modulate the availability of IGFs and may act as targeting factors. Our results show clear, increased expression of IGFBP-3 in the ligated hemisphere, 3–5 days following HI injury. The expression of IGFBP-3 mRNA was confined to the lateral cerebral cortex, striatum and dentate gyrus.

Combined immunohistochemistry and *in situ* hybridization suggests that some of the expression of IGFBP-3 is by microglia. In contrast we found no change in the low constitutive expression of IGFBP-1 mRNA after HI (Gluckman *et al.*, 1992). Subsequent experiments have shown increased expression of IGFBP-2 (Klempt *et al.*, 1992a; Klempt *et al.*, 1993), IGF-II (Beilharz *et al.*, 1995b) and IGFBP-5 but not IGFBP-4 in the brain following HI (Beilharz *et al.*, 1993). In contrast to the earlier and more widespread expression of IGF-I, IGF-II expression was not induced until 6–10 days following HI injury and expression was limited to the region of the cortical infarct (Beilharz *et al.*, 1995b). Immunohistochemistry demonstrated that IGF-II was expressed by microglia and accumulated on dividing astrocytes suggesting a role for IGF-II in glial proliferation and wound repair after injury (Beilharz *et al.*, 1995b). IGFBP-2 is markedly induced in similar regions to IGF-I with a similar time-course, primarily in reactive astrocytes particularly those situated adjacent to degenerating neurons (Klempt *et al.*, 1992a; Klempt *et al.*, 1993). While IGFBP-4 expression is suppressed on the ligated side, IGFBP-5 expression increases within white matter tracts and thalamic neurons but not until 6–10 days after injury (Beilharz *et al.*, 1993). Recent data (unpublished observations) suggests that expression of IGFBP-6 may increase following HI in blood borne neutrophils which enter the brain, in ependymal cells which line the lateral ventricles and in the choroid plexus.

Two related growth factors, transforming growth factor-β1 (Klempt *et al.*, 1992b) and activin (Lai *et al.*, 1996) are also strongly induced in the infant brain following HI. There are, however, important differences in the induction of these two related genes. While expression of TGF-β1 increases following both severe and moderate HI on the ligated side, activin expression is only increased after severe HI. However, after severe HI activin is expressed on both the ligated and non-ligated hemisphere in non-nerve and nerve cells respectively. As previously mentioned, moderate HI is associated with selective neuronal loss in the frontoparietal cortex (pyramidal neurons of layers 4 and 5 in middle cerebral artery territory) and hippocampal CA1 region of the ipsilateral hemisphere over several days post-HI insult. At 3–5 days following moderate HI, but not earlier, TGF-β1 mRNA expression was elevated in those regions undergoing selective neuronal death in the ligated hemisphere. In contrast, in the severe model, widespread expression of TGF-β1 was seen around the infarct at 3–5 days post-HI. However, TGF-β1 expression was also seen at earlier time-points (1–5 hr) in hippocampus, choroid plexus, striatum and cortex of the ligated side (Klempt *et al.*, 1992b). Like, TGF-β1, the related activin growth factor was induced by HI. In contrast to TGF-β1 however, activin expression was limited to the severe model, where its expression increased in both neurons of the non-ligated side (at early time-points 1–24 hr post HI) and in non-nerve cells of the ligated side [at later time-points of 3–5 days post-HI; Lai *et al.* (1996)]. Rapid induction in neurons (hippocampus, piriform cortex and amygdala) was due to increased

seizure activity (see previous sections) since expression could be prevented with anti-convulsants while the delayed induction in non-nerve cells (of meningeal membrane and microvessels in infarct region) was related to neuronal injury processes and could not be prevented by anti-convulsants (Lai *et al.*, 1996). Activin βA (βAβA) is the predominant form of activin induced by HI since the βA but not βB subunit was expressed. Delayed (3–5 days) co-incident limited expression of the inhibin α subunit after HI raises the possibility that inhibin ($\alpha\beta$A) might exist in the injured brain, albeit with a more limited distribution than activin (Lai *et al.*, 1996).

In addition to characterizing the expression of constitutive and ITFs and growth factors after HI we have also been able to show increased expression of neuropeptides [i.e. calcitonin gene-related peptide (CGRP), Dragunow *et al.* (1992b)], glycoproteins [i.e. sulphated glycoprotein-2 (SGP-2), Walton *et al.* (1996b)], phospholipid enzymes [prostaglandin H synthase-2 and phospholipase A$_2$, Walton *et al.* (1997b)] and phospholipid binding proteins [Annexin V, Walton *et al.* (1997c)] after HI in rats.

In summary, from these studies we believe that it is possible to identify at least three distinct phases of gene expression associated with HI which are preceded by changes in the phosphorylation states of various constitutively expressed TFs such as CREB (to active pCREB). The first phase of gene expression is the initial, rapid (within hours), activity-induced expression of genes in neurons of the non-ligated hemisphere due to seizure activity. These genes include most ITFs and the neurotrophins (NGF, BDNF and NT-3). The second phase of gene expression (within 24–72 hr) is the selective and delayed but prolonged expression of certain ITFs (i.e. c-Jun, nur-77 and p53, see Section 4.2) in neurons undergoing apoptosis and the expression of certain growth factors (IGF-I, TGF-β1) in reactive astrocytes and microglia surrounding these regions of neuronal death on the ligated side. The release of these factors may lead to either neuroprotection (rescue) or neurotoxicity (induction of apoptosis) depending upon the factor. The third most delayed phase of gene expression (5–10 days following HI) is associated with the non-nerve cell expression of other growth factors (i.e. IGF-II) by microglia and by dividing astrocytes which may play a role in glial proliferation/scar formation and wound repair after injury.

4.2. Prolonged Seizure Activity

In addition to using brief hippocampal seizures or ADs (see Section 4.1) to investigate plasticity-related gene expression during kindling, we have also developed seizure models which have allowed us to investigate changes in gene expression in response to prolonged seizure activity (i.e. status epilepticus, SE) and associated neuronal injury. The three main models are the MK801/pilocarpine model (Hughes *et al.*, 1993c), electrically-induced status epilepticus via continuous hippocampal stimulation [CHS, Young and Dragunow (1993); Young and Dragunow (1994)] and an MK801/kainic acid model (Dragunow and Preston, 1995). In the MK801/pilo-carpine model, MK801 (5 mg kg^{-1} i.p.) is injected into rats 30 min before one-tenth of a normally convulsant dose of pilocarpine (i.e. 25 mg kg^{-1} i.p.) is injected (Turski *et al.*, 1984). We had previously discovered that MK801 pretreatment would sensitize rats to pilocarpine induced seizure activity (Hughes *et al.*, 1993c) similarly to lithium (Jope *et al.*, 1986). This model produces limbic status epilepticus (LSE) lasting for up to 10 hr with no generalization of the seizure in most rats. Although seizure activity is severe no neuronal injury occurs because of the presence of MK801 (Hughes *et al.*, unpublished observations but see Fig. 5).

In the CHS model, rats are stimulated continuously within the hippocampus via a chronic stimulating electrode until continuous self-sustaining LSE develops. The stimulating electrode then can be used to record the duration of the LSE. In the CHS model, however, we terminated the LSE with sodium pentobarbital after 30 min so that all rats had a consistent amount of seizure activity (Dragunow and Preston, 1995). CHS resulting in 30 min of LSE is associated with reliable neuronal degeneration of CA1 and hilar but not dentate gyrus neurons within the hippocampus. In the MK801/kainic acid model rats were either injected with 1 or 5 mg kg^{-1} i.p. MK801 followed 30 min later with 10 mg kg^{-1} i.p. kainic acid. LSE lasting for hours is induced in both groups, however, in this model selective neuronal degeneration (as seen in the CHS model) is confined to the 1 mg kg^{-1} MK801 group with the 5 mg kg^{-1} MK801 group having prolonged LSE but no neuronal injury as seen in the MK801 pilocarpine model. We harvested brains from rats in these models at time-points ranging from 30 min to 6 days following the start of the seizure activity.

We have found that all three models are associated with a similar pattern of gene expression that is biphasic with the first wave (within hours) associated with increased neuronal (seizure) activity as characterized previously (Dragunow and Robertson, 1987b, 1988; Morgan *et al.*, 1987; Saffen *et al.*, 1988) and the second delayed (1–3 days) phase of gene expression being associated with neuronal injury. Firstly, in all three models we find early (30 min to 1 hr) and transient induction of all ITFs examined (Fos, c-Jun, Jun-B, Krox-20, Krox-24) in all hippocampal neuronal population (CA1/dentate gyrus/hilus) similar to the expression of ITFs seen in kindling. This early expression of ITFs lasts from 30 min to 12 hr. In contrast beginning within 12–24 hr of LSE we began to see more selective expression of ITFs in select regions of the hippocampus in only those models (MK801, 1 mg kg^{-1}/kainic acid and CHS) where selective neuronal degeneration occurs (Dragunow and Preston, 1995). We found increased expression of Fos, c-Jun and Jun-D at 24 hr in neuron destined to die (CA1 pyramidal and hilar neurons) but at 3 days when these neurons are undergoing maximal apoptosis expression was limited to c-Jun and to a lesser extent Jun-D. Fos, Jun-B and Krox-20 were not expressed by these neurons which had also lost their basal expression of Krox-24 (Hughes *et al.*, 1992) at this 72 hr time-point (Dragunow and Preston, 1995). In
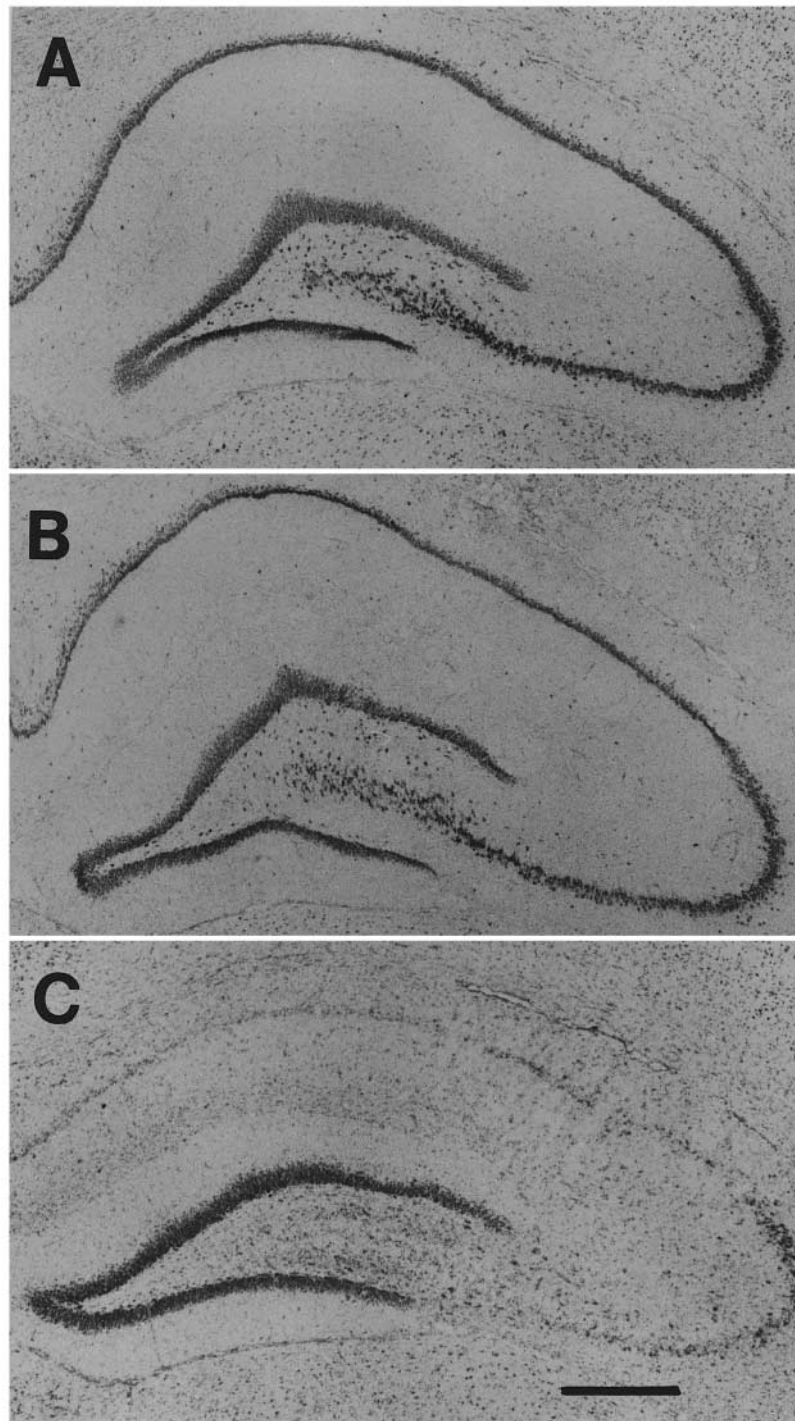
Fig. 5. Cresyl-Violet stain of hippocampal sections from (A) sham rat (B) rat that had long-lasting hippocampal seizures (> 5 hr) induced by MK801/pilocarpine drug combination (see Hughes *et al.*, 1993c) or (C) rat that had long-lasting hippocampal seizures induced by CHS (see Dragunow and Preston, 1995) 6 days after the end of seizure activity. Notice the lack of typical (see C) seizure-induced neuronal loss in animals that had LSE-induced by MK801/pilocarpine even though it lasted 10–12 times longer than CHS-induced LSE. Bar = 250 μm.

contrast, in those models were neuronal degeneration is absent (MK801/pilocarpine, MK801 5 mg kg^{-1}/kainic acid) we saw only the early expression of all ITFs and not the later selective delayed expression of c-Jun/Jun-D in hippocampal CA1/hilar neurons (Dragunow *et al.*, 1993b; Dragunow and Preston, 1995; Hughes *et al.*, unpublished observations). These results are similar to those of others (Kaminska *et al.*, 1994). Further experiments which examined ITF gene expression at

much later time-points (up to 2 weeks) following prolonged seizure activity have identified perhaps a third phase of ITF expression (increased Fra expression specifically) that may be related to gliosis (Hope *et al.*, 1994; Pennypacker *et al.*, 1994).

4.3. Focal Hippocampal Injury

To further examine gene expression *in vivo* and to specifically identify transcription factor/target gene linkages we have developed a model of focal hippocampal injury. In this model unilateral needle insertion and fluid injection into hippocampus (i.e. stab wound injury) produces a now well characterized unilateral, NMDA receptor-dependent, induction of various genes in neocortical (piriform cortex especially) and hippocampal (dentate gyrus) neurons (see Fig. 6). In this model, adult rats are anaesthetized before being positioned in a sterotaxic frame. A burr hole is drilled through the skull to allow unilateral sterotaxic guided insertion of a hamilton syringe through the neocortex into the underlying hippocampus. Once the syringe is in place, 5 μl of saline is infused into the hippocampus (at 0.5 μl min^{-1}). At the end of the infusion period, the syringe is removed carefully over 5 min to prevent reflux of injected solution into the needle tract. Once the syringe is removed the animal is sutured and allowed to recover.

While this model is associated with increased unilateral expression of many genes in cortical neurons on the ipsilateral side, the strongest and most consistent gene expression is seen within hippocampal neurons of the ipsilateral dentate gyrus (Fig. 6). Genes expressed in ipsilateral dentate gyrus neurons following focal hippocampal injury include ITFs such as Fos, Fos-B, c-Jun, Jun-B, Jun-D, Krox-20, Krox-24 (Dragunow *et al.*, 1990b,c; Hughes *et al.*, 1993a; Dragunow and Hughes, 1993; Hughes *et al.*, 1997b) and nur-77 (Dragunow *et al.*, 1996), neurotrophins such as NGF and BDNF (Ballarin *et al.*, 1991; Hughes *et al.*, 1993a) and trkB and trkC neurotrophin receptors (Mudo *et al.*, 1993; Hughes and Dragunow, 1995b), growth factors such as activin βA (Lai *et al.*, 1997) and other zinc finger transcription factors (Honkaniemi *et al.*, 1995). Pretreatment of rats with the NMDA receptor antagonist drug MK801 dose-dependently prevents gene expression in dentate gyrus neurons following focal hippocampal injury (Hughes *et al.*, 1993a; Dragunow *et al.*, 1990b; Lai *et al.*, 1997) suggesting that NMDA receptor activation (likely related to spreading depression, Lauritzen, 1987; McLachlan, 1992) leads to increased gene expression in dentate gyrus neurons. In addition with this model we also see NMDA-receptor independent expression of various ITF genes in non-nerve cells around the wound margin including Fos, c-Jun, Jun-B, Jun-D and Krox-24 but not Fos-B or Krox-20 (Dragunow and Hughes, 1993; Dragunow *et al.*, 1990a).

This model has proven useful since it has allowed us to examine whether the increased expression of ITFs such as Fos and c-Jun in dentate gyrus neurons after injury is responsible for regulating the expression of other genes such as BDNF, trkB, trkC or activin βA which are also induced in this model

(Hughes, 1994). In these experiments we pretreated rats with the protein synthesis inhibitor cycloheximide (CHX, 1 hr prior to injury) before performing focal hippocampal injury. Pretreatment with CHX prevented the translation of ITF proteins in dentate gyrus neurons 1 hr after injury even in the presence of super-induction of their mRNAs [see figures in Hughes *et al.* (1993a, 1997b) and Won *et al.* (1997) who show super-induction and prolonged expression of c-fos and c-jun mRNAs with CHX after kainic acid-induced seizures]. With the translation of ITF proteins prevented, this allowed us to examine whether the expression of mRNAs of putative ITF target genes was also inhibited at this 1 hr time-point. Inhibition of expression by CHX would strongly suggest that the gene was regulated by ITFs. Conversely, a lack of effect of CHX would classified the gene as an IEG, that is, a gene whose expression occurs independently of prior protein-synthesis. In our initial experiments we investigated whether the focal hippocampal injury-induced expression of BDNF mRNA in dentate gyrus neurons was regulated by ITFs since we and others had found numerous instances of selective coexpression of ITFs with BDNF mRNA [for example, Hughes *et al.* (1993b)]. In this experiment we found that CHX pretreatment prevented ITF protein expression but failed to prevent the increased expression of BDNF mRNA in dentate gyrus neurons. This showed that BDNF was induced as an IEG following NMDA receptor activation (Hughes *et al.*, 1993a). This result has now been confirmed by others using CHX, anisomycin and BDNF mRNA expression in dentate gyrus neurons induced by kainic acid-initiated seizure activity (Lauterborn *et al.*, 1996). Recently however, we have found a gene, activin βA, whose expression at 1 hr following injury is completely blocked by CHX in this model (Lai *et al.*, 1997) suggesting that, in contrast to BDNF, the expression of this gene is likely regulated by ITFs such as Fos. This is supported by the identification of AP-1 sites in regulatory regions of the activin gene (Tanimoto *et al.*, 1991, 1996) and by similar results showing that CHX prevents activin βA expression induced by maximal electroconvulsive shock (Andreasson and Worley, 1995). Interestingly activin βA mRNA also appears to be induced in dentate gyrus neurons during LTP perhaps suggesting that it may be one of the, as yet, unidentified downstream genes whose expression is regulated by ITFs during LTP (Inokuchi *et al.*, 1996; Andreasson and Worley, 1995).

While the focal injury-induced, early (at 1 hr) expression of a gene (activin βA) was prevented by CHX pretreatment in our model it appeared more likely that the expression of other ITF target genes would be somewhat delayed with respect to ITF expression. For this reason we also examined the CHX sensitivity of trk receptor expression in dentate gyrus neurons after hippocampal injury, since peak trk receptor mRNA expression does not occur until 4 hr following focal hippocampal injury (Mudo *et al.*, 1993) in contrast to the expression of ITFs mRNAs which peak 30 min to 1 hr following injury (Hughes *et al.*, 1993a). Using our focal hippocampal injury model we found significantly increased ex-
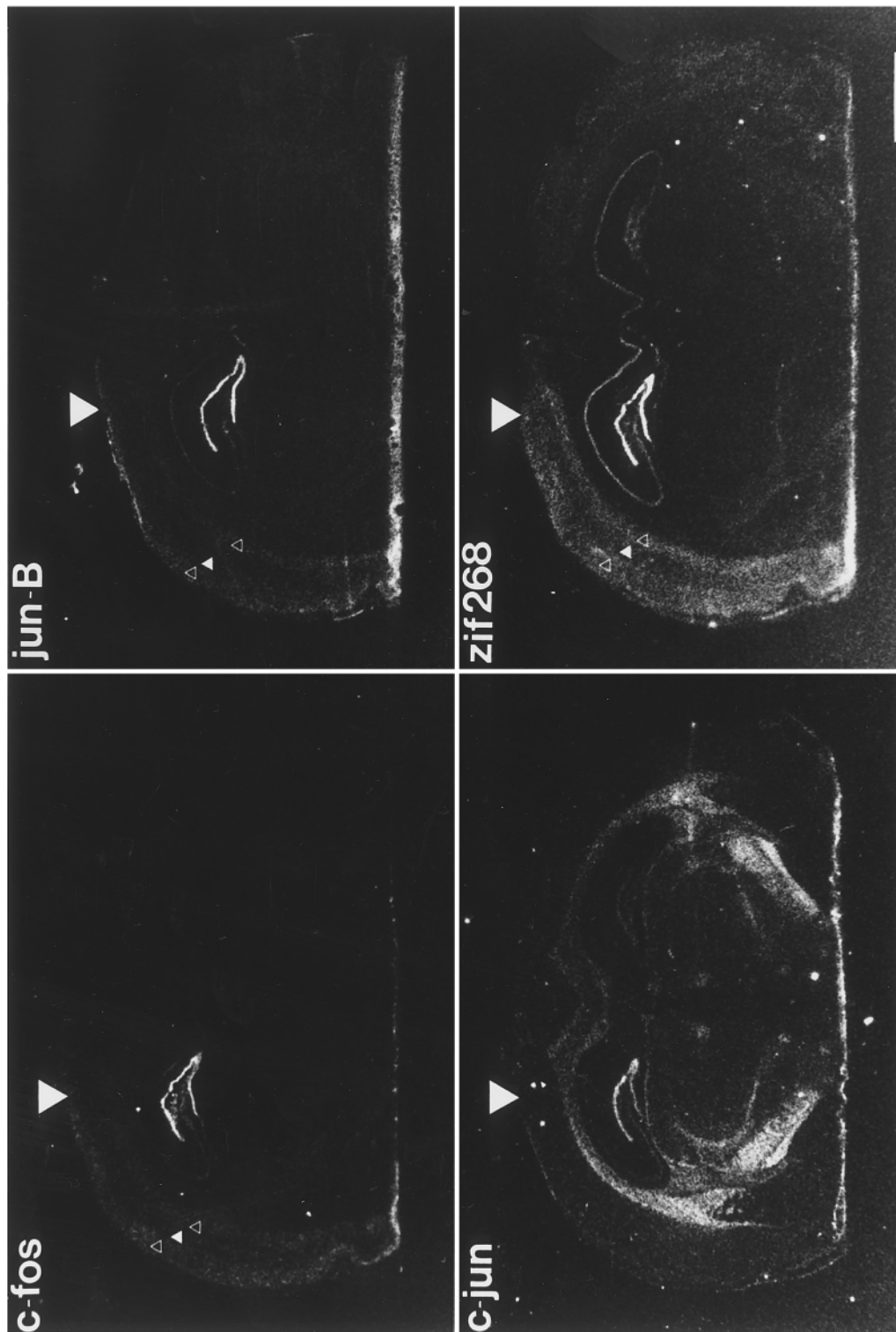
Fig. 6. Increased expression of IEGs in rat brain following focal injury to hippocampus. Focal brain injury to the hippocampus produced by needle insertion through cortex and fluid ejection into hippocampus induces increased ipsilateral expression of *c-fos*, *jun-B*, *c-jun* and *zif268* (*krox-24*) mRNA in hippocampus and neocortex on the injured side 1 hr following injury (large filled triangle shows injection site). Notice that the highest expression of IEG mRNA occurs in dentate granule neurons in hippocampus and that expression in neocortex is laminar (increased expression in superficial layers 2, 3 and deep layers 5, 6, small open triangles but not layer 3, small filled triangle). Although not clear in this figure *c-fos*, *jun-B*, *c-jun* and *zif268* mRNA was also induced strongly in piriform cortex on the injured but not uninjured side. Bar = 1500 μm.

pression of trkB and trkC but not trkA mRNA in dentate gyrus neurons peaking 4 hr following injury (Hughes and Dragunow, 1995b). Therefore to examine the CHX sensitivity of trk receptor expression we had to prevent ITF protein synthesis for at least 4 hr following focal hippocampal injury. Whereas as single pretreatment dose of CHX (1 hr prior to injury) had proved sufficient to clearly block ITF protein expression in dentate gyrus neurons at 1 hr post-injury [and in fact will block ITF protein expression for up to 2 hr following injury, Hughes et al. (1997b)] it failed to prevent ITF protein expression at later time-points (3–4 hr post-injury) and in fact appeared to super-induce ITF protein expression at these later time-points (Hughes et al., 1997b). We believe the reason for this involves the persistent superinduction of ITF mRNA induced by CHX pretreatment in this model (Hughes et al., 1993a, 1997b) coupled with the short effective half-life of CHX, so that as levels of CHX decline (3–4 hr post-injury), translation of super-induced mRNAs occurs. We have therefore suggested that a single pretreatment dose of CHX merely phase-shifts, but will not prevent, de novo expression of ITF proteins at later time-points (Hughes et al., 1997b). Therefore it appeared that a single pretreatment dose of CHX would not prevent ITF protein expression for the necessary 4 hr as required in our experiment necessitating multiple dosing with CHX (1 hr before and 1, 2 and 3 hr after injury) to fully prevent ITF protein expression for up to 4 hr. Using this protocol we were able to show that CHX almost completely prevented the expression of trkC but not trkB mRNA following hippocampal injury (Hughes and Dragunow, 1995b). This result firstly suggests that trkC may be an ITF target gene, but also demonstrates conclusively that the trkB gene is induced in a protein synthesis independent manner. Thus, both BDNF and the BDNF receptor coded for by trkB are induced in neurons after injury as IEGs (Hughes et al., 1993a; Hughes and Dragunow, 1995b).

4.4. Axotomy

The transection of a neurons axon (axotomy) initiates a complex series of events which will either lead to neuronal atrophy, degeneration or regeneration depending upon the particular circumstances associated with the axotomy (Kelly, 1991; Bahr and Bonhoeffer, 1994). For example, in many instances within the peripheral nervous system (PNS) following axotomy, the neuron may successfully regenerate its axon and reconnect to its previous synaptic sites. Within the CNS, however, the glial scar that forms at the site of axotomy prevents any regeneration and reconnection of axons with their targets. After CNS axotomy the neuron will usually degenerate or at best remain in an atrophied state (Kelly, 1991; Bahr and Bonhoeffer, 1994). We and others have specifically investigated changes in ITF gene expression associated with axotomy. In contrast to the expression of multiple ITFs following increased neuronal activity (associated with LTP and kindling) or neuronal injuries (i.e. stab wound or hypoxia/ischemia) axotomy is associated with a much more selective and prolonged expression of ITFs in the axotomized neuron. Specifically, axotomy in either the PNS or CNS is associated with selective and prolonged expression of c-Jun and Jun-D with much weaker and less consistent expression of Krox-24 but no increased expression of Fos, Fras, Fos-B, Jun-B or Krox-20 in the axotomized neurons (Dragunow, 1992; Leah et al., 1991, 1993; Gold et al., 1993; Brecht et al., 1995; Gass and Herdegen, 1995; Butterworth and Dragunow, 1996; Haas et al., 1996; Hughes et al., 1997c; Isenmann and Bahr, 1997; Blotttner and Herdegen, 1998). The expression of Jun proteins (most significantly c-Jun) in axotomized nerve cells (see Fig. 7 top panel) usually begins within 24–48 hr of the axon being transected (Dragunow, 1992; Leah et al., 1993; Brecht et al., 1995; Butterworth and Dragunow, 1996; Isenmann and Bahr, 1997) and in certain situations within the CNS may last for up to 100 days post-axotomy (Brecht et al., 1995). It is our belief that the length of time that c-Jun expression occurs in the axotomized neuron will determine its eventual fate. For example, in CNS neurons that do not successfully regenerate and which do not undergo apoptosis in response to axotomy [i.e. basal forebrain medial septal cholinergic neurons following transection of the fornix-fimbria, see Haas et al. (1996); Butterworth and Dragunow (1996); Hughes et al. (1997c)] c-Jun expression is long-lasting (Brecht et al., 1995). In contrast, in PNS neurons which regenerate successfully c-Jun expression is much shorter only being expressed by the axotomized neuron until successful target re-innervation is achieved (Leah et al., 1991). In other neurons that undergo apoptosis in response to axotomy, c-Jun expression is short-lived and may play some role in initiating the neuron to undergo apoptosis (Isenmann and Bahr, 1997). For these reasons it has been suggested that the c-Jun transcription factor may play a role in both regeneration and degeneration of axotomized neurons depending upon the particular circumstances [Herdegen et al. (1997) and Section 5].

Thus c-Jun and to a lesser extent Jun-D selectively (other ITFs do not appear to be involved) form part of the cell body response of the axotomized neuron in response to axotomy. While nuclear c-Jun expression appears to represent one of the earliest molecular responses to axotomy, the signal(s) that trigger prolonged c-Jun expression in axotomized neurons remain obscure. Molecules derived from the lesioned nerves themselves or perhaps loss of target-derived factor(s) as a consequence of axotomy might be involved. Reports now published by ourselves (Hughes et al., 1997c) and others (Haas et al., 1997) demonstrate that nerve growth factor (NGF) regulates c-Jun expression in axotomized cholinergic medial septal diagonal band (MSDB) neurons following fornix-fimbria lesion (FFL) perhaps suggesting that loss of target-derived trophic support triggers c-Jun expression (see Figs 7 and 8).

Axotomized MSDB cholinergic neurons express nuclear c-Jun within 48 hr after transection of their projections to the hippocampal formation and expression persists for weeks to months as mentioned (Dragunow, 1992; Brecht et al., 1995). We find that intraventricular injection of NGF (2.5 μg per day)
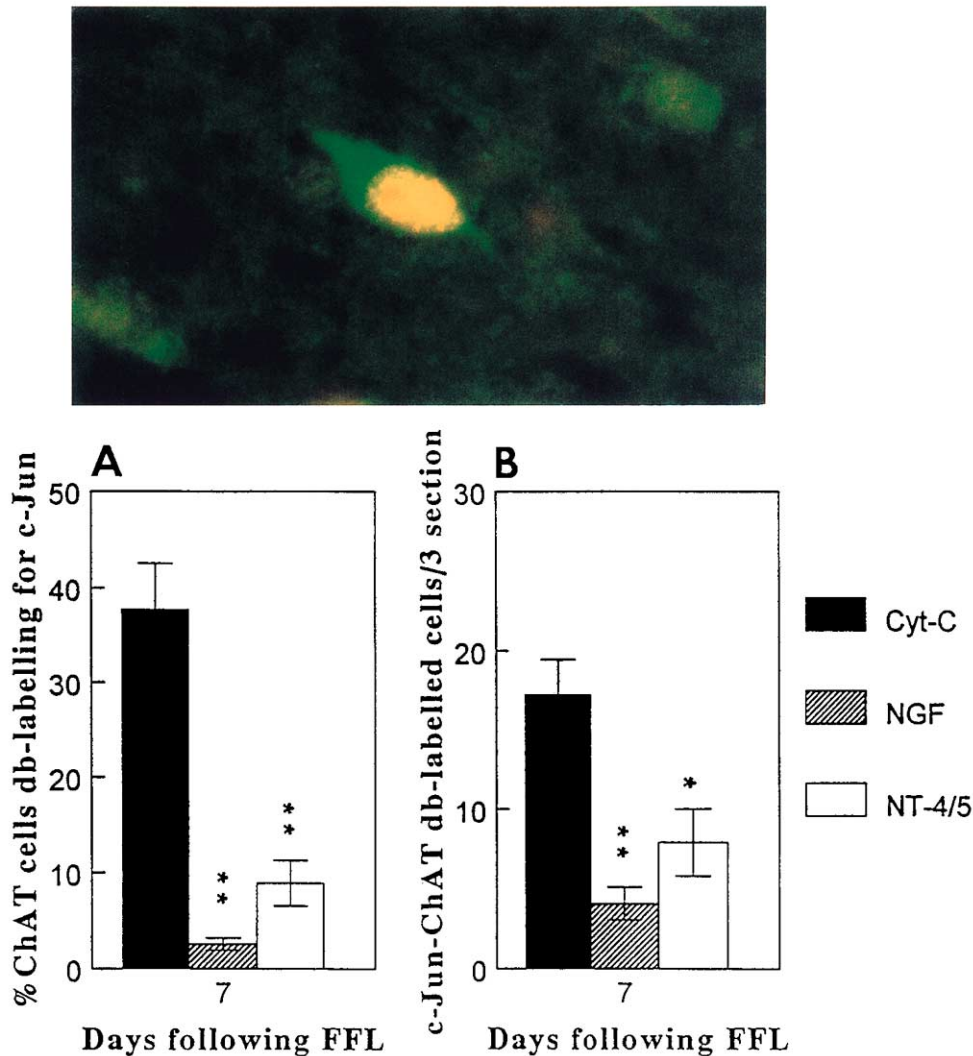
Fig. 7. Top panel, strong and persistent increased expression of c-Jun protein (yellow) in the nucleus of an axotomized MSDB cholinergic neuron (green) 7 days following lesion of the fornix-fimbria. These neurons become axotomized after FFL since their axons project from the septum through the fornix-fimbria to the hippocampus. Intraventricular administration of NGF or NT-4/5 each day for the 7 days following FFL strongly prevented the expression of c-Jun (A and B) in cholinergic MDB neurons. (A) Shows the effect of NGF or NT-4/5 treatment on the percentage of axotomized MSDB ChAT neurons coexpressing c-Jun 7 days after FFL while (B) shows the effect of NGF and NT-4/5 treatment on the total number of ChAT/c-Jun double-labelled MSDB neurons counted in three 16 μm brain sections per rat. Notice that NGF significantly prevents axotomy-induced expression of c-Jun in cholinergic neurons following FFL [for full methods see Hughes *et al.* (1997c)].

to rats for 7 days [when the number of neurons expressing c-Jun is near maximal, Brecht *et al.* (1995)] prevents both the axotomy-induced loss of cholinergic phenotype as previously reported (Hefti, 1986) and axotomy-induced expression of c-Jun in cholinergic but not GABAergic neurons [Hughes *et al.* (1997c) and see Figs 7 and 8]. In addition, Hass *et al.* demonstrate that while MSDB cholinergic neurons fail to express c-Jun in response to axotomy when examined in organotypic slices, addition of NGF antibody to the slice preparation will restore the c-Jun response in a dose-dependent manner (Haas *et al.*, 1997). This suggests that the presence of NGF in the preparation acts to inhibit the normal axotomy-induced increase in c-Jun expression.

Similarly, NGF suppresses axotomy-induced c-Jun expression in adult dorsal root ganglion (DRG) neurons following sciatic nerve crush (Gold *et al.*, 1993), while fibroblast growth factor-2 down-regulates c-Jun expression in axotomized sympathetic preganglionic neurons (Blotttner and Herdegen, 1998). Because blockade of axonal transport through the medial forebrain bundle using colchicine also induces c-Jun expression within substantia nigra pars compacta neurons comparable to axotomy (Leah *et al.*, 1993) it is tempting to speculate that loss of retrograde transport of target-derived trophic factor(s) subsequent to axotomy may be the trigger for c-Jun expression in many different populations of axotomized neurons within both the PNS
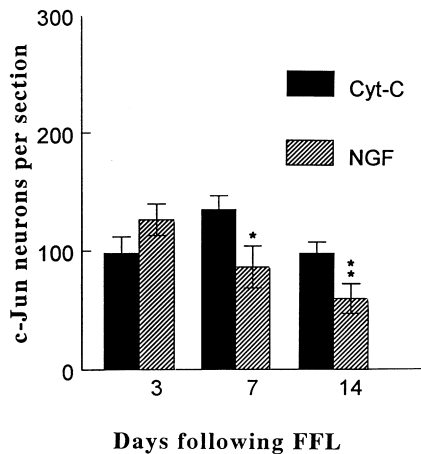
Fig. 8. Quantitative effect of daily NGF treatment on the total number of MSDB neurons expressing c-Jun at 3, 7 and 14 days following lesion of the fornix-fimbria. Notice that NGF administration only partially reduces the *total* number of neurons expressing c-Jun. This might occur because while c-Jun is expressed in cholinergic (ChAT-positive and NGF-responsive neurons) it is also likely expressed in non-cholinergic (and therefore non-NGF responsive) MSDB (GABA?) neurons that also become axotomized following FFL. $*P < 0.05$, $**P < 0.01$. [For full methods see Hughes et al. (1997c)].

and CNS. Since c-Jun expression may be important for initiating either regeneration or degeneration of axotomized neurons depending upon the particular situation, the ability of trophic factors to modulate axotomy-induced c-Jun expression clearly requires further investigation. Additionally because c-Jun expression occurs as part of the cell-body reaction to axotomy and in some situations may be important for regeneration of the axon with subsequent successful target re-innervation (Leah et al., 1991), factors (such as NGF) which reduce the cell body response to axotomy (Gold et al., 1991) and also c-Jun expression (Hughes et al., 1997c) may interfere with the ability of the neuron to regenerate. This idea of course is opposite to that of others who postulate that neurotrophins will increase nerve regeneration after axotomy *in vivo* because of their neurite outgrowth promoting effects *in vitro* (i.e. the strong effects of NGF on PC12 cells and sensory neurons as an example). Support for the idea that neurotrophins (such as NGF) might inhibit regeneration by inhibiting the cell-body response to axotomy has recently been published. Gold has shown that axonal regeneration of sensory nerves is delayed by continuous intrathecal infusion of NGF (Gold, 1996).

5. ROLES OF THE DIFFERENT WAVES OF GENE EXPRESSION AFTER INJURY AND ACTIVITY

5.1. The Possible Role of ITFs, BDNF and trkB in Neuronal Plasticity

5.1.1. *Learning, Memory and LTP/LTD*

Over the past few years major advances have been made in understanding the biochemical basis of

early events in memory formation (Alberini et al., 1994; Stevens, 1994; Bliss and Collingridge, 1993; Abraham and Mason, 1988). In contrast, less is known about the processes responsible for stabilizing new memories. The process seems to require new mRNA and protein synthesis (Nguyen et al., 1994; Otani et al., 1989; Huang et al., 1994), but exactly how this is switched on is unclear. As we have discussed in depth above using the hippocampal LTP model of memory formation we and others have recently identified transcription factors that might be involved in stabilizing memories (Abraham et al., 1992; Barnes et al., 1994; Cole et al., 1989; Dragunow et al., 1989a,b; Fordyce et al., 1994; Wisden et al., 1990; Worley et al., 1993). In particular, we have shown that the Krox family of ITFs (Krox 20 and Krox 24) are the ITFs most likely involved in stabilizing LTP (Abraham et al., 1993; Williams et al., 1995), whereas members of the Fos and Jun families are less likely to be involved in this process. Krox proteins may also play a role in stabilizing LTD (Abraham et al., 1994). LTP appears to be a useful model for long-term memory. For example, Bourtchuladze et al. (1994) have shown that CREB mutant mice have deficient long-term memory but intact short-term memory, and similarly showed only transient early phase LTP lasting 90 min. This study suggests that molecules involved in stabilizing LTP are also likely to be involved in stabilizing long-term memories. Thus, ITFs from the Krox family are candidate memory consolidation molecules. It will be important to now demonstrate a causal relationship between their expression and memory consolidation/LTP durability, and to try to identify down-stream genes regulated by their expression. These down-stream genes are likely to code for critical components of the physical substrate of memory.

In addition to ITFs, we have recently shown that the mRNA for both BDNF and its full length receptor TrkB (Klein et al., 1990, 1991; Middlemas et al., 1991) are also induced in dentate granule cells after LTP and that the induction correlates with durable LTP production (Dragunow et al., 1993a, 1997a). Furthermore, although it remains to be tested, it appears likely that both BDNF and TrkB will be induced as IEGs by LTP (Hughes et al., 1993a; Hughes and Dragunow, 1995b; Lauterborn et al., 1996). What then are the functional implications of this activity-dependent (but perhaps protein synthesis independent) expression of neurotrophins such as BDNF? Clearly, in the LTP model they may play important roles in synaptic enhancement, since BDNF can itself induce LTP, via tyrosine kinase receptors [Kang and Schuman (1995); Levine et al. (1995) see also Lebmann et al. (1994)], and this effect requires immediate protein synthesis (Kang and Schuman, 1996). However, whether this is merely a pharmacological effect, or whether it also occurs physiologically is presently unclear because although BDNF mRNA has been shown by us and others to be induced after LTP (Dragunow et al., 1993a; Castren et al., 1993; Patterson et al., 1992), BDNF protein levels have not been examined. A recent paper, however, shows that antisense DNA to BDNF inhibits LTP expression suggesting that

BDNF might have a physiological role in LTP (Ma *et al.*, 1998). Furthermore, in this report the researchers also showed that performance in a memory task was also impaired by BDNF antisense DNA at a step that might involve memory consolidation. However, we must be aware that there are potential specificity problems with the use of antisense DNA which might impact on the implications of these results. Other studies however in BDNF knock-out mice have also demonstrated a role for this neurotrophin in memory processes (Korte *et al.*, 1995; Patterson *et al.*, 1996). These mice show reduced LTP which can be overcome by exogenous delivery of BDNF protein. The receptor for BDNF, TrkB, is also induced after LTP and its induction profile suggests that it might be involved in LTP durability (Dragunow *et al.*, 1997a). Again only mRNA expression was studied and it will be important to ascertain whether LTP stimulation induces TrkB receptor protein changes that predict the durability of LTP. Strong supporting evidence for a role of the TrkB receptor in LTP comes from recent studies showing that TrkB antibodies prevent various forms of LTP in hippocampal slices (Kang *et al.*, 1997).

Taken together, these results suggest that the BDNF/TrkB neurotrophin system likely plays an important role in learning and memory processes. An early report showing that intracerebroventricular BDNF did not reverse spatial memory problems in aged rats (Fischer *et al.*, 1994) was likely due to poor brain penetration of BDNF via this route of administration (Yan *et al.*, 1994). Because this BDNF/TrkB system is also neuroprotective (Acheson *et al.*, 1995; Beck *et al.*, 1993; Cheng and Mattson, 1994; Ghosh *et al.*, 1994) BDNF and/or TrkB receptor agonists might provide an ideal treatment for Alzheimer's disease to treat both the cognitive decline and the ongoing neuronal death (see Dragunow *et al.*, 1998; Conner and Dragunow, 1998). This neuroprotective cognitive enhancement strategy might also be activated physiologically, since levels of BDNF mRNA (protein levels have not yet been studied) increase in brain with increased motor activity (Neeper *et al.*, 1995, 1996) and in enriched environments (Falkenberg *et al.*, 1992).

5.2. The Role of Gene Expression in Neuronal Pathology and in the Response to Brain Injury

5.2.1. *Epileptogenesis*

There is considerable interest in understanding the mechanisms involved in the development of kindling since it models the development of human temporal lobe epilepsy (McNamara, 1994). While the underlying brain changes that lead to the kindled state have not been fully elucidated, work over the last 10 years has established several important brain changes that occur during kindling development. For example, within the hippocampus kindling has been shown to induce a variety of permanent structural changes in the brain including sprouting of the mossy fibre pathway that originates from the dentate granule cells [Sutula *et al.* (1988)

although see Elmer *et al.* (1997)]. The axons of dentate gyrus neurons (i.e. the mossy fibres) project on the proximal segment of the apical dendrites of CA3 pyramidal neurons but invade the supragranular layer of the dentate gyrus after kindling (as assessed using the Timm's stain for zinc). Kindling also induces neuronal loss in specific populations of limbic neurons, particularly of the hilar cells in the hippocampus (Cavazos and Sutula, 1990). Furthermore recent results show that hippocampal kindling induces both neurogenesis and apoptosis of dentate gyrus neurons (Bengzon *et al.*, 1997). The relationships between kindling-induced apoptosis, sprouting and neurogenesis and their importance in kindling presently remains unclear although it has been suggested that kindling-induced hilar neuronal loss may induce sprouting of mossy fibres as a secondary consequence of axonal degeneration in their target regions (Cronin *et al.*, 1992). However, kindling-induced mossy fibre sprouting has been found in cases where no hilar loss is seen (Represa *et al.*, 1993). It is tempting to speculate that mossy fibre sprouting is important for the development of kindling since it is also seen in temporal lobe epilepsy (Sutula *et al.*, 1989), however, there is considerable uncertainty about the overall effects of sprouting of these fibres on excitatory hippocampal pathways although it has been suggested that mossy fibre sprouting might increase recurrent excitation (Waurin and Dudek, 1996).

Kindling is, however, clearly associated with both short (Dragunow and Robertson, 1987a) and longer term changes in gene expression (Perlin *et al.*, 1993). These changes in gene expression and the concomitant increased synthesis of new proteins appears necessary for kindling to develop since, as we have previously discussed, inhibitors of protein synthesis significantly retard kindling development (Jonec and Wasterlain, 1979). We find that kindling induces complex, transient changes in both ITFs and neurotrophin/neurotrophin receptor gene expression [Hughes *et al.* (1994, 1998); Dragunow and Robertson (1987a); Dragunow *et al.* (1988, 1992a) and see Figs 2 and 3]. Since these gene changes are transient they are unlikely to be involved in the long-term maintenance of the kindled state. Rather it is believed that they may be involved in the development of kindling. Specifically we have found increased expression of ITFs in dentate gyrus (Fos, c-Jun, Jun-B, Jun-D, Krox-20 and Krox-24) and hippocampal hilar interneurons (Fos, Jun-D and Krox-24 but not any others) following hippocampal ADs (Hughes *et al.*, 1998). We also found transient expression of all ITFs examined within the amygdala with amygdala kindling (Hughes *et al.*, 1994). Support for the role of ITF gene expression in the development of kindling comes from a study showing that null mutation of *c-fos* retards both kindling development and mossy fibre sprouting (Watanabe *et al.*, 1996). The authors suggest that in the 'c-fos-less phenotype' there is impaired AD-induced transcriptional activation of one or more growth (sprouting)-related genes since the AD-inducible Fos transcription factor is lost. Strong candidates for these ITF/Fos-regulated growth/sprouting-inducing genes are the neurotrophins NGF and BDNF whose

expression (both mRNA and protein) is strongly increased in the hippocampus by kindling ADs as we and others have shown (Ernfors et al., 1991; Hughes et al., 1998; Bengzon et al., 1992). In contrast to NGF and BDNF the expression of NT-3 remains unchanged or decreases in dentate gyrus neurons with kindling (Ernfors et al., 1991; Bengzon et al., 1993). It has also been shown that neurotrophin receptor mRNAs encoding trkB and trkC but not trkA receptors also increase in dentate gyrus neurons with kindling (Hughes et al., 1998; Bengzon et al., 1993). Support for a role of these neurotrophins in kindling is strong. Firstly and obviously their expression increases within hippocampal neurons during kindling. The AD-induced expression of BDNF and the BDNF receptor, trkB (but not trkC) is also sensitive to blockade of the NMDA receptor by MK801 [Hughes et al. (1998) and see Fig. 3] as is the development of kindling and mossy fibre sprouting (Sutula et al., 1996). Secondly, the neurotrophins are known to induce sprouting and axon branching and remodelling. These effects are seen in cultured neurons (i.e. the neurite outgrowth promoting effects of NGF on PC12 cells) and extend to the mature nervous system (Cohen-Cory and Fraser, 1995; Van der Zee et al., 1992). Mossy fibre synaptosomes contain neurotrophic activities (Taupin et al., 1994) suggesting that the expression of NGF and/or BDNF could be responsible for mossy fibre sprouting induced by kindling. Additionally, in support of a role for NGF are results demonstrating that blockade of NGF activity using intraventricular injections of NGF antiserum or peptide antagonists not only retard hippocampal and amygdala kindling (Rashid et al., 1995; Funabashi et al., 1988) but also inhibit mossy fibre sprouting (Rashid et al., 1995; Van der Zee et al., 1995). Further, intraventricular infusions of NGF itself accelerates hippocampal kindling, enhances mossy fibre sprouting and attenuates AD-induced neuronal injury (Adams et al., 1997). The role of BDNF in kindling is less clear, however, although a role is supported by results which show that the development of hippocampal kindling is markedly suppressed in BDNF (±) mutant mice (Kokaia et al., 1995). However, AD-induced mossy fibre sprouting was augmented in mutant vs wild type mice in this study and in another study in which rats received intrahippocampal infusion of BDNF during kindling the development of kindling was prevented (Larmet et al., 1995). Further work is obviously required to determine what role BDNF plays in kindling.

5.2.2. c-Jun, a Regenerative or Degenerative Gene or is It Both?

As we have previously discussed, our results show very strong, near selective expression of the ITF c-Jun in both degenerating neurons following HI and prolonged seizure activity (Dragunow et al., 1993b, 1994; Dragunow and Preston, 1995) and in axotomized neurons which do not die but in fact may attempt to regenerate (Dragunow, 1992; Butterworth and Dragunow, 1996; Hughes et al., 1997c). The observation that c-Jun expression appears to be associated with both degeneration and

regeneration of neurons has also been made by other groups [for a recent review see Herdegen et al. (1997) and Dragunow and Lawlor (1998)]. In addition to our in vivo results, in vitro work with cultured NGF-dependent sympathetic neurons supports an important role for the c-Jun transcription factor in regulating neuronal degeneration. In this now well characterized model of neuronal PCD (Martin et al., 1988) sympathetic neurons are initially grown in media containing NGF. NGF withdrawal from the media initiates degeneration of sympathetic neurons in a protein-synthesis dependent manner. The timely addition of inhibitors of transcription and translation can prevent neuronal death. These inhibitor drugs (including CHX) must be given within 18 hr of NGF withdrawal to prevent degeneration. This suggests that there are protein(s) synthesized within the sympathetic neuron within this critical window of time that ultimately lead to its demise (Martin et al., 1988). Subsequent experiments performed to identify the protein(s) responsible, demonstrated that the death of cultured sympathetic neurons following NGF withdrawal was associated with strong de novo expression of c-Jun (Estus et al., 1994; Ham et al., 1995). The functional role of c-Jun expression in programmed neuronal death was clearly demonstrated by experiments showing that microinjections of antibodies specific for c-Jun (Estus et al., 1994) or of an expression vector encoding a dominant-negative mutant of c-Jun (Ham et al., 1995) completely prevented the death of sympathetic neurons. These experiments linking NGF and c-Jun expression in vitro directly lead to us to ask whether a similar link existed in vivo in the adult CNS between axotomy-induced c-Jun expression in NGF-responsive cholinergic MSDB neurons of the basal forebrain and NGF. The results of these experiments show that exogenously administered NGF can prevent the usual expression of c-Jun seen in axotomized cholinergic but not GABAergic MSDB neurons [Hughes et al. (1997c) see Figs 7 and 8] suggesting that the loss of retrograde transport of hippocampal-derived NGF due to transection of the fornix-fimbria may be the trigger for c-Jun expression. This result compares favourably with the earlier in vitro work demonstrating the increase in c-Jun expression seen in cultured sympathetic neurons following withdrawal of NGF from the media. Of course our finding made it important to examine the question of whether adult MSDB neurons that where expressing c-Jun actually underwent neuronal death following axotomy in vivo. Our results (Hughes et al., 1997c; Butterworth and Dragunow, 1996) which are supported by others (Haas et al., 1996) show that adult MSDB neurons do not undergo apoptosis following axotomy [although see Wilcox et al. (1995)]. This also demonstrates that neurons can express c-Jun for prolonged periods without undergoing apoptosis, suggesting that while c-Jun expression may be necessary, its expression is not sufficient to induce neuronal death/apoptosis. It is of interest then at this point to mention that c-Jun also appears to be selectively expressed in Alzheimer's post-mortem brain tissue. This suggests that a component of the neuronal loss in Alzheimer's may be due to PCD

[Anderson *et al.* (1996); MacGibbon *et al.* (1997b) for review see Dragunow *et al.* (1998)] and also raises intriguing questions about the relationship between the observed decrease in retrograde transport of NGF to nucleus basalis in AD (Crutcher *et al.*, 1993; Mufson *et al.*, 1995; Scott *et al.*, 1995) and the increased expression of c-Jun with AD pathology (Hughes *et al.*, 1997c).

Since neurons can express high levels of c-Jun for prolonged periods of time (i.e. after axotomy) and still fail to undergo apoptosis it seems likely that c-Jun must interact with other protein(s) to induce neuronal apoptosis and that the partner protein(s) will regulate the functioning of c-Jun. One partner might function with c-Jun to initiate apoptosis while another might function with c-Jun to induce cellular genes associated with a regenerative response. For example, in neurons undergoing apoptosis following HI or prolonged seizure activity both c-Jun and to a lesser extent Fos are expressed (Dragunow and Preston, 1995; Dragunow *et al.*, 1994). Fos and Jun family members and other proteins of the b-zip family (including Myc and CREB) interact via a leucine zipper to form heterodimer complexes which then may act as transcription factors to regulate gene expression. Additionally Jun but not Fos family members also form homodimers (i.e. Jun–Jun). The composition of the dimer regulates the transactivational ability (i.e. functioning) of the transcription factor complex (the AP-1 transcription factor) with Fos–Jun heterodimers having significantly increased transactivational ability when compared with Jun/Jun homodimers (Hughes and Dragunow, 1995a). In degenerating neurons after HI or seizures Fos/c-Jun heterodimers will likely predominate and Fos expression has also been associated with neuronal PCD (Smeyne *et al.*, 1993; Gonzalez-Martin *et al.*, 1992). In contrast in axotomized MSDB neurons, c-Jun and to a lesser extent Jun-D (but not Fos) are expressed (Gass and Herdegen, 1995; Brecht *et al.*, 1995) suggesting that c-Jun/c-Jun homo- or c-Jun/Jun-D heterodimers will form. Therefore perhaps the different dimers formed allow c-Jun to function in a specific fashion depending on the circumstance. The specificity inherent in ITF expression is further demonstrated by examining the pattern of expression of ITFs subsequent to stimulation of neuronal cholinergic receptors by pilocarpine injection to rats (a treatment which induces neither neuronal apoptosis nor regeneration). In this case pilocarpine induces robust expression of Fos and Jun-B but not c-Jun (Hughes and Dragunow, 1994). It is likely then that the functioning of c-Jun is differentially regulated by other protein(s) which interact with c-Jun and may be induced specifically in certain situations. The regulation of c-Jun may occur in numerous other ways so that depending upon the circumstance the expression of c-Jun might be involved in either regeneration and degeneration of CNS neurons. Some possibilities include regulation by phosphorylation via JNKs [c-Jun N-terminal kinases, Virdee *et al.* (1997)] and redox regulation via Ref-1 (Dragunow, 1995). Finally, we have recently found that ectopic expression of c-Jun in PC12 cells drives process out-

growth, but does not cause apoptosis (Dragunow *et al.*, 1997b; Xu *et al.*, 1997).

5.2.3. *Does Growth Factor Expression in the Brain After Injury Represent An Endogenous Neuroprotective Strategy? Use of Growth Factors for 'Neuronal Rescue'*

Both the distribution and timing of growth factor expression, as shown by our results, suggests distinct roles for different growth factors after injury. For example both TGF-β1 and IGF-1 are strongly induced in the vicinity of the lesion and their expression increases at time-points following HI injury when cell death rate is declining (McNeill *et al.*, 1994; Gluckman *et al.*, 1992; Beilharz *et al.*, 1995a). This raises the possibility that these growth factors might function as endogenous 'neuronal rescue' or neuroprotective agents. For our purposes neuronal rescue is distinguished from neuronal protection by the time of administration of the factor relative to the injury. For example, if the factor is given before the injury and it reduces neuronal injury then it is neuroprotective. If the factor can be given post-injury and it is effective in reducing neuronal damage then we say that it is acting as a neuronal rescue agent. Obviously, neuronal rescue agents will be clinically more useful for treating acute brain injuries than neuroprotective factors, since the use of neuroprotective factors will require chronic prophylactic treatment of at risk individuals while administration of neuronal rescue agents would begin only after the injury had occurred. We have tested the hypothesis that growth factors such as IGF-I and TGF-β1 may be useful neuronal rescue agents by administering pharmacological doses of these two growth factors i.c.v., 2 hr after a transient HI injury to the adult rat. At this time-point many of the neurons destined to die have recovered plasma membrane function and neuronal cellular DNA remains intact (Beilharz *et al.*, 1995a; Williams *et al.*, 1991). We found significant neuronal rescue following administration of either IGF-1 (Williams *et al.*, 1991; Gluckman *et al.*, 1992) or TGF-β1 (McNeill *et al.*, 1994) but not of IGF-II, NGF-β or insulin at the doses studied (Guan *et al.*, 1996b). IGF-I and TGF-β1 demonstrated a broad rescue effects on populations of neurons and glia in multiple brain regions. The broad neuronal rescue effect of IGF-I and TGF-β1 on different populations of cells contrasts with the limited specificities of target derived neurotrophic factors such as NGF which tend to have highly specific sites of action and thus highly specific neuronal rescue effects after global injuries such as hypoxia-ischemia. Some recent studies have begun to determine the underlying mechanisms responsible for the potent neuronal rescue abilities of IGF-I and TGF-β1. Like other growth factors, both IGF-I and TGF-β1 may exert some of their effects by inhibiting neuronal apoptosis following brain injury. Further, while IGF-I may act classically via its type-I IGF receptor, it has also been suggested that a tripeptide fragment, GPE, derived from the n-terminal end of IGF-1 may be responsible for some of the effects of IGF-I since GPE also has neuronal rescue effects (Guan *et al.*, 1996b;

Guan *et al.*, 1998). *In vitro* studies have also clearly demonstrated the antiapoptotic effects of both factors. For example TGF-β1 upregulates Bcl-2 gene expression and reduces Ca^{2+} overloading (Prehn *et al.*, 1994). Likewise IGF-1 may be able to prevent apoptosis by acting at multiple points along the apoptotic pathway which may include activating phosphatidylinositol-3-, mitogen activated- and akt-kinases or by upregulating Bcl-x(L) (D'Mello *et al.*, 1997; Kulik *et al.*, 1997; O'Connor *et al.*, 1997; Parrizas *et al.*, 1997; Parrizas and LeRoith, 1997). If neuroprotective growth factors such as IGF-I are to be used therapeutically in treating brain injuries such as stroke it is important to consider several factors that modulate the bioavailability of these growth factors to specific populations of injured cells *in vivo*. These include: cerebrospinal fluid (CSF) and extracellular fluid kinetics, the presence of binding or targeting proteins and proteolytic cleavage of the binding protein growth factor. A prevailing view suggests that growth factors administered centrally into the CSF have severely limited penetration into the parenchyma (Pardridge, 1991). This, however, tends to contradict the neuroprotective effects of CSF administered growth factors such as IGF-I that we have seen (Gluckman *et al.*, 1992; McNeill *et al.*, 1994; Guan *et al.*, 1998). In our studies we have recently shown that CSF borne IGF-1 rapidly flows through the cerebral parenchyma. This flow appears to share similarities with lymph flow and is accelerated through injured regions (Guan *et al.*, 1996a). Two pathways of CSF flow appear to be involved; the perivascular pathway presumably driven via peristaltic pulsations of the arteriole perivascular space (Rennels *et al.*, 1990). The other involves flow along the periventricular white matter fiber tracts (i.e. corpus callosum) and throughout the external and internal capsules (Guan *et al.*, 1996a). Binding proteins may also modulate the availability of growth factors to cells. For example IGFBP-2 exists in relatively high concentrations in the CSF and is thought to be involved in targeting of IGF from the CSF to receptors (Guan *et al.*, 1996a; Russo *et al.*, 1997). Subsequent proteolytic cleavage of either the binding protein or the growth factor (i.e. IGF-I) may reduce the affinity of the binding protein for the growth factor potentially making the factor more available for interreaction with its receptor(s). On this note recent results show that agents which displace IGFs from their binding proteins are neuroprotective after stroke (Loddick *et al.*, 1998).

5.2.4. A Specific Role for the Tumour-Suppressor Gene P53 in Regulating Neuronal Apoptosis

The tumour-suppressor gene, p53, is a nuclear transcription factor protein whose correct functioning is crucial for an appropriate cellular response to DNA damage. The p53 tumour-suppressor was identified initially through its association with the SV40 large T antigen in SV40-transformed cells (Lane and Crawford, 1979). The p53 protein is predominantly nuclear and has been shown to act as a transcription factor to regulate the expression of other p53-responsive genes [i.e. WAF/p21, Gadd-45 or Bax. El-Deiry *et al.* (1993); Yoshida *et al.* (1996);

Selvakumaran *et al.* (1994); MacGibbon *et al.* (1997a)] by binding as a tetramer to its DNA consensus sequence [5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′; p53 binds to two copies of this 10bp sequence separated by 0–13bps, El-Deiry *et al.* (1992)]. Mutation or deletion in p53 has been shown to be one of the most common features of human cancer (Levine *et al.*, 1991). The precise manner in which p53 inactivation contributes to tumourigenesis is not fully understood, however, a loss in the ability of wild-type p53 to halt the cell cycle and to induce cellular apoptosis are likely involved. DNA damage is a strong inducer of p53 and it has been said that p53 functions to act as the 'guardian of the genome' (Lane, 1992). When DNA damage is mild, p53 halts cell cycle progression by increasing expression of the cyclin-dependent kinase (Cdk) inhibitor, WAF/p21 (El-Deiry *et al.*, 1993) allowing DNA repair to occur before progression through the cell cycle. This prevents 'fixing' of lesions into the genome via replication. However, when DNA damage is severe and irreversible, p53 induces cellular apoptosis, although the mechanism(s) by which p53 induces apoptosis remain speculative and p53 transcriptional activity may or may not be involved (Selvakumaran *et al.*, 1994; Caelles *et al.*, 1994). For example, p53 expression is known to induce apoptosis in a wide range of cell types including oligodendrocytes, thymocytes, lens epithelial and myeloid leukaemic cells (Eizenberg *et al.*, 1995; Clarke *et al.*, 1993; Nakamura *et al.*, 1995; Yonish-Rouach *et al.*, 1991).

Several recent findings suggest that p53 may also play a role in initiating apoptosis in post-mitotic neurons following different types of brain injuries [for a review see Hughes *et al.* (1997a)]. For example, recent results have demonstrated DNA fragmentation and increased expression of p53 within neurons after injury. Chopp *et al.* initially investigated whether p53 protein might be expressed within the injured rat brain (Chopp *et al.*, 1992). In this report they found increased p53 protein expression in regions of neuronal 'necrosis' 12 hr following ischemia induced by 120 min of middle cerebral artery occlusion. It was suggested that p53 protein might serve some function in promoting cell death in ischemic neuronal tissue (Chopp *et al.*, 1992). Subsequent reports by this author and others show that both p53 mRNA and protein are induced in neurons after different brain injuries (Sakhi *et al.*, 1994; Manev *et al.*, 1994; Hughes *et al.*, 1996; Sakhi *et al.*, 1997). In studies that have looked at both p53 expression and DNA fragmentation (indicative of apoptosis) within the injured brain or in organotypic brain slice cultures exposed to excitotoxins, it has been shown that neuronal p53 expression precedes DNA fragmentation (Sakhi *et al.*, 1994; Manev *et al.*, 1994; Hughes *et al.*, 1996; Sakhi *et al.*, 1997). For example, Hughes *et al.* have clearly demonstrated, using intrastriatal injections of the excitotoxin quinolinic acid, that p53 mRNA is induced in both striatum and neocortex by 6 hr post-injection (see Fig. 9), a time-point when TUNEL-positive cells have yet to appear (Hughes *et al.*, 1996). In addition, Sakhi *et al.* have recently shown that the expression of p53 mRNA precedes DNA fragmentation, detected using *in situ* nick translation
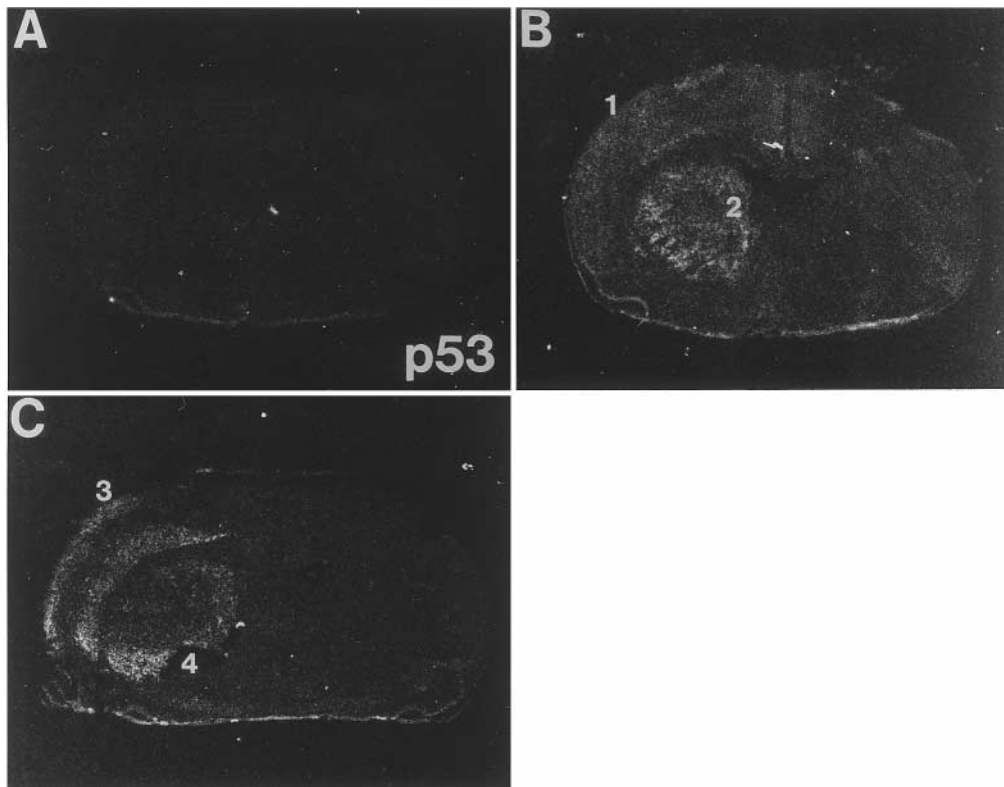
Fig. 9. Excitotoxic lesion of adult rat brain with intrastriatal injection of quinolinic acid increases p53 mRNA in striatum and neocortex (intrastriatal injections: left side = QA, 150 nmol in PBS; right = PBS). (A) Shows p53 mRNA expression in untreated rat brain (p53 mRNA was detected using an antisense p53 cRNA transcribed from a 0.95 kb insert corresponding to the coding region of murine wild type p53 [see Hughes *et al.* (1996) for full methods]. (B) and (C) show expression of p53 mRNA at 24 and 48 hr post-QA injection. Note the strong increase in p53 expression on the QA but not PBS side. Sense probes hybridized on adjacent brain sections showed no signal. Bar = 230 μm.

(ISNT), in injured neurons of organotypic hippocampal slice cultures induced by either NMDA or kainic acid (Sakhi *et al.*, 1997). These findings suggest that p53 induction might be a more sensitive indicator of DNA damage than the methods that have been utilized to date to detect it, namely TUNEL which detects free 3′-OH ends (damaged DNA with blunt, 3′ overhang or recessed 3′-OH overhangs) and/or ISNT which detects recessed 3′-OH ends in damaged DNA. Since p53 expression increases within neurons before DNA fragmentation becomes detectable, it has been suggested that increased p53 expression, induced by mild (undetectable?) DNA damage, might initiate neuronal apoptosis which would then lead to further (detectable) apoptotic-related DNA fragmentation (see Fig. 10). This raises the possibility that attenuation or prevention of p53 expression or function might reduce or abolished neuronal death/apoptosis.

Recent *in vitro* studies have addressed this question by examining the effect of p53 gene deletion on neuronal survival after lesion or insult. These studies have utilized p53 null mice (Donehower *et al.*, 1992) and have cultured neurons that are p53 gene-deficient (p53$^{-/-}$), heterozygous for p53 (p53$^{\pm}$) or homozygous for p53 (p53$^{+/+}$, Xiang *et al.*, 1996; Enokido *et al.*, 1996a,b). In one study, primary neuronal cultures of hippocampal or cortical neurons containing at least one copy of the p53 gene (p53$^{+/+}$ or p53$^{\pm}$) were severely damaged following exposure to the excitotoxins, kainic acid or glutamate. In contrast, neurons lacking the p53 gene (p53$^{-/-}$) exhibited little or no damage in response to excitotoxin treatment (Xiang *et al.*, 1996). Furthermore, in other studies when cerebellar granule cells from 15–16 day postnatal mice were treated with γ-irradiation, DNA damaging agents or cytosine arabinoside (which induces apoptosis in these cells through an uncharacterized pathway), p53$^{+/+}$ neurons underwent massive cell death, while p53$^{-/-}$ neurons were resistant to injury (Enokido *et al.*, 1996a,b). These results show that under these conditions, the p53 gene is necessary for neuronal apoptosis *in vitro*. Wood and Youle have also demonstrated an effect of p53 gene deletion on cerebellar granule cell apoptosis *in vivo*. Cerebellar granule cells are also highly sensitive to genotoxic agents such as γ-irradiation during the first 2 weeks of postnatal development *in vivo* and undergo apoptosis in response to irradiation. In p53$^{-/-}$ mice however, whole body γ-irradiation failed to induce cerebellar granule cell apoptosis (Wood and Youle, 1995). In mice heterozygous for the p53 allele (p53$^{\pm}$) granule cell apoptosis was delayed, indicating an intermediate response while massive granule cell apoptosis was observed in p53$^{+/+}$ mice (Wood and
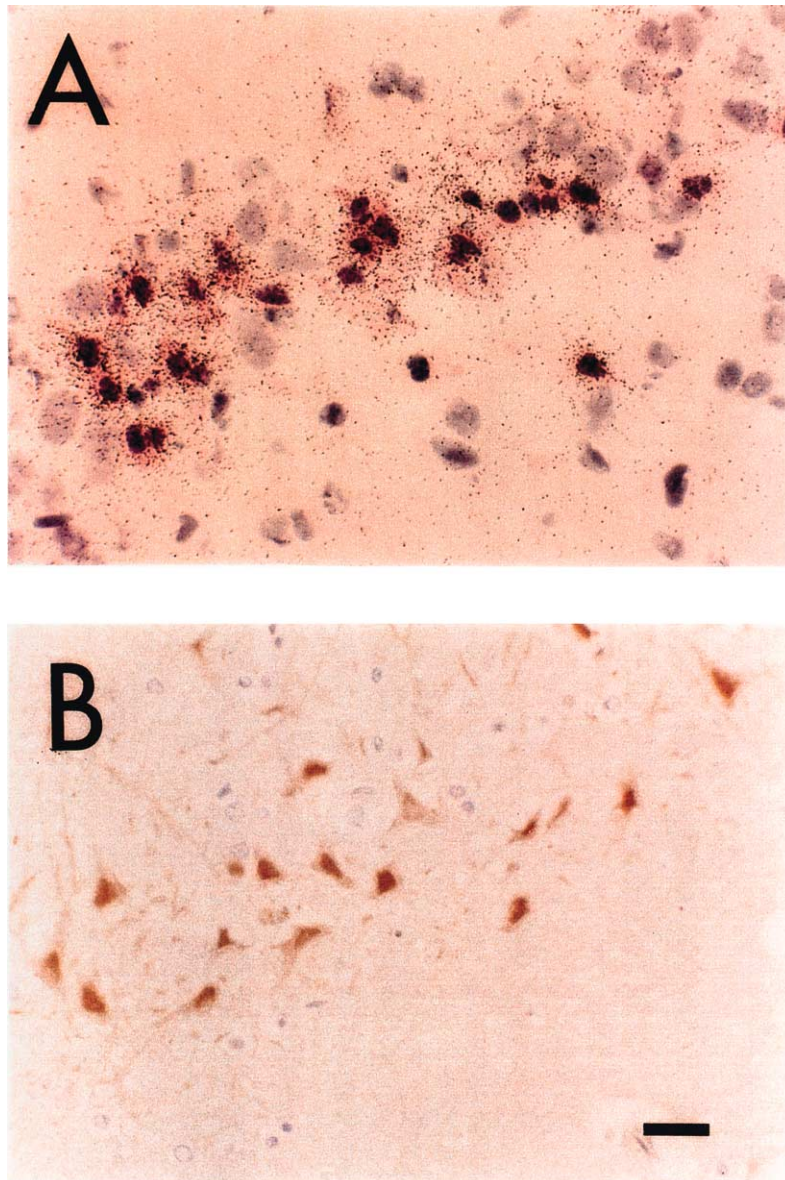
Fig. 10. Association of p53 expression with injured hippocampal CA1 pyramidal neurons 16 hr after kainic acid induced seizures. (A) Dual *in situ* hybridization for p53 and histochemistry (Hematoxylin and eosin) shows that increased p53 expression is associated with injured neurons. Injured eosinophilic neurons stain pink while p53 mRNA expression is represented by the black dots over these cells. (B) Immunostaining for p53 using a rabbit polyclonal antibody to p53 (CM-I Novocasta Labs) on adjacent sections. Notice the nuclear localization of p53 protein. Bar = 70 μm.

Youle, 1995). Other reports *in vivo* have shown that dopamine neurons from transgenic mice with a knockout of the p53 gene resist *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity (Trimmer *et al.*, 1996), that ischemic damage is attenuated in p53 knockout vs p53 normal-copy mice (Crumrine *et al.*, 1994) that methamphetamine-induced neurotoxicity is reduced (Hirata and Cadet, 1997) and that loss of p53 protects hippocampal pyramidal neurons from seizure-mediated injury induced by kainic acid (Morrison *et al.*, 1996) and hippocampal granule neurons from apoptosis induced by adrenalectomy (ADX, Sakhi *et al.*, 1996).

Furthermore, overexpression of p53 alone induces neurons to undergo apoptosis. In cortical and hippocampal neurons cultured from p53 null mice (p53$^{-/-}$), adenovirus-mediated transfection of neurons with the p53 gene resulted in nuclear expression of p53 protein, detectable by 4 hr post-infection. Thereafter, the number of p53 immunoreactive neurons continuously increased over time so that by 3 days after infection, *ca* 90% of all neurons exhibited intense p53 immunoreactivity. Neurons expressing p53 protein showed a dramatic decline in viability first detected 24 hr after infection. By 5 days post-infection only 5% of neurons survived. Neurons declined with a morphology suggestive of

death by apoptosis, with blebbing and dissolution of the neuritic processes, followed by shrinking in the soma and condensation of the nucleus (Xiang *et al.*, 1996). Additionally, transfection of the p53 gene into p53$^{+/+}$ hippocampal neurons resulted in the death of 75% of neurons within 3 days of infection, again with a morphology suggestive of apoptosis. Although constitutively, the expression of p53 protein was undetectable in these neurons, by two days post-infection approximately half of the neurons expressed p53. In comparison transfection of hippocampal neurons with β-galactosidase, the Cdk-inhibitor WAF/p21 or the retinoblastoma (Rb) gene resulted in only a small increase in neuronal death at 3 days [10–20%, Jordan *et al.* (1997)]. These results indicate therefore that increased p53 expression causes post-mitotic neurons to undergo apoptosis, consistent with its effects in a variety of dividing cells.

Taken together these findings suggest that p53 expression induced by DNA damage may regulate neuronal apoptosis following various brain injuries. It has been suggested that p53 may initiate apoptosis by increasing the expression of Bax (Selvakumaran *et al.*, 1994; Miyashita and Reed, 1995) and decreasing the expression of Bcl-2 (Selvakumaran *et al.*, 1994) [although see Caelles *et al.* (1994)], while reactive oxygen species (ROS) and ICE (interleukin-1β converting enzyme)-like proteases may be further down-stream mediators of p53-dependent apoptosis (Johnson *et al.*, 1996; Lotem and Sachs, 1996). Further studies will be required to fully understand the role of the tumour-suppressor gene p53 in regulating neuronal apoptosis.

6. CONCLUSIONS

This review has attempted to demonstrate the role that neuronal gene expression likely plays in both normal and abnormal functioning of the nervous system. Understanding the precise roles of specific genes will allow greater understanding of the processes underlying both neurodegeneration, regeneration, epileptogenesis and learning and memory within the CNS. The hope is that greater understanding of these processes will lead to advances in the treatment of the many diseases and disorders of the brain.

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