University of St. Andrews

A Developmental Study Of Glutamate-Mediated Excitotoxicity In Primary Cultures Of Mouse Cerebellar Granule Cells: Evaluation Of Receptor-Effector Coupling, Proto-Oncogene And Transcription Factor Activation, And Cell Death As Neurochemical Endpoints

A thesis submitted for the degree of Ph.D. by Kathleen M. Lidwell on

22nd October 1999.

DECLARATIONS

I, KATHLEEN M. LIDDELL, hereby certify that this thesis, which is approximately 50,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

date 22/10/99 signature of candidate

I was admitted as a research student in October 1995 and as a candidate for the degree of Ph.D. in September 1996; the higher study for which this is a record was carried out in the University of St. Andrews between 1995 and 1999.

date 22/10/99 signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

date 20-10-99 signature of supervisor

In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker.

date 22/10/99 signature of candidate

ABSTRACT

The excitotoxic effects of L-glutamate (Glu) on cultured cerebellar granule cells have been studied. Excitotoxicity was only observed following exposure of mature cells to Glu at high levels, but not at low levels, and not following exposure of immature cells to either concentration of Glu. This toxicity was accompanied by an increase in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. Using selective antagonists of the various Glu receptor subtypes, it was shown that Glu-mediated toxicity in cerebellar granule cells is largely mediated by NMDA receptor activation rather than via AMPA/KA preferring receptors. In contrast, high levels of KCl failed to cause any toxicity at either age of cell, but did exert a similar increase in $[Ca^{2+}]_i$ to that of Glu.

Using cerebellar granule cells as a model *in vitro* system, induction of the c-*fos* gene in a delayed, elevated fashion has been established as a biomarker of excitotoxicity. It was found that induction of c-*fos* mRNA in this manner was indeed stimulated by high Glu in 7 DIV cells, and that low levels of Glu induced a transient profile of c-*fos* mRNA induction.

Investigation of the composition of the AP-1 transcription factor complex in terms of protein members of the Fos and Jun families revealed a complex array of regulation. Parameters studied were age, [Glu], and culture medium used at the time of exposure. Each of these parameters had effects on the resultant AP-1 transcription factors. Briefly, JunD was found to be present in the pool of AP-1 dimers in all cases studied, whilst a heterodimer consisting of FosB and JunD appeared to be exclusively expressed under toxic conditions. KCl (55 mM) was also able to induce expression of FosB protein, but under

these conditions, FosB was unable to participate in the AP-1 transcription factor complex. It can therefore be suggested that FosB, rather than c-Fos, may be a more appropriate marker of Glu-mediated excitotoxicity in cultured cerebellar granule cells.

ACKNOWLEDGEMENTS

My thanks go to my supervisor, Dr. Roger Griffiths, for help and advice, and also to the other members of the lab over the years - Lyndsay Ritchie, Gus Grieve, Jennifer Inglis, Helen Reavey and Colin Sinclair. Thanks are also due to those in other labs - Mary Scott and Dr. Clive Meredith at BIBRA, Dr. Laurette Boutillier and Dr. J-P. Loeffler at the University of Strasbourg, and Dr. Sinead Boyce, Dr. Danni Zisterer and Prof. Clive Williams at Trinity College, Dublin. I dedicate this work to my amazing husband, Simon, without whose prayers and support it would have been a lot more difficult.

CONTENTS

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	activator protein-1
APV	2-amino-5-phosphonovaleric acid
ATF	activating transcription factor
BDNF	brain-derived neurotrophic factor
bZIP	basic region leucine zipper
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CaMK	Ca^{2+}/calmodulin dependent protein kinase
cAMP	cyclic adenosine monophosphate
CaRE	cAMP response element
CAT	chloramphenicol acetyl transferase
cIEG	cellular immediate early gene
CRE	Ca^{2+} response element
CREB	CRE binding protein
CSBP	cytokine-supressive anti-inflammatory drug binding protein
DAG	diacylglycerol
DEPC	diethylpyrocarbonate
DIV	days *in vitro*
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FRK	Fos regulating kinase
GABA	γ-aminobutyric acid

Gln	glutamine
Glu	L-glutamic acid
GSK-3	glycogen synthase kinase
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
HRP	horseradish peroxidase
IgG	immunoglobulin G
IP_3	inositol 1,4,5-trisphosphate
JNK	Jun N-terminal kinase
KA	kainic acid
MAP kinase	mitogen-activated protein kinase
mGluR	metabotropic glutamatergic receptor
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NBQX	6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione
NDGA	norhydroguariaretic acid
NEM	*N* -ethylmaleimide
NLS	nuclear localisation signal
NMDA	*N* -methyl-D-aspartic acid
NT	neurotrophin
PCV	packed cell volume
L-*trans*-PDC	L-*trans*-pyrrolidine-2,4-dicarboxylate
PDGF	platelet-derived growth factor
PEI	polyethylenimine
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A/cAMP-dependent protein kinase
PKC	protein kinase C/Ca^{2+}-dependent protein kinase
PLA_2	phospholipase A_2
PMA	phorbol 12-myristate-13-acetate
PMSF	phenylmethyl sulphonyl fluoride
polydIdC	polydeoxyinosinic-deoxycytidylic acid

SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SIE	*sis*-inducible element
SRE	serum response element
SRF	serum response factor
T3	3,3', 5-triiodo-L-thyronine
TBP	TATA binding protein
TCA	tricarboxylic acid
TCF	ternary complex factor
TCP	N-[1-(2-Thienyl)cyclohexyl]piperidine
TEMED	N,N,N'N'-tetramethylethylenediamine
TF	transcription factor
TNF	tumour necrosis factor
TPA	12-O-tetradecanoylphorbol 13-acetate
TRE	TPA response element
VGCC	voltage-gated Ca^{2+} channel

Chapter 1

Introduction

The mammalian system comprises many different cell types, each of which are able to control homeostasis, phenotypic change in response to the environment, and prevention of the spread of cell death by initiation of apoptosis via a host of molecular mechanisms. Cellular signalling involves, initially, interaction between a ligand and its receptor. In turn, these receptors are able to convert extracellular cues into intracellular cascades using cations, enzymes and proteins, e.g. Ca^{2+}, kinases, phosphatases and transcription factors.

Each of the many cell types can be host to several hundred types of receptor, each of which can cause either an inhibitory or a stimulatory signal. In the central nervous system (CNS), much use is made of excitatory (e.g. glutamate (Glu), aspartate) and inhibitory (e.g. γ-aminobutyric acid (GABA), dopamine) ligands. Excitation leads to membrane depolarisation and subsequent Ca^{2+} influx, whereas binding of an inhibitory ligand to its receptor causes influx of anions, e.g. Cl^-, thus maintaining the cell membrane in a polarised state, and preventing excitation. However, it is not the case that one receptor type is confined to a specific response. The same response can be driven by several different receptor types, due to the fact that messages can converge downstream, causing activation of similar intracellular signal molecules. Long-term effects can be activated by transient ligand binding of a receptor via regulation of gene transcription, an endpoint achievable by all receptor classes. Similarly, the concept of a single signalling cascade, formed by each component relaying the same message all the way down the line, is not a common one. Rather, several different ligands bind to a cell via specific receptors, causing various cascades which connect with each other at various points allowing crosstalk, branched pathways and feedback loops.

Obviously, this level of complexity requires high levels of co-ordination in order to achieve correct growth, differentiation and development of an organism, and regulation of gene expression ensures this. Transcription factor proteins are the means by which genes are targeted and their transcription is regulated. Again, the complexity of the task is mirrored by a vast array of transcription factor proteins. These proteins can be grouped together into families, members of which perform similar functions on different genes. Indeed, some transcription factor proteins require to dimerise in order to bind to their target sequences, whilst others need also to bind with

associated proteins, e.g. ternary complex factors (TCFs), without which regulation cannot take place. Disruption of the timing or specificity of transcription factor function can lead to a myriad of disease or pathological states. This thesis attempts to provide insight into the function of one group of transcription factors, the Fos and Jun families, in the context of comparing Glu- and K^+-mediated effects on cell viability, intracellular Ca^{2+} concentration and immediate-early gene induction and product protein expression in developing cultures of mouse cerebellar granule cells.

1.1 Glu and its receptors

L-Glutamate (Glu) is an important, endogenous amino acid which acts as an excitatory neurotransmitter within the brain. As with all compounds in the mammalian system, the distribution of Glu inside and outside of the cell must be tightly regulated. Disruption of the regulation of Glu has been implicated in a number of acute (*e.g.* hypoglycaemia, stroke) and chronic (*e.g.* Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, acquired immunodeficiency syndrome (AIDS) related dementia) neuropathologies (Choi, 1988; Lipton, 1992). In order to act as a neurotransmitter, Glu is packaged in the pre-synaptic cell into vesicles, which translocate to the pre-synaptic membrane in response to an action potential arriving at the synaptic bouton. Glu is then released into the synaptic cleft and interacts with either a post-synaptic cell via glutamatergic receptor binding or with astrocytes via transporter proteins. Astrocytes convert the Glu to glutamine (Gln), which is taken back into the pre-synaptic cell for re-processing into Glu or other functions (Figure 1.1) (for a review, see Nicholls and Sanchez-Prieto, 1998). In addition to the Glu-Gln cycle, it has been suggested that TCA cycle intermediates are synthesised in astrocytes and then released, allowing neuronal uptake and conversion of Glu (Hertz *et al.*, 1992).

Regulation of the concentration of Glu in the synaptic cleft is performed by sodium-dependent, high-affinity transporters. Four subtypes have been identified to date: in humans these are named excitatory amino acid transporter 1 (EAAT1), EAAT2, EAAT3 and EAAT4. The nomenclature for the animal equivalent are known as GLAST, GLT-1 and EAAC1. EAAT1 (GLAST) and EAAT2 (GLT-1) are widely accepted as being located in glial cells (Rothstein *et al.*,

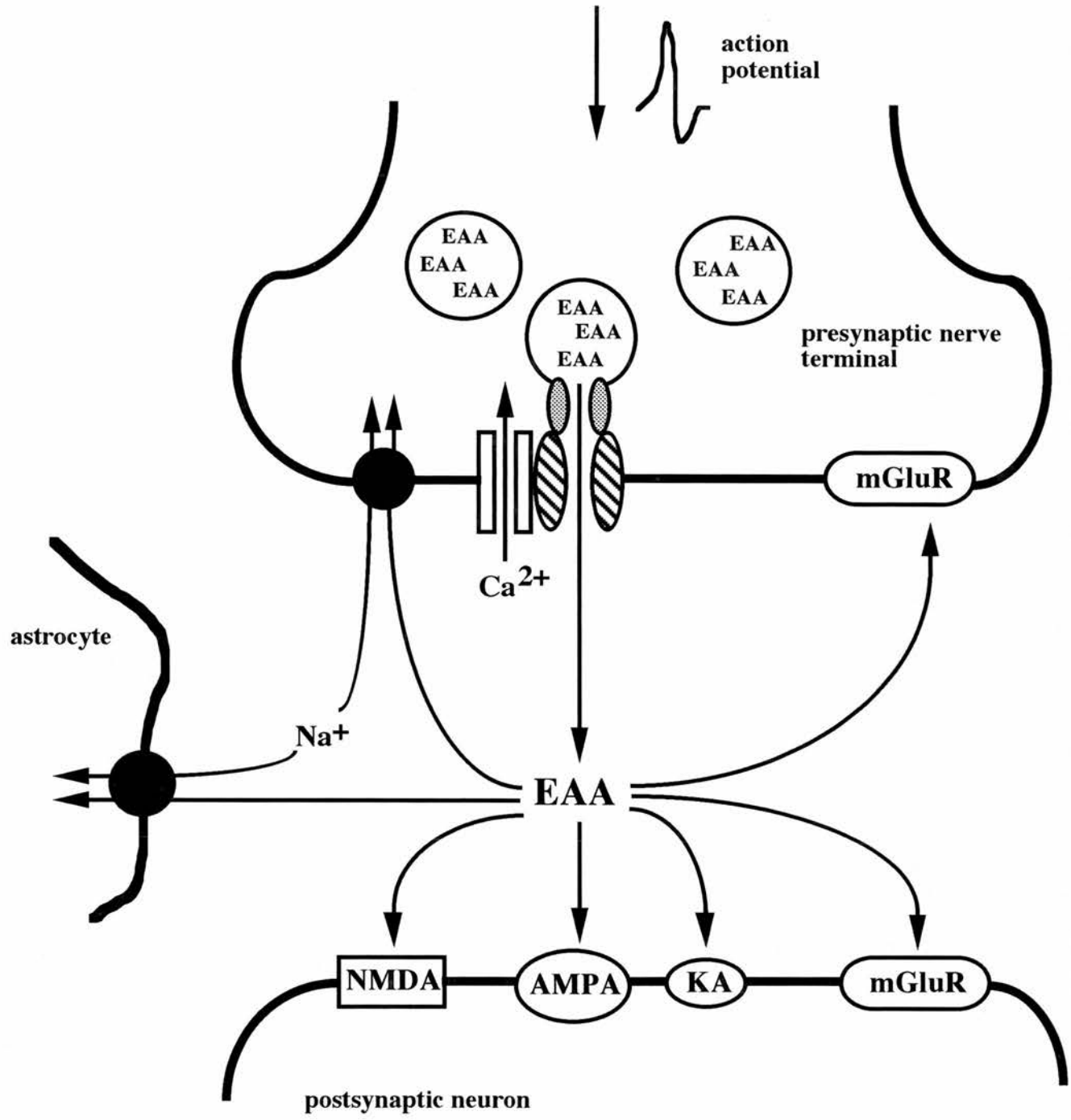


Figure 1.1 Synaptic processing of excitatory amino acids.

In response to an action potential with consequent Ca2+ influx, synaptic vesicles containing Glu are translocated to the cell membrane where Glu is released into the synaptic cleft. Glu is then able to bind and activate one of several glutamatergic receptor subtypes on the post-synaptic membrane. Ionotropic receptor subtypes (NMDA, AMPA and KA) comprise an ion channel, which, upon activation, fluxes cations (i.e. Ca2+, Na+) into the cell. Metabotropic glutamatergic receptor subtypes (mGluR) are linked to G-proteins, which in turn are linked to cellular enzymes (e.g. phospholipase C) and can cause release of Ca2+ ions from intracellular stores. Once dissociated from the receptor, Glu can be taken into astrocytes or back into the pre-synaptic neuron via high-affinity transporters. Once inside the astrocyte, Glu is processed into glutamine (Gln), which is then released into the synaptic cleft for uptake by a neuronal high-affinity transporter. Here, the Gln is reprocessed into Glu and repackaged into vesicles.

1994, 1995; Chaudhry *et al.*, 1995; Lehre *et al.*, 1995) and EAAT3 (EAAC1) and EAAT4 are neuronally-specific transporters. However, Bar-Peled and colleagues have reported the presence of GLAST and GLT-1 in neuronal cells during development, due to the failure of these proteins to co-localise with vimentin, a compound which is expressed by immature glial cells (Bar-Peled *et al.*, 1997). They explain this by suggesting that GLT-1 is required to prevent the accumulation of extracellular Glu in toxic amounts during development (Bar-Peled *et al.*, 1997). In addition, granule cell migration has been found to be regulated by Ca^{2+} ion influx via activation of NMDA receptors (Komuro and Rakic, 1993, 1996). Since GLT-1 facilitates removal of Glu from the synaptic cleft, it is involved in regulation of this important part of neuronal development (Bar-Peled *et al.*, 1997).

Various substances may be able to cause neurotoxicity by a mechanism which involves these transporters, rather than by interaction with glutamatergic receptors themselves. L-*trans*-pyrrolidine-2,4-dicarboxylate (L-*trans*-PDC), itself unable to interact with NMDA receptors, is a Glu transporter inhibitor, and causes neurotoxicity in rat cortical cultures by stimulating release of Glu from the cells via sodium-dependent transporters (Blitzblau *et al.*, 1996). Blockage of Glu reuptake via this method causes neurotoxicity and Ca^{2+} accumulation in cultured cortical neurons (Sattler *et al.*, 1998). This L-*trans*-PDC-mediated release of Glu has been shown to involve active heteroexchange, rather than passive blockage of Glu transporters (Griffiths *et al.*, 1994; Volterra *et al.*, 1996).

The post-synaptic membrane of a glutamatergic neuron contains many receptors designed to bind Glu and thus allow the signal to be transmitted into the cell. There are two main subtypes of Glu receptor; ionotropic and metabotropic (Figure 1.2). Ionotropic Glu receptors are directly linked to ion channels, and when activated will allow the flux of ions (*e.g.* Ca^{2+}) into the cell. The two classes of ionotropic receptor are named after the specific compound which binds each of them; hence the *N*-methyl D-aspartate (NMDA) receptor class and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainic acid (KA) receptor class. Binding of either of these receptor classes by an agonist causes cation flux in and out of the cell. NMDA receptor activation can cause influx of Ca^{2+} ions directly through the associated ion channel (Mayer *et al.*,

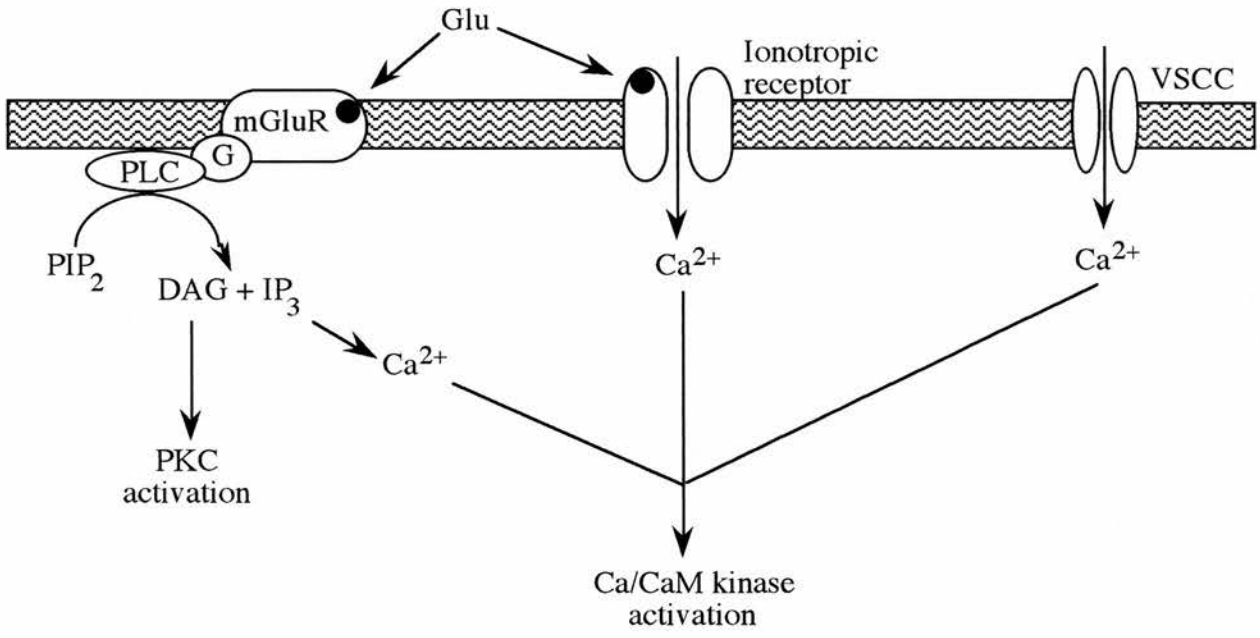


Figure 1.2 Glutamatergic receptor activation
Binding of Glu to metabotropic receptors (mGluR) activates secondary messages via G proteins. In the example shown above, phospholipase C is activated, catalysing the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to 1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG). DAG is able to activate protein kinase C (PKC), whilst IP3 binds to a specific receptor in the endoplasmic reticulum, causing liberation of calcium ions from intracellular stores. Activation of ionotropic receptors causes influx of calcium ions (in the case of NMDA receptors and those AMPA receptors which contain unedited GluR2 subunits) or sodium ions (in the case of AMPA receptors containing edited GluR2 subunits).

1987), as well as an exchange of sodium (Na^+) and potassium (K^+) ions into and out of the cell, respectively. In the same way, AMPA/KA receptors flux Na^+ and K^+ ions in response to activation (Mayer and Westbrook, 1987); however, only some sub-types have been shown to flux Ca^{2+} ions (Hollmann *et al.*, 1991). There are at least five KA receptor subunits (GluR5, 6 and 7; KA-1 and KA-2) and four AMPA receptor subunits (GluR1, 2, 3 and 4, also known as GluRA, B, C and D respectively) (Seeburg, 1993; Hollmann and Heinemann, 1993). Receptors are formed by several of these subunits joining together to form a pore. However, KA-1 and KA-2 are unable to form active homomeric channels (Coyle and Puttfarcken, 1993). GluR1, 2, 3 and 4 have almost identical DNA sequences encoding the second transmembrane region (TMII). The GluR2 subunit is important since it was found that only those AMPA receptors which do not contain this subunit are highly permeable to Ca^{2+} ions. The reason for this is that most GluR2 subunits contain an arginine residue (R) in the second transmembrane region (TMII), whereas GluR1, GluR3 and GluR4 contain a glutamine residue (Q), although the genomic sequences are identical for all four subunits at this codon. The process by which a different protein is translated from that which was encoded by the genome, called RNA editing, has been reported in GluR2, GluR5 and GluR6 (Sommer *et al.*, 1991). In receptors which contain an unedited form of GluR2, where the glutamine codon is faithfully translated, high Ca^{2+} ion permeability is conferred, but not in the case where a GluR2 which has undergone RNA editing forms part of the receptor (Hume *et al.*, 1991; Sommer *et al.*, 1991; Seeburg, 1993). However, at least in cultured hippocampal cells, the edited form of GluR2 was found to be predominant; in addition, GluR2 is a constituent of most naturally-occurring AMPA receptors (Sommer *et al.*, 1991). GluR5 and GluR6 also contain the so-called Q/R site. GluR6, a kainate receptor subunit, is also a candidate for RNA editing in the first transmembrane region (TMI). GluR6 pre-mRNA can be edited to change an isoleucine (I) codon to a valine (V) and a tyrosine (Y) codon to a cysteine (C). These changes in the first transmembrane region alter the effect of the Q/R editing (which takes place in the second transmembrane, pore-forming region) on Ca^{2+} ion permeability in those receptors which contain a GluR6 subunit (Burnashev *et al.*, 1992; Hume *et al.*, 1991). Sommer and colleagues argue that there are different editing functions for GluR2 and GluR6, since GluR6

exists in both edited and non-edited forms, and GluR1, 3 and 4 are never found in edited form, despite being almost identical in genomic sequence to GluR2 (Sommer *et al.*, 1991). DRADA and RED1, both double stranded RNA deaminases, have been identified as being the most likely mediators of the RNA editing of GluR5 and GluR6 (Belcher and Howe, 1997).

In contrast to the ionotropic receptors, metabotropic receptors do not contain ion channels within their structure, and instead are linked via G proteins to effectors (*e.g.* cytoplasmic enzymes), and are thus slower-acting than ionotropic receptors (Drejer *et al.*, 1986; Greenamyre and Porter, 1994). The subtypes are named mGluR1-8, and are classified in three groups, according to expression patterns and receptor function. mGluR1 and mGluR5 (Group 1) are involved in phosphoinositol metabolism (via phospholipase C activation), which yields inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 activates Ca^{2+} release from intracellular stores, whilst DAG stimulates activation of protein kinase C (Sugiyama *et al.*, 1987; Masu *et al.*, 1991; Abe *et al.*, 1992). In contrast, mGluR2, 3, 4, 6,7 and 8 (Groups 2 and 3) are involved in inhibition of adenylate cyclase, with mGluR2 and 3 being most effective. mGluR7 is thought to be involved in regulation of Glu release from neurons (Lafon-Cazal *et al.*, 1999). Metabotropic receptor subtypes are also susceptible to alternative splicing; three variants of mGluR1 are known, named mGluR1a, b and c. Localisation of these receptor subtypes is also important; for instance, mGluR1 is expressed in high levels in Purkinje cells of the cerebellum, whilst mGluR6 is found only in the retina (for review see Henley *et al.*, 1998; Michaelis, 1998).

1.2 Glu-mediated cytotoxicity

There is much evidence for the cytotoxic actions of Glu (*e.g.* Choi, 1988; for review see Choi, 1992; Dessi *et al.*, 1993; Thomas, 1995; Chen *et al.*, 1995a). The toxic effects of high levels of extracellular Glu on many types of cells in culture as well as in *in vivo* preparations have been studied (*e.g.* Garthwaite and Garthwaite, 1986a; 1989; Chen *et al.*, 1995a). There are several ways in which Glu is believed to effect cytotoxicity from its contact with the cell surface; binding to ionotropic receptors activates the receptor channels to flux Na^+ and Ca^{2+} ions, while activation of metabotropic receptors causes switch-on of second messenger systems. One

important endpoint of many of these pathways involves the induction of the immediate-early gene, c-*fos* (see Section 1.4 for more detail about c-*fos*). Induction of this immediate-early gene is implicated in cell death in many cell types (Colotta *et al.*, 1992; Smeyne *et al.*, 1993; Vendrell *et al.*, 1993; Dragunow and Preston, 1995; Walton *et al.*, 1998), although it is not believed that c-*fos* induction is a causal event of cytotoxicity.

The term excitotoxicity was first coined by Olney *et al.* (1971), in the context of Glu- and other related excitatory amino acid-mediated neuronal destruction. The four different subtypes of glutamatergic receptor have been described above. All of these receptor subtypes (NMDA, AMPA, KA and metabotropic) are thought to be involved in excitotoxicity in various cell types, where mediation by Ca^{2+} ion influx is a common denominator. The main route of excitotoxicity is via NMDA receptor channels, but in some cell types, *e.g.* striatal neurons, blockage of the NMDA receptor does not prevent all of the toxicity (Chen *et al.*, 1995a). There is evidence for the involvement of non-NMDA receptors in Glu-mediated excitotoxic insult in cortical neurons, caused by the influx of huge amounts of Ca^{2+} ions (Lu *et al.*, 1996). In these neurons, this method of toxicity, i.e. that mediated via AMPA receptors, has been shown to increase during development (Jensen *et al.*, 1998b).

Of course, different neuronal cultures have different susceptibilities to certain excitotoxins, and age of culture is another key factor. Cortical neurons at 5-7 DIV are less susceptible to excitotoxicity than those at 14-18 DIV (Choi *et al.*, 1987). Striatal (Chen *et al.*, 1995a) and cortical (Koh *et al.*, 1990) neurons are vulnerable to non-NMDA receptor-mediated Glu-induced toxicity, whereas the excitotoxicity exerted on hippocampal and cerebellar granule neurons is primarily via NMDA receptor-mediated mechanisms (Lehmann, 1987; Lin *et al.*, 1997), although Garthwaite and Garthwaite have reported AMPA/KA receptor-mediated toxicity in cerebellar granule neurons (Garthwaite and Garthwaite, 1986b).

1.3 Involvement of Ca^{2+} ions in Glu-induced excitotoxicity

Many of the effects that occur as a consequence of activation of EAA receptors, whether directly or indirectly, are as a result of the influx of Ca^{2+} ions; the resting extracellular Ca^{2+} ion

concentration is ~1.3 mM, whereas the resting intracellular concentration is 0.05-0.2 μM. NMDA-induced ischaemia can be prevented by voltage-gated Ca^{2+} channel blockers (Frandsen and Schousboe, 1993), suggesting that the damage is communicated by influx of Ca^{2+} ions via these ion channels. However, KA-induced increases in extracellular Ca^{2+} cannot be prevented by these channel blockers. Thus the increase in intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) may not be due primarily to influx through Ca^{2+} channels (Frandsen and Schousboe, 1993). Rather, the initial influx of Ca^{2+} ions through the channels can stimulate release of Ca^{2+} ions from intracellular stores (Mody and MacDonald, 1995). However, those workers also report that Ca^{2+} channel blockers and antagonists of both NMDA and non-NMDA receptors are effective in protecting neurons from excitotoxicity, and therefore postulate that Ca^{2+} ion entry via these channels plays a highly important and instrumental part in excitotoxicity (Mody and MacDonald, 1995). Ca^{2+} ions are also the means by which depolarisation of cell membranes by high K^+ occurs, and in PC12 cells maintained in a Ca^{2+}-deficient medium, the induction of c-*fos* mediated by K^+ was prevented (Morgan and Curran, 1986).

There are a variety of targets for Ca^{2+} ions once inside the cell membrane. The influx of calcium causes activation of calcium-dependent enzymes (e.g. protein kinase C, Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases)), which in turn activate intracellular cascades, leading to overproduction of free radicals and over-activation of degradative enzymes. Since the remit of this study did not involve work in this area, this brief summary is sufficient. Several reviews outline the important role of Ca^{2+} ions in the response of neuronal cells to stimuli (Bito *et al.*, 1997; Van Haasteren *et al.*, 1999).

In addition to the regulation via Ca^{2+} ions mentioned above, there are other mechanisms by which the effect of Glu on a cell can be modulated using these ions. Binding of Glu to NMDA receptors causes influx of Ca^{2+} ions, which stimulates activation of the enzyme phospholipase A_2 (PLA_2), which produces arachidonic acid and platelet-activating factor among its metabolites (Lerea and McNamara, 1993; see Figure 1.8). Platelet-activating factor is able to increase neuronal Ca^{2+} levels by stimulating Glu release, and is, along with arachidonic acid, implicated in the increase of c-*fos* mRNA levels observed in several different cell types (Bazán, 1970).

Arachidonic acid also potentiates NMDA-evoked currents and inhibits reuptake of Glu into astrocytes and neurons (Lipton and Rosenberg, 1994).

Changes in the cell can be mediated by two main levels of control: post-translational processing, including phosphorylation of existing proteins, causing short-term regulation, and regulation of gene expression, which can cause long-lasting effects. The following sections explain the involvement of a particular family of immediate-early genes in this second, long-term method of regulation. Immediate-early genes are so-called because they are rapidly expressed as a result of extracellular stimulation without the requirement for *de novo* protein synthesis (Angel and Karin, 1991; Ginty *et al.*, 1992; Karin, 1995). The proteins encoded by these immediate-early genes are themselves subject to both levels of control. Extracellular stimuli can modulate gene expression in three basic ways (Figure 1.3):

i) direct receptor interaction by membrane-permeant molecules causes transcriptional regulation;

ii) binding of non-channel receptors causes alteration of second messenger levels, thus changing the activity of existing transcription factors;

iii) and immediate-early gene activation allows protein expression and thus formation of transcription factors which can then go on to affect target genes.

1.4 The c-*fos* proto-oncogene

The v-*fos* oncogene was first described in Finkel-Biskis-Jinkins murine osteosarcoma virus (Finkel *et al.*, 1966), and its cellular counterpart, c-*fos*, was described by Curran and Teich (1982). These and other RNA tumour viruses require to stimulate cellular transformation in order to cause oncogenesis (e.g. v-*fos*). These oncogenes were found to have formed as a result of recombination between viral and cellular DNA (Bishop, 1985). The cellular equivalent of oncogenes, termed proto-oncogenes or cellular immediate-early genes (cIEGs), encode many vital proteins including extracellular growth factors and transcription factors. The c-*fos* gene includes a promoter region which has several sequences which are recognised by transcription factors. These sequences, or response elements, are recognised by specific binding proteins. The Ca^{2+}/cAMP response element binding protein (CREB) binds to the corresponding CRE on many

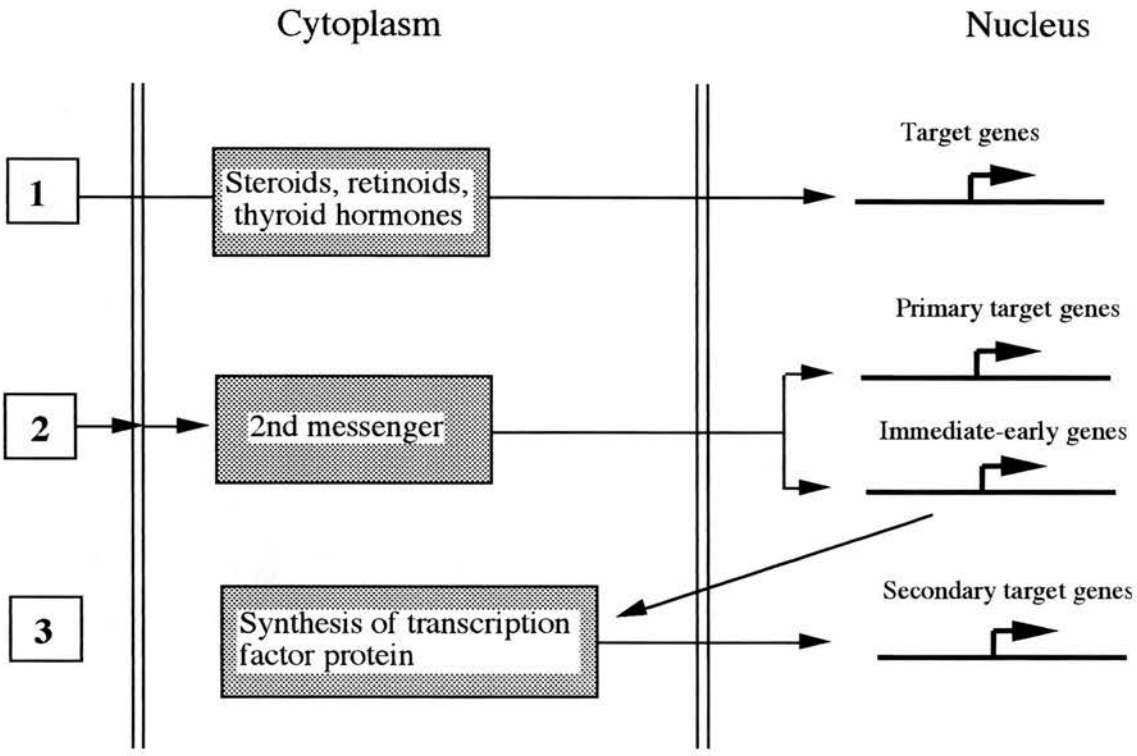


Figure 1.3 Extracellular stimuli modulate gene expression in three basic ways:
1: membrane-permeant molecules bring about alterations in transcription rates by direct interaction with nuclear receptor proteins
2: stimuli that cannot enter cell bring about direct changes in target gene transcription via changes in 2nd messenger levels which alter activity of constitutive transcription factors, or
3: indirect changes by rapidly activating cellular immediate-early genes encoding transcription factors; these genes can be viewed as '3rd messengers' in a stimulus-transcription coupling cascade effectively coupling short term signals elicited by cell surface stimulation to long term alterations in cellular phenotype.

genes, including c-*fos* (Figure 1.4). Serum can also affect the transcription of many genes via the serum response element (SRE), which is bound by the serum response factor (SRF). The 12-O-tetradecanoylphorbol 13-acetate (TPA) response element (TRE) was named after the phorbol ester which is able to stimulate its binding by the activator protein-1 (AP-1) transcription factor complex. This important transcription factor is formed by heterodimerisation between a member of the Fos family and a protein belonging to another family of cIEG proteins, the Jun family. In this way, Fos is able to regulate its own transcription (Schöntal *et al.*, 1988; Sassone-Corsi *et al.* 1988a,b), but Sonnenberg *et al.* (1989a) suggest that the phase of c-*fos* repression in brain correlates with expression of Fos-related antigens but not c-Fos itself, so there is likely to be a higher order of organisation of the cIEG cascade than simple feedback inhibition.

The c-Fos protein, encoded by the c-*fos* gene, has several domains of note: the DNA-binding domain, made up of a region of basic amino acid residues which is situated near the N-terminal (Nakabeppu and Nathans, 1989), the leucine zipper region which is required for dimerisation and is situated immediately C-terminal to the DNA-binding domain (Agre *et al.*, 1989), and the activation domain, which is an acidic region situated N-terminal to the DNA-binding domain, containing four conserved Glu residues (Figure 1.4) (Abate *et al.*, 1990a). Dimerisation of c-Fos with a member of the Jun family via the leucine zipper structure brings the DNA binding domains of each protein into the correct position (Kouzarides and Ziff, 1988; Gentz *et al.*, 1989). Proteins which contain this leucine zipper region belong to the basic region leucine zipper (bZIP) family (Landschultz *et al.*, 1988).

Expression of the c-Fos protein can be induced by a number of stimuli; e.g. phorbol esters, serum, NMDA and KA receptor agonists, UV radiation (Kasof *et al.*, 1995). In the case of NMDA and KA receptor activation, different mechanisms are stimulated and thus the outcome of c-Fos expression is reached via independent pathways (Sonnenberg *et al.*, 1989b). After translation, the c-Fos protein is translocated to the cytoplasm where it is able to dimerise with the products of other cIEGs to form the AP-1 transcription factor complex. This dimer is then translocated back to the nucleus in order to bind to and regulate the transcription of a

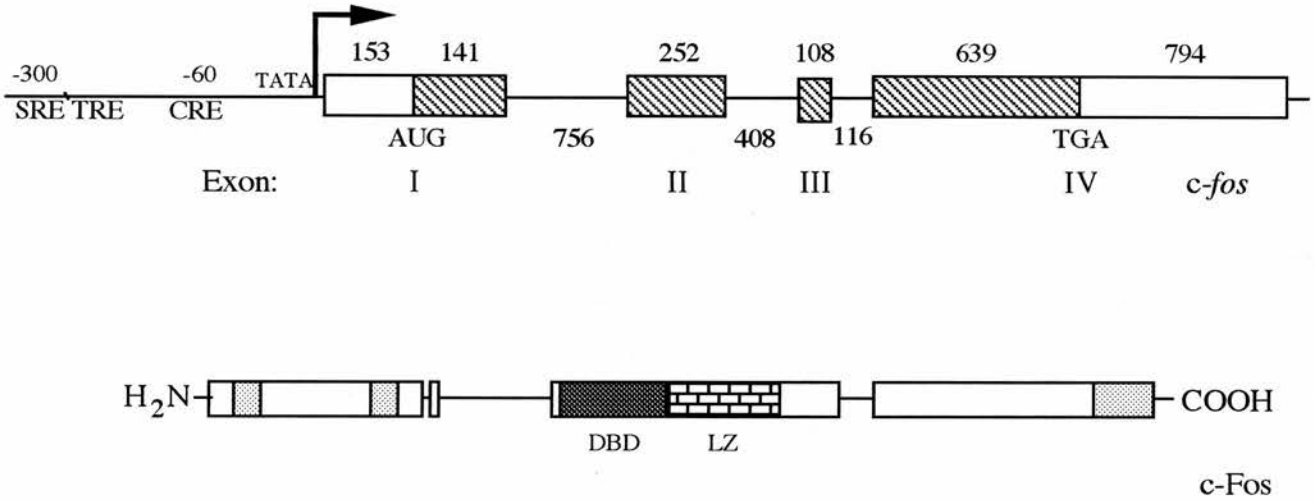


Figure 1.4 Structure of the c-*fos* gene and its product, c-Fos

The c-*fos* gene comprises 4 exons, indicated by boxes and numbered I-IV. Within each exon, the coding sequence is indicated by shading. Numbers (e.g. 153) refer to size of exons, introns and non-coding sequences in base pairs. The promoter region contains binding sites for CREB (CRE), AP-1 (TRE) and SRF (SRE).

The c-Fos protein has three main areas of note: the basic, DNA-binding domain (DBD), the leucine zipper (LZ) and the activation domain, situated N-terminal to the DNA-binding domain. The dotted areas denote highly-conserved regions throughout the Fos family.

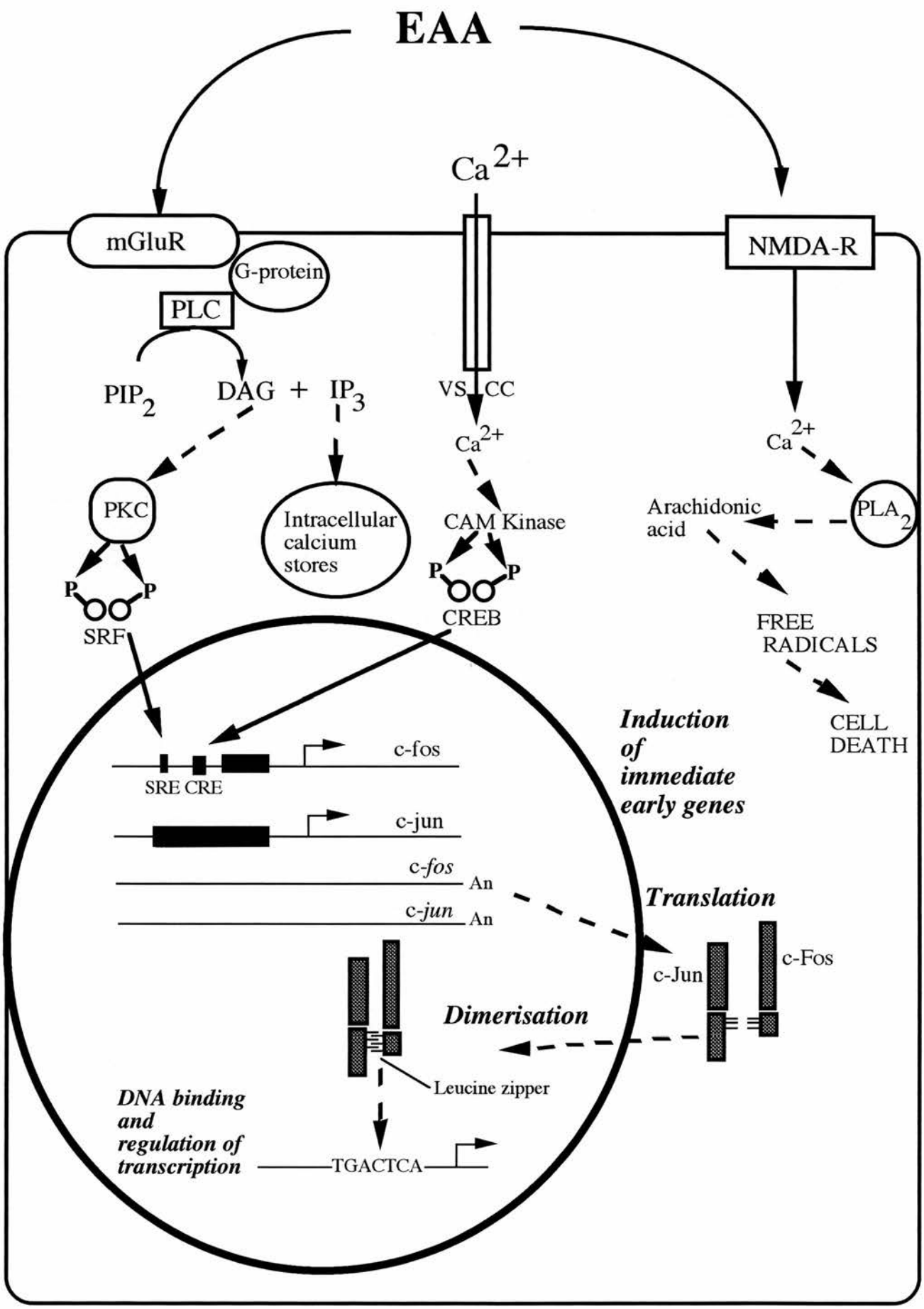
particular gene (Figure 1.5[1]). In addition to the Jun family, the Fos family member proteins are able to bind other bZIP families with varying affinities. The c-Fos protein is able to form heterodimers with activating transcription factor (ATF)-4, but not with ATF-3. This is explained as being due to differences in amino acid residues present between the leucine residues in the leucine zipper region (Hai and Curran, 1991).

The induction of c-*fos* mRNA has been studied particularly in the phaeochromocytoma cell line, PC12 (Greenberg and Ziff, 1984; Müller *et al.*, 1984; Morgan and Curran, 1986; Ghosh *et al.*, 1994). This was found to be an excellent model for the study c-*fos* mRNA induction in response to a variety of stimuli including growth factors and ion flux (Morgan and Curran, 1986). Morgan and Curran (1986) have shown that induction of c-*fos* mRNA by K^+ ion-mediated membrane depolarisation requires that Ca^{2+} ions are present in the extracellular medium, whereas induction of c-*fos* mRNA by addition of nerve growth factor (NGF) occurs in the absence of extracellular calcium. Thus different pathways are involved in switch-on of cIEG expression in this system, leading to distinct regulatory effects.

In addition to the PC12 model, studies on c-*fos* mRNA induction have been reported in a number of *in vivo* and *in vitro* preparations; this includes primary cultures of mouse cerebellar granule cells, the model used in the present study (Müller *et al.*, 1984; Treisman, 1985; Morgan and Curran, 1986; Stumpo and Blackshear, 1986; Dony and Gruss, 1987; Sassone-Corsi *et al.*, 1988c; Szekely *et al.*, 1989; Grayson *et al.*, 1990; Monstein and Folkesson, 1991; Schreiber *et al.*, 1992; Gillardon *et al.*, 1994; Barthel and Loeffler, 1995; for review see Finkbeiner and Greenberg, 1998). Taken together, these results paint a very varied picture of the ways in which transcriptional regulation can be activated (for a summary of the different stimuli and pathways involved in transcriptional regulation of Fos and Jun, see Figure 1.7). In the rat pineal gland, for example, induction of cIEGs can be differentially regulated via adrenergic receptors. The c-*fos*

---

[1] **Figure 1.5 Schematic showing signal transduction from receptor to nucleus.**
Several different stimuli are able to activate receptor- or voltage-gated channel-mediated increase in $[Ca^{2+}]_i$, leading to activation of transcription factors and subsequent expression of Fos and Jun family proteins, which in turn dimerise and regulate transcription of target genes.


EAA
Ca 2+
mGluR
G-protein
PLC
NMDA-R
PIP2
DAG + IP3
VS CC
Ca2+
Ca2+
PKC
Intracellular calcium stores
CAM Kinase
Arachidonic acid
PLA2
P P
SRF
P P
CREB
FREE RADICALS
CELL DEATH
Induction of immediate early genes
c-fos
SRE CRE
c-jun
c-fos An
c-jun An
Translation
c-Jun
c-Fos
Dimerisation
Leucine zipper
DNA binding and regulation of transcription
TGACTCA

gene is induced by activation of the adrenergic α_1-type receptor, while *jun*B can be induced via both the α_1- and β-type receptors (Carter, 1992). Oligodendrocyte progenitors induce c-*fos* gene expression after stimulation of non-NMDA glutamatergic ionotropic receptors (Liu and Almazan, 1995).

In summary, the endpoint of cIEG induction can be reached by a number of different signalling pathway types. Activation of a kinase (often by an upstream kinase kinase) can cause phosphorylation of a transcription factor which is then able to elicit a regulatory effect on a target gene. Alternatively, a cytosolic transcription factor can itself be phosphorylated and thus translocated to the nucleus where it can activate or repress gene activity. A third scenario is that a transcription factor may be constitutively repressed by an inhibitory subunit, which is removed upon phosphorylation, thus activating the transcription factor. Examples of these three scenarios are reviewed in Edwards (1994).

1.5 Activator Protein 1 (AP-1) is a dimeric transcription factor comprising cIEG-encoded proteins

The AP-1 protein was first described as a nuclear factor which bound to *cis* regions of the SV40 and human metallothionein IIA gene (Angel *et al.*, 1987; Lee *et al.*, 1987) in order for transcription induced by growth factors and phorbol esters to occur. AP-1 activity is induced by growth factors, cytokines, T cell activators, neurotransmitters and UV irradiation (Angel and Karin, 1991). The DNA sequence which is bound by the AP-1 factor is named after one such phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate (TPA), which activates protein kinase C, and hence the binding site is called the TPA response element (TRE) (Angel *et al.*, 1987). Its consensus sequence is 5'-TGACTCA-3'. This DNA sequence is palindromic, thus allowing the AP-1 transcription factor to bind both strands of DNA simultaneously (Abate *et al.*, 1990b). It was thought that Fos and Jun would bind to each side of the DNA with equal affinity, but UV crosslinking studies have shown that Fos favours the lower strand and Jun favours the upper strand (Risse *et al.*, 1989). These workers suggest that the nucleotides on either side of the consensus sequence serve to determine the binding of proteins.

Subsequently, it was discovered that the AP-1 transcription factor was actually a dimer, which could be formed from proteins encoded by members of the Fos and Jun immediate-early gene families (Rauscher *et al.*, 1988a; Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988b; Gentz *et al.*, 1989). Figure 1.6 shows the possible combinations of Fos and Jun family members which form functional AP-1 transcription factor complexes. These dimers are formed via a heptad of leucine residues on each of the proteins coming together to form a "leucine zipper" (Landschultz *et al.*, 1988; Gentz *et al.*, 1989). The complex formation process clearly requires more than just the repeat of leucine residues (Vinson *et al.*, 1989; Kerppola and Curran, 1991), since Fos family members are unable to form stable homodimers (Cohen *et al.*, 1988; Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988). One possible area of specificity is the overall charge of the leucine repeat; those of both c-Jun and JunB have a net charge of +4, whereas that of c-Fos is -4 (Schütte *et al.*, 1989). However, since Jun homodimers are possible, this cannot explain the instability of Fos homodimers. Fos-Jun heterodimers do, however, have a much higher affinity for the TRE than Jun-Jun homodimers (Halazonetis *et al.*, 1988; Rauscher *et al.*, 1988b; Kovary and Bravo, 1991; Lazo *et al.*, 1991). An interesting finding goes some way to explaining this difference in affinity for the TRE; Jun-Jun homodimers bend the DNA towards the minor groove in the process of binding, whilst Fos-Jun heterodimers bend the DNA towards the major groove (Kerppola and Curran, 1991).

The Fos and Jun families are now classified as being members of a larger gene family, the bZIP or basic-zipper family, the member genes of which all contain a leucine zipper and a basic DNA binding domain. Along with the Fos and Jun families, activating transcription factors (ATFs) and the cAMP response element binding protein (CREB) belong to this superfamily (Vendrell *et al.*, 1993). Heterodimers between Fos or Jun family members and ATF family members are known to occur (Aronheim *et al.*, 1997), and in fact the c-Jun/ATF-2 dimer is thought to be responsible for autoregulation of c-Jun by binding to the c-*jun* promoter (Angel *et al.*, 1988; Ham *et al.*, 1995). The TRE located in the c-*jun* promoter is constitutively occupied by this dimer (Rozek and Pfeifer, 1993), and then, in response to a stimulus, the proteins are phosphorylated *in situ* by mitogen-activated protein kinases (MAP kinases), causing upregulation

Family	Member	Partners
Jun	c-Jun	c-Jun, **JunB**, JunD, c-Fos, FosB, **Δ-FosB**, Fra-1, Fra-2, ATF-2, -3 & -4, NFkB
	JunB	**c-Jun**, JunB, JunD, c-Fos, FosB, **Δ-FosB**, Fra-1, Fra-2, ATF family
	JunD	c-Jun, JunB, JunD, c-Fos, FosB, **Δ-FosB**, Fra-1, Fra-2, ATF-2
Fos	c-Fos	c-Jun, JunB, JunD, ATF-4
	FosB	c-Jun, JunB, JunD
	Fra-1	c-Jun, JunB, (JunD), ATF-4
	Fra-2	c-Jun, JunB, JunD

Figure 1.6 Formation of dimers by AP-1 proteins

The possible combinations of Fos and Jun family members which form active dimers are shown above (underlined = homodimer, **bold** = suppressive dimer). Amount and composition of AP-1 dimer changes with time, and different stimuli will cause formation of specific complexes, which will act on distinct sets of genes. Thus at any one time, a single neuron may contain several distinct molecular species of AP-1, raising the possibility that these different species could have different activities. In addition, some complexes are repressive to particular genes (e.g. the c-*fos* gene is auto-repressed by the c-Fos/c-Jun heterodimer), thus different complexes can bind to the same gene and have different effects.

of the transcription of c-Jun (Karin, 1995). This is unusual; although studies using cycloheximide have shown that much of the cIEG response occurs without *de novo* protein synthesis (Angel and Karin, 1991; Ginty *et al.*, 1992; Karin, 1995), in this case, the dimerisation of proteins occurs in response to a stimulus and only then does binding occur, causing transcriptional regulation. Other combinations of Fos or Jun family members dimerising with ATF family members are common; two in particular are known to bind to the TRE: c-Jun/ATF-3 and Fra-1/ATF-4 (Hai and Curran, 1991). In addition, autoregulation of the c-*fos* promoter is thought to be mediated via Fos/Jun heterodimers and CREB/ATF heterodimers binding to the TRE site situated close to the SRE (Sassone-Corsi *et al.*, 1988c). Repression of the c-*fos* promoter by c-Fos (Sassone-Corsi *et al.*, 1988b) only occurs, however, when the transcription of c-*fos* is induced by serum (Sassone-Corsi *et al.*, 1988a). Maf and Nrl (neural retina specific gene) proteins can also bind to Fos, Jun and ATF proteins to form dimers which in turn bind to TRE and CRE, the response element specific for Ca^{2+} and cAMP (Kerppola and Curran, 1994; Aronheim *et al.*, 1997).

1.6 TRE and CRE/CaRE - response elements which are bound by AP-1

In addition to the TRE, there are several other response elements present in many genes, including c-*fos*, which, when bound by specific transcription factors, are involved in regulation of gene transcription (Figure 1.7). The cAMP response element and the Ca^{2+} response element were identified initially as separate elements (Montminy *et al.*, 1986; Sheng *et al.*, 1988), but were then shown to be indistinguishable (Sheng *et al.*, 1990), and it is now accepted that one element exists which is sensitive to activation by pathways beginning with the elevation of either Ca^{2+} or cAMP. Its consensus sequence is similar to that of the TRE, differing only by the addition of one base pair: 5'-TGAC**G**TCA-3'. Due to the similarities between the two sequences, the CRE/CaRE can be bound by AP-1 dimers; indeed, heterodimers between the Fos and Jun families and/or the ATF family have a high affinity for the CRE (Hai and Curran, 1991). However, the CRE binding protein, CREB, has a higher affinity for CRE than does AP-1. Like AP-1, CREB is a transcription factor, but it must be phosphorylated in order to be activated. This

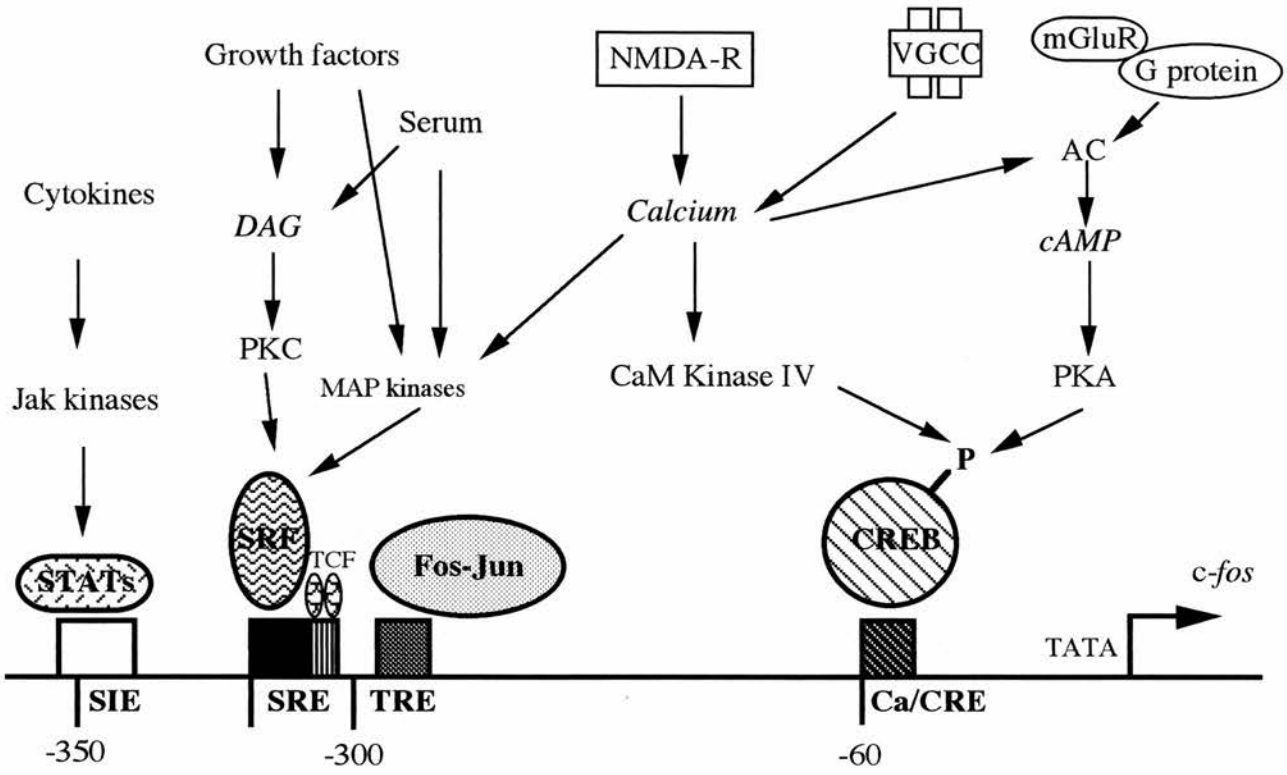


Figure 1.7 Schematic of 5'-flanking regulatory region of the c-*fos* gene indicating major upstream regulatory elements.

The c-*fos* gene includes at least four regulatory element sequences, which, when bound by their specific activation factors, cause transcriptional activation. Cytokines activate Janus kinases (Jaks), causing binding of STAT proteins to the SIS-inducible element (SIE). Growth factors and serum can cause production of diacyl glycerol (DAG), which activates protein kinase C (PKC), which phophorylates the serum response factor (SRF), causing it to bind and activate the serum response element (SRE). Ternary complex factors (TCFs) are also involved in this activation. Serum and growth factors are also able to activate MAP kinases, leading to SRF phosphorylation. An increase in calcium ions, either via NMDA receptor activation or voltage-gated calcium channels (VGCC), can activate the MAP kinase cascade as well as activating CaM kinase IV, implicated in the phosphorylation of CREB, which binds and activates the Ca2+/cAMP response element (Ca/CRE). CREB can also be phosphorylated by cAMP-dependent protein kinase (PKA), which is activated when cAMP levels are increased via adenylate cyclase (AC) activation (either by NMDA-mediated Ca2+ increases or via mGluR binding).

phosphorylation, which occurs in response to depolarisation and increasing cAMP levels (Sheng *et al.*, 1990), can be performed by certain isoforms of both cAMP-dependent protein kinase (PKA) and Ca^{2+}-dependent protein kinase (CaM kinase). Thus both cAMP and Ca^{2+} are able to control the transcriptional regulation of CRE/CaRE, as the name suggests (Sheng *et al.*, 1991). Activation of TRE- and CRE-binding activities induced by NMDA and KA is dependent on the presence of extracellular calcium (Sakurai *et al.*, 1992).

CREB is activated via phosphorylation of a specific serine residue, Ser 133 (Yamamoto *et al.*, 1988; Gonzales and Montminy, 1989). CaMKIV activates CREB in this way in response to Ca^{2+} or calmodulin. CaMKII, however, phosphorylates both Ser 133 and Ser 142. Since phosphorylation of CREB on Ser 142 inhibits CREB-mediated expression, this enzyme is much less effective at stimulating activation of CREB (Sun *et al.*, 1994). CaMKIV is expressed in high levels in the cerebellum (Ohmstede *et al.*, 1989), and thus activation of CREB and therefore induction of genes whose promoters contain the CRE is likely in the event of cell depolarisation or an increase in Ca^{2+} ions due to *e.g.* EAA stimulation. CREB is also thought to form heterodimers with c-Jun and bind to the TRE, thereby regulating gene transcription relating to CNS growth and differentiation (Pennypacker *et al.*, 1995). Since CaMKIV can be localised in the nucleus of cerebellar granule cells (Jensen *et al.*, 1991), it is well placed to activate CREB and thus allow this heterodimerisation to occur.

Expression of the c-*fos* gene itself can be induced in PC12 cells by voltage-gated calcium influx (Morgan and Curran, 1986; Ghosh *et al.*, 1994) and by neurotransmitters (Greenberg *et al.*, 1986), both of which act through the CRE/CaRE via phosphorylation of CREB. Indeed, KCl-induced c-*fos* transcriptional activation was inhibited by the addition of 3 mM EGTA to the medium of PC12 cells (Greenberg *et al.*, 1986).

Ca^{2+}-dependent mechanisms also have an effect on growth and development in neurons. A high level of granule cell-enriched CaM kinase-IV, termed CaM kinase-Gr, has been measured in the rat cerebellum during the first two postnatal weeks, and is thought to be linked to the development of granule cells (Ohmstede *et al.*, 1989; Jensen *et al.*, 1991). In addition, early postnatal AP-1 DNA binding activity may be due to the CREB/c-Jun heterodimer. CREB

transcription factors, acting at AP-1 and CRE DNA sites, may regulate gene transcription relating to CNS growth and differentiation. The cerebellum has higher levels of AP-1 DNA binding activity than any other brain region during development. In general, AP-1 DNA binding activity appears to be high in developing tissues and decreases to basal as growth and differentiation continues (Pennypacker *et al.*, 1995).

1.7 Serum affects activation of transcription

The serum response element (SRE) functions as an inducible enhancer element, and is situated 300 bp 5' to the mRNA start site of the human c-*fos* gene (Norman and Treisman, 1988; Figure 1.4). The core consensus sequence, found by these workers to be present in a number of different SREs tested, is 5'-CC[A/T]$_6$GG-3'. The SRE is bound by a 67 kDa nuclear protein, named the serum response factor (SRF) (Treisman, 1985). Binding can occur in a constitutive manner, or in the presence of growth factor stimulation, and often involves an accessory protein which either recognises the SRF when bound to the SRE or binds to the SRF thus enabling the SRF/accessory protein complex to bind to the SRE (Norman and Treisman, 1988). This accessory protein is now known to be one of a family of ternary complex factors (TCFs), which bind to the Ets site (CAGGAT) which lies just 5' of the SRF binding site (CC[A/T]$_6$GG). p62/TCF, Elk-1 and SAP-1 are members of this family, whose polypeptide sequences contain three conserved regions, known as box A, box B and box C. Boxes A and B mediate ternary complex formation and nuclear location, whereas box C contains five sets of a serine/threonine residue immediately followed by a proline residue, whose phosphorylation is instrumental in activation of transcriptional regulation (for reviews, see Treisman, 1992, 1994). p62/TCF, in particular, is phosphorylated by MAP kinase, causing an increase in the affinity for SRF and thus enhancing ternary complex formation (Gille *et al.*, 1992). SRF is phosphorylated by ribosomal S6 kinase (RSK or MAPKAP kinase-1) in response to mitogenic stimulation which then enhances its affinity for the SRE (Karin and Hunter, 1995). As well as mediating serum-stimulated transcriptional responses, via PKC-dependent and -independent signals but not PKA-dependent signals (Gilman, 1988), the SRE contributes to basal transcription levels in resting cells (Mohun

et al., 1987; Konig *et al.*, 1989). The SRE is also thought to be involved in cellular responses to insulin (Stumpo and Blackshear, 1986) and nerve growth factor (Visvader *et al.*, 1988). The c-*fos* gene contains, among other response elements, the SRE, and transcriptional activation via this response element is regulated in particular by the presence of differing levels of Ca^{2+} ions (Ghosh *et al.*, 1994).

1.8 The Fos family of immediate-early genes

In addition to c-Fos, there are three other members of the Fos family of cellular immediate-early genes (Table 1.1). All the genes are highly homologous, and many commercially-available antibodies recognise all four gene-encoded proteins, suggesting that they have similar accessible epitopes (for information on the antibodies used in this study, see Appendix 1). In this way, large numbers of possible dimers can be formed, from a relatively small pool of proteins, by dimerisation of a Fos family member with the Jun family or other members of the bZIP superfamily. There are some important differences, however, in the abilities of the Fos-related proteins to bind to the various other members of the bZIP superfamily, and also, as detailed in subsequent chapters, in their responses to toxicity and depolarisation. The variety of different AP-1 factor combinations may allow for specific targeting to different genes containing the TRE sequence (Zerial *et al.*, 1989).

Protein name	**Protein size**	**Reported in:**
c-Fos	~ 62 kDa	Curran and Teich, 1982
FosB	~ 48 kDa	Zerial *et al.*, 1989
Fra-1	~ 35 kDa	Cohen and Curran, 1988
Fra-2	~ 46 kDa	Nishina *et al.*, 1990

Table 1.1 Fos family members

The *fos*B gene was cloned in 1989 and found to have a 70% homology with c-*fos* (Zerial *et al.*, 1989). The gene-encoded protein, FosB, was found to be located in the nucleus,

suggesting that this protein, like c-Fos, played a part in transcriptional regulation. FosB was also found to positively influence the binding of c-Jun and JunB to AP-1 binding sites, and is thus implicated in control of gene expression (Zerial *et al.*, 1989). When the genomic structure was determined (Lazo *et al.*, 1991), it was discovered that the *cis* elements CRE, SRE and AP-1 are in the 5' upstream region in both c-*fos* and *fos*B. Further information on the role of FosB as a transcriptional regulator was reported by Metz *et al.* (1994), who described how FosB associates with the TATA binding protein (TBP) and thus the multiprotein complex transcription factor IID (TFIID). Transcriptional activation by FosB is dependent on its phosphorylation within a cluster of serine residues in the C-terminal activation domain (Skinner *et al.*, 1997). The kinase responsible is thought to be distinct from the mitogen-activated protein kinase (MAP kinase) and stress-activated protein kinase (SAP kinase) groups of enzymes.

In addition, a naturally-occurring, truncated form of FosB (FosB/SF (short form), Δ-FosB or FosB2) has been isolated (Dobrzanski *et al.*, 1991; Nakabeppu and Nathans, 1991; Yen *et al.*, 1991). This protein, formed by alternative splicing of exon 4, does not contain the 101 amino acids found at the C-terminal end of FosB. Included in this section of the gene is the TATA box binding protein (TBP) binding motif, or TBM, which is thought to be involved in transformation of cell lines. Thus, unlike FosB and c-Fos, FosB/SF is unable to interact with TBP or transform these cell lines (Metz *et al.*, 1994). Like FosB, FosB/SF is able to form heterodimers with members of the Jun family, and bind to the TRE. However, heterodimers containing FosB/SF are unable to activate transcription. In this way, FosB/SF can competitively prevent Jun and Fos/Jun dimers from activating transcription at AP-1 binding sites, and also prevents the repression of the c-*fos* promoter which occurs in the case of binding of c-Fos or FosB protein.

In 1988, Cohen and Curran described a protein which was antigenically related to Fos, and which was induced by serum. In their study using fibroblast cultures, they found that the gene encoding this Fos-related antigen, *fra-1*, had significant sequence homology to c-*fos* (Cohen and Curran, 1988). The protein, Fra-1, was also very similar to c-Fos, having large sections of highly conserved residues. It was suggested that these areas (which also corresponded with those

of Jun) might be involved in DNA binding, and that the non-conserved, hydrophilic regions might confer specificity to each protein. It is thought that the expression of Fos-related antigens is induced later than that of c-Fos, and that translation of Fra proteins continues after that of c-Fos has terminated (Sonnenberg *et al.*, 1989b). One important dimerisation is between Fra-1 and ATF-4, which has a higher dimerisation affinity than c-Jun/ATF-4 or c-Fos/ATF-4, and binds to the CRE more readily than it binds to the TRE. This heterodimer can bind to the TRE and thus regulate transcription (Hai and Curran, 1991).

The second Fos-related antigen, Fra-2, was named by Nishina *et al.* (1990). It too contains a leucine zipper and a basic region (DNA-binding site), and functions in a similar way to other Fos family members. Like Fra-1, it is serum-inducible (Nishina *et al.*, 1990).

1.9 The Jun family of immediate-early genes

Sequenced and named by Maki *et al.* (1987) from avian sarcoma virus 17, c-Jun had been previously identified as a 39 kDa protein (named p39) which interacted with c-Fos and bound the AP-1 recognition site (Chiu *et al.*, 1988; Rauscher *et al.*, 1988a; Sassone-Corsi *et al.*, 1988b). Like all the other bZIP proteins, c-Jun has a basic DNA-binding domain and a leucine zipper region. In addition, c-Jun contains a proline- and glutamine-rich activation domain which lies N-terminal to the DNA-binding domain. The combination of this domain of c-Jun and the acidic activation region of c-Fos causes a more than additive stimulation (Abate *et al.*, 1990a).

Dimerisation of Fos and Jun proteins is known to give rise to an AP-1 transcription factor complex with much higher affinity (up to 30-fold higher) for the TRE than simply a c-Jun homodimer (Kouzarides and Ziff, 1988; Sassone-Corsi *et al.*, 1988d; Zerial *et al.*, 1989). Earlier studies claimed that c-Jun was the DNA-binding protein and that c-Fos merely enhanced the affinity of c-Jun without contacting the DNA itself (Chiu *et al.*, 1988; Rauscher *et al.*, 1988b). However, Abate *et al.* (1990b) have reported that in a dimer comprised of c-Fos and c-Jun, both proteins contact the DNA directly. Like c-Fos, c-Jun binds to other leucine zipper proteins to form dimers (Sassone-Corsi *et al.*, 1988d), including ATFs 2, 3, and 4 (Hai and Curran, 1991). In fact, a c-Jun/ATF heterodimer has been shown to bind the TRE of the c-*jun* gene constitutively

(Rozek and Pfeifer, 1993). Expression of c-Jun protein is induced by a variety of factors including growth factors and excitatory amino acids. Induction of c-*jun* by kainate has been shown to be part of the process of apoptosis in certain cell types (Cheung *et al.*, 1998).

The *jun*B gene is a member of the Jun family of immediate-early genes due to the high level of homology which it shares with c-*jun*. However, the human *jun*B sequence shows more sequence homology to the mouse *jun*B gene than it does to the human c-*jun* gene (Schütte *et al.*, 1989). Sequence is highly preserved in the C-terminal, DNA-binding domain as well as the N-terminal, activator domain of the *jun*B and c-*jun* genes (Ball *et al.*, 1988; Vogt and Bos, 1989). Association between the gene-encoded protein, JunB, and c-Fos has been demonstrated *in vitro* (Nakabeppu *et al.*, 1988). One interesting role of the JunB protein is its inhibitory action on the c-*jun* promoter (Schütte *et al.*, 1989), which is thought to prevent c-*jun* from being permanently activated (Chiu *et al.*, 1989), which would presumably happen in the absence of JunB due to the binding of c-Jun/ATF-3.

JunD was isolated in 1988 by two groups (Ryder *et al.*, 1988; Hirai *et al.*, 1989). It has two main regions of homology with JunB and c-Jun; the first includes a DNA-binding domain and dimer formation sequences, and the second contains an acidic region thought to be involved in gene activation. It is thought to be produced constitutively in many areas (Hope *et al.*, 1994; Hou *et al.*, 1997), although levels in the brain are less than in other organs (*e.g.* spleen, lung, heart). Unlike JunB and c-Jun, expression of JunD is only slightly stimulated by serum growth factors, suggesting that JunD is regulated differently from the other family members (Ryder *et al.*, 1988). Further evidence for this is found in a report by Castellazzi *et al.* (1991), where fibroblasts were stimulated to enter the cell cycle and the transcription of the Jun family members was measured. They found that there was a rapid but transient activation of transcription of c-*jun* and *jun*B, but that *jun*D transcription was only weakly activated over basal levels. In addition, the Jun family members respond differently to activation of kinase-dependent signal transduction pathways and hormone receptors.

1.10 Phosphorylation affects the activity of Fos and Jun

Much work has been done to study the mechanisms involved in regulation of the AP-1 transcription factor complex. Phosphorylation of proteins is a particularly effective and widespread mechanism for regulation of gene expression as a result of stimulation on the cell surface (Hill and Treisman, 1995; Karin and Hunter, 1995). It was discovered that much of the c-Jun protein present in resting cells is phosphorylated on one or more of three sites in the DNA-binding domain (Boyle *et al.*, 1991). Serine and threonine residues are thought to be phosphorylated by glycogen synthase kinase (GSK-3), and on phosphorylation, in response to phorbol esters via protein kinase C, the protein becomes more likely to take part in the AP-1 transcription factor complex and thus bind to specific DNA sites (Boyle *et al.*, 1991). However, it is also known that phosphorylation can have a positive effect on the activity of c-Jun. In this case, the serine residues involved are situated near the N-terminal of the protein (Ser 62 and 72 (human)), and are thought to be phosphorylated by mitogen-activated (MAP) kinases, due to the presence of proline residues immediately following both serine sites (Pulverer *et al.*, 1991). In this way, the phosphorylation state of c-Jun must be finely balanced in order for dimerisation and AP-1 activation to occur. Similarly, Thr-232 of c-Fos (analogous to Ser-73 in c-Jun) is phosphorylated, but not by a MAP kinase. The enzyme responsible in this case is a Ras-activated protein kinase known as Fos regulating kinase, or FRK (Deng and Karin, 1994), which causes a stimulation of the transcriptional activity of c-Fos in response to growth factors but not phorbol esters.

1.11 Several pathways are implicated in the induction of cIEGs, mediated by various extracellular stimuli and second messenger systems and leading to different results

The induction of immediate-early genes can be stimulated by a large number of agents such as: growth factors, mitogens, EAAs, UV radiation, cytokines, T cell activators (Angel and Karin, 1991). These diverse stimuli act through different receptors and second messenger systems in order to achieve their objective. It is beyond the remit of this thesis to delve too deeply

into the myriad of pathways which lead to cIEG induction. However, a brief overview of some of the more common pathways follows (for a review, see Karin and Hunter, 1995).

cAMP-dependent protein kinase (PKA) is a tetramer of two of each of a regulatory subunit and a catalytic subunit. In response to activation of G-protein-coupled receptors, ATP is converted to cAMP, which binds to PKA, causing the release of the catalytic subunits. These are translocated to the nucleus, where they are able to phosphorylate CREB on Ser 133. CREB, already constitutively bound to the CRE of the target gene, is then activated and able to stimulate transcriptional activation.

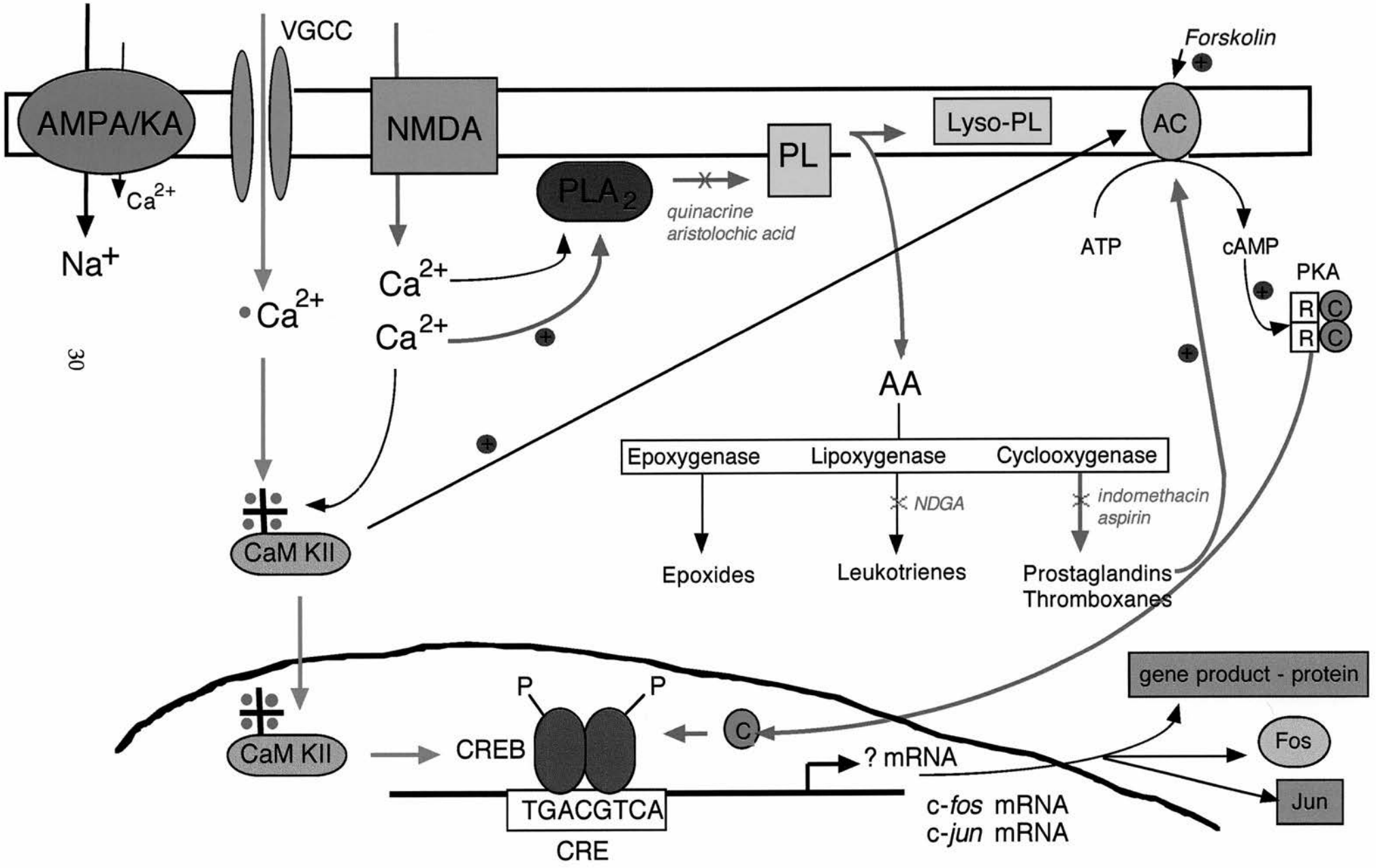
Mitogen-activated protein kinases (MAPKs) are involved in the signalling mechanism which begins with activation of growth factor receptors on the cell membrane and which culminates in the induction of cIEGs. There are several types known in vertebrate systems; extracellular signal-regulated kinases (ERKs), Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs) and p38/Mpk2/cytokine-suppressive anti-inflammatory drug binding protein (CSBP). These protein kinases are activated by phosphorylation on threonine and tyrosine residues by MAPK kinases (MAPKKs). These kinase cascades are involved in AP-1 formation and activation in several ways. ERKs are involved in phosphorylation of the ternary complex factor (TCF) which is part of the complex involving the SRE situated in the promoter of the c-*fos* gene. JNK/SAPKs, in contrast, phosphorylate c-Jun (on Ser 63 and 73, situated in the activation domain) and ATF-2, both of which are constitutively bound to the TRE, in order to enhance their transcriptional activities and cause transcription of c-*jun*. In this way, after translation of each of the proteins, the amount of AP-1 factor present is increased. In addition, the transcriptional activity of this complex is enhanced by phosphorylation of c-Jun protein by JNK/SAPK (Karin and Hunter, 1995).

Figure 1.8[2]. shows a diagrammatic representation of the pathway which involves

---

[2] **Figure 1.8 Schematic showing the involvement of phospholipase A_2 (PLA_2) in the NMDA receptor-mediated induction of c-*fos* and c-*jun* mRNA, leading to expression of proteins which are possible components of the AP-1 transcription factor.**

Enzyme inhibitors studied in this work are marked in red.


AMPA/KA
VGCC
NMDA
Forskolin
Lyso-PL
AC
PL
PLA2
quinacrine
aristolochic acid
Ca2+
Na+
Ca2+
Ca2+
Ca2+
ATP
cAMP
PKA
R
C
R
C
AA
Epoxygenase
Lipoxygenase
Cyclooxygenase
NDGA
indomethacin
aspirin
Epoxides
Leukotrienes
Prostaglandins
Thromboxanes
CaM KII
CaM KII
P
P
CREB
C
? mRNA
gene product - protein
Fos
Jun
TGACGTCA
CRE
c-fos mRNA
c-jun mRNA

phospholipase A_2 (PLA_2) and finally culminates in induction of cellular immediate-early gene expression or production of arachidonic acid. Stroke or head trauma can lead to increased release of Glu into the extracellular space which is then bound by the NMDA receptor and leads to PLA_2 activation. This in turn results in arachidonic acid and free radical production, finally leading to cell death either by apoptosis or necrosis (Nicotera *et al.*, 1996). Katsuki *et al.* (1995) claim that neuronal death due to arachidonic acid toxicity is entirely due to the production of free radicals via lipoxygenase activity, and that the death is necrotic rather than apoptotic since high levels of K^+ and cAMP are unable to protect against this toxicity. Tumour necrosis factor (TNF) is able to activate this pathway and cause release of arachidonic acid, leading to the induction of c-*fos* mRNA (Haliday *et al.*, 1991) in its attempt to cause cancerous cells to commit suicide via apoptosis. Kacich and colleagues were the first to report induction of c-*fos* via a pathway not involving protein kinase C (Kacich *et al.*, 1988). Lipoxygenase inhibitors (*e.g.* norhydroguariaretic acid (NDGA), see Figure 1.9 for structure) are able to prevent TNF-mediated induction of c-*fos*; however, inhibitors of cyclooxygenase (*e.g.* aspirin, indomethacin) have no effect. NMDA receptor activation has been reported to stimulate release of arachidonic acid, an endpoint of the PLA_2 pathway, in striatal cells, cerebellar granule cells and hippocampal cells (Okuda *et al.*, 1994).

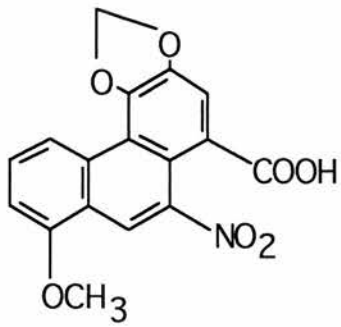
Several stages in the PLA_2 activation pathway can be blocked by specific inhibitors (Figure 1.8). Since the release of high levels of arachidonic acid is known to be involved in cytotoxicity (Carine and Hudig, 1984; Okuda *et al.*, 1994), attempting to block the production of arachidonic acid using inhibitors would be an obvious next step. Bazán (1989) reports that an increase in the synthesis of prostaglandins and leukotrienes (metabolites of arachidonic acid) is a consequence of epileptic and other cerebral pathologies, thus linking eicosanoid production to cell death. Prostaglandins, products of cyclooxygenase activity, are able to stimulate adenyl cyclase to produce cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA), causing phosphorylation of CREB and induction of cellular immediate-early gene expression. Production of c-Fos is thought to have a role in cell death under certain circumstances (Colotta *et al.*, 1992; Gillardon *et al.*, 1994; 1995; Ham *et al.*, 1995; Meredith *et al.*, 1996). Thus

if inhibition of this pathway could also prevent Glu-induced cytotoxicity, a link could be made between toxic Glu and the production of immediate-early gene products. Lerea and McNamara (1993) have reported the involvement of the phospholipase A_2 pathway via cyclooxygenase activity in NMDA receptor-mediated induction of c-*fos* mRNA in primary cultures of dentate gyrus cells. The structures of the inhibitors quinacrine; aristolochic acid, which directly inhibits phospholipase A_2 (Vishwanath *et al.*, 1988; Rosenthal *et al.*, 1989); indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid; Ferreira and Vane, 1974; Hong and Levine, 1976); aspirin (acetylsalicylate; 2-acetyloxy-benzoic acid), which inhibits prostaglandin synthesis by cyclooxygenase (Vane, 1971); and norhydroguariaretic acid (NDGA; Chang *et al.*, 1984; Miyazawa *et al.*, 1985; Van Wauwe and Goossens, 1983) are shown in Figure 1.9.

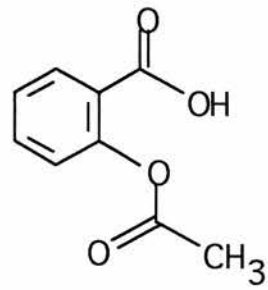
In some of the experiments reported here (Western blotting, transfection studies), forskolin or phorbol 12-myristate-13-acetate (PMA, a phorbol ester) were used as positive controls for c-Fos expression. Forskolin activates cAMP accumulation, and PMA is a protein kinase C activator, causing activation of two different cascades, both of which eventually lead to transcription of immediate-early genes (Monstein and Folkesson, 1991; Barthel *et al.*, 1996).

1.12 Aims and objectives

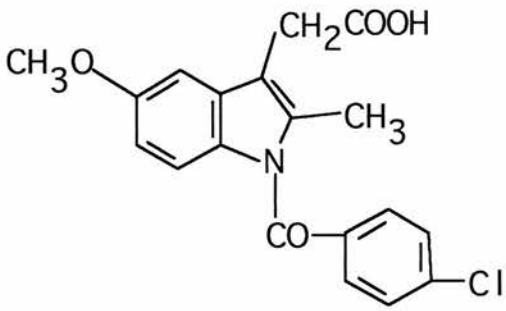
The aim of this study was to integrate with a collaborative project which was already ongoing in the laboratory where the author carried out the work. This EC-funded BIOTECHNOLOGY project was concerned with the development of a predictive *in vitro* test for excitotoxicity, following evidence which suggested a link between c-*fos* mRNA induction and excitotoxicity (Gorman *et al.*, 1995). The model system used for these studies was primary cultures of cerebellar granule cells. These cells express NMDA, non-NMDA and metabotropic glutamatergic receptors, and have been widely used to investigate the mechanism of toxicity induced by excitatory amino acid stimulation. More precisely, in cerebellar granule cells cultured for 7 days (7 DIV cells), treatment with low and high levels of Glu were found to elicit differing responses in terms of c-*fos* mRNA induction. After treatment with low, non-toxic (10 μM) levels of Glu, the time-course of mRNA induction was transient, peaking at 1h after treatment and



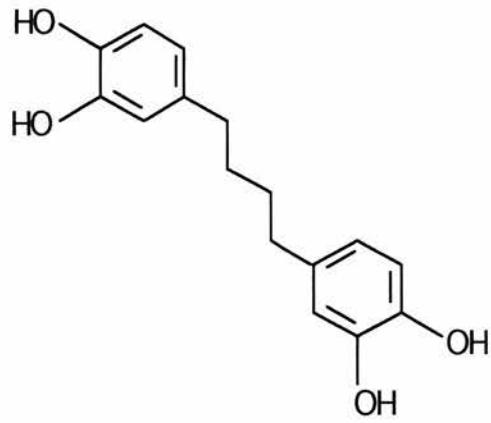
ARISTOLOCHIC ACID



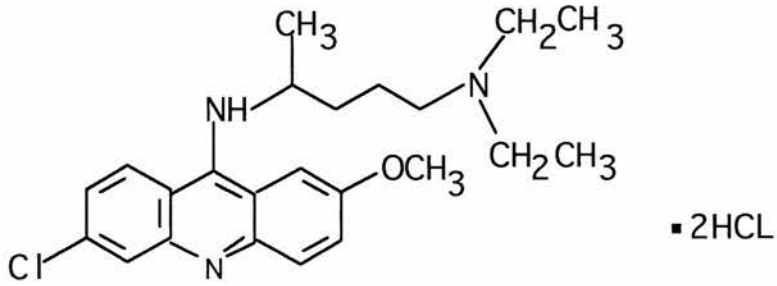
ACETYLSALICYLATE (ASPIRIN)



INDOMETHACIN



NORHYDROGUARIARETIC ACID



QUINACRINE DIHYDROCHLORIDE

Figure 1.9 Structures of compounds involved in inhibition of the PLA_2 pathway.

Aristolochic acid and quinacrine dihydrochloride inhibit the action of PLA_2 on phospholipids to create lyso-phospholipids and arachidonic acid.
Acetylsalicylate (aspirin) and indomethacin inhibit the production of prostaglandins and thromboxanes from arachidonic acid by cyclooxygenase.
Norhydroguariaretic acid (NDGA) inhibits the processing of arachidonic acid into leukotrienes by lipoxygenase.

returning to basal at 4h. However, if the cells were treated with high, toxic (250 μM) levels of Glu, the mRNA induction profile was quite different: the increase from basal was delayed and peaked at a higher level than with the low level of Glu, and the response did not return to basal until much later (6h). The test that was developed involved measurement of the c-*fos* mRNA levels at two distinct time points, namely 30 and 240 min after treatment with an excitatory amino acid (EAA). The ratio of these values (defined as the $Q^{240/30}$ ratio) was suggested as a predictive value, where concentrations of EAAs giving a $Q^{240/30}$ value of < 1 were non-excitotoxic and those giving a $Q^{240/30}$ value of > 2 were excitotoxic (Gorman *et al.*, 1995; Malcolm *et al.*, 1997; Griffiths *et al.*, 1997). The project reported herein was initiated to study the protein levels of the Fos and Jun family members produced in these cells but predominantly to explore the composition of the AP-1 transcription factor complex in response to toxic and non-toxic events and at different stages of development in culture. In particular, the aims were as follows:

- Objective 1: To evaluate the dynamic changes in AP-1 transcription factor complex composition in 7 DIV cultures of murine cerebellar granule cells, using the electrophoretic mobility shift assay and Western blotting, thus demonstrating the translation of c-*fos* mRNA to protein.
- Objective 2: To evaluate the functionality of these protein complexes using transient transfection technology.
- Objective 3: To further probe the Glu-mediated induction of cIEGs in 7 DIV cultures by exploring a specific pathway.
- Objective 4: To determine possible developmental differences in the responses of 7 DIV and 2 DIV granule cells to stimulation with Glu by studying cytotoxicity as well as $[Ca^{2+}]_i$, mRNA and protein levels, and, where appropriate, comparing the effects of K^+ ions to those of Glu.

In Chapter 2, the methods and sources of materials used are described. The work carried out is reported in Chapters 3 and 4 of this thesis. Chapter 3 outlines the studies on mature, 7 DIV murine cerebellar granule cell cultures, from Glu-induced toxicity and Ca^{2+} influx to the effect of

Glu on mRNA and protein expression. In order to carry out a comparative study, it was decided to explore the induction patterns of the gene responsible for encoding the fos-related antigen, *fos*B. The hypothesis of this part of the work was that FosB may be a more valid marker of toxicity, since in preliminary work using gel supershift analysis it was found that FosB was only a component of the AP-1 transcription factor complex under toxic circumstances (7 day-old cells which were stimulated with 250 μM Glu in the presence of serum) and not after membrane depolarisation by high K^+ levels. In addition, the effect of the PLA_2 pathway inhibitors described in Section 1.11 on Glu-induced responses in these cells was investigated.

In Chapter 4, the response of immature, 2 DIV cultured cerebellar granule cells to Glu and KCl was studied, again by monitoring toxicity, intracellular calcium concentration ($[Ca^{2+}]_i$), mRNA induction and protein expression. In Chapter 5, the results of the previous two chapters are discussed together, in order to draw some conclusions on the developmental effects on Glu-mediated immediate-early gene induction and expression in these cells.

Publications arising from this work to date are:

Lidwell, K., Boutillier, L., Loeffler, J-Ph., Malcolm, C., Meredith, C. and Griffiths, R. (1997) Differential profiles of c-*fos*/c-*jun* mRNA induction and of AP-1 transcription factor composition during the development of cerebellar neurons *in vitro* following exposure to physiologic and excitotoxic levels of glutamate. *Journal of Neurochemistry* **69**: S36A (Suppl.).

Griffiths, R., Ritchie, L., Lidwell, K., Grieve, A., Malcolm, C.S., Scott, M. and Meredith, C. (1998) Calcium influx via L-type voltage-gated channels mediates the delayed, elevated increases in steady-state c-*fos* mRNA levels in cerebellar granule cells exposed to excitotoxic levels of glutamate. *Journal of Neuroscience Research* **52**: 641-652.

Chapter 2

Materials and Methods

2.1 Materials

Mice (CD1 strain) were obtained from Charles River UK Ltd (Kent, UK). Cell culture plasticware was obtained from Nunc A/S (Roskilde, Denmark) and Corning (Corning, NY). Guanidinium isothiocyanate, Neurobasal™ medium, B-27 medium supplement and neural cell medium was from Life Technologies Ltd. (Paisley, UK). Foetal calf serum was supplied by Sera-Lab Ltd. (Sussex, UK). D-glucose, HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid), KCl, $MgSO_4$, sodium acetate and sodium citrate (all Analar grade) were obtained from Merck Ltd. (Dorset, UK). Acetylsalicylic acid, *p*-aminobenzoic acid, aprotinin, cytosine arabinoside, DNAse, DTT (dithiothreitol), Ficoll, forskolin, L-glutamine, indomethacin, insulin, 2-mercaptoethanol, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), quinacrine, penicillin G, phenol red (phenolsulfonphthalein), polydIdC (polydeoxyinosinic-deoxycytidylic acid), poly-L-lysine, putrescine hydrochloride (tetramethylenediamine), sarkosyl, bovine serum albumin, sodium selenite, spermidine trihydrochloride, T3 (3,3',5-triiodo-L-thyronine), TEMED (N,N,N',N'-tetramethylethylenediamine), apo-transferrin, trypsin, soybean trypsin inhibitor, Trizma base (tris[Hydroxymethyl]aminomethane) and Tween 20 (polyoxyethylenesorbitan monolaurate), were obtained from Sigma Chemical Co. (St. Louis, MO). PEI (Polyethylenimine) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Chloroform (Analar), iso-amyl alcohol (Analar), isopropanol (Analar) and Triton X-100 were from Fisons Scientific Equipment (Leicestershire, England). Phenol (water-saturated, HPLC grade) was from Camlab Ltd. (Cambridge, UK). Ethanol (HPLC grade) was obtained from Rathburn Chemicals Ltd. (Walkerburn, UK). All antibodies for electrophoretic mobility shift assay (EMSA) and primary antibodies for Western blotting were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibody (rabbit anti-goat) for Western blotting was supplied from Dakopatts (Sweden) and tertiary antibody (anti-rabbit-HRP conjugated) and ^{14}C-chloramphenicol and γ-^{32}P ATP were from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). Prestained SDS-PAGE standards and nitrocellulose membrane were obtained from Biorad Laboratories (Hercules, CA). Acrylamide/Bisacrylamide solution was supplied by Scotlab (Coatbridge, UK). Luciferase assay system was supplied by Promega

(Madison, WI). T4 polynucleotide kinase and buffer were obtained from New England Biolabs, Inc. (Beverley, MA). L-glutamate, D,L-2-amino-5-phosphonopentanoic acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX) were obtained from Tocris Cookson Ltd. (Bristol, UK). N-[1-(2-thienyl)cyclohexyl]-piperidine (TCP) and nifedipine were obtained from Research Biochemicals, Inc. (Natick, MA). The acetoxymethyl ester of Fluo-3 (Fluo-3/AM) and Pluronic F-127 were purchased from Molecular Probes, Inc. (Eugene, Oregon). A23187 was obtained from Fluka Chemicals Ltd. (Dorset, UK). Aristolochic acid and nordihydroguaiaretic acid were obtained from Biomol Research Laboratories, Inc. (distributed by Affiniti research products Ltd., Mamhead, Exeter, UK). X-Ray film was from Fuji Photo Film Co., Ltd. (Tokyo, Japan). All other chemicals were of the purest grade available from regular commercial sources.

2.2 Methods

2.2.1 Granule cell culture

Primary cultures of cerebellar granule cells were prepared essentially as described by Schousboe *et al.* (1989). Cerebella from 7-day-old CD1 mice were dissected and the tissue roughly macerated using a sterile razor blade. The tissue was transferred to a centrifuge tube containing Solution 1 (14 mM D-glucose; 3 mg/ml bovine serum albumin; 1.2 mM $MgSO_4$; 15 μg/ml phenol red in PBS [0.137 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4]) and centrifuged at 100g for 5 seconds. The supernatant was removed and the pellet was resuspended in Solution 2 (as Solution 1 but with added: 0.25 mg/ml trypsin; 0.04 mg/ml deoxyribonuclease I type IV) and incubated at 37°C for 15 min with gentle agitation every 5 min. Solution 4 (Solutions 1 and 3 [as Solution 1 but with added: 0.52 mg/ml soybean trypsin inhibitor; 0.04 mg/ml deoxyribonuclease I type IV; 1.5 mM $MgSO_4$] at 6.25:1 ratio) was added and the mixture was centrifuged for 5 min at 100g. The supernatant was removed and the pellet was triturated 10 times in 7 ml of Solution 3, keeping the pipette tip firmly pressed against the bottom of the tube during expulsion.

6 ml of N-DMEM (Dulbecco's modified Eagle medium supplemented with 19.5 mM KCl, 30 mM D-glucose, 0.8 mM L-glutamine, 7 μM *p*-aminobenzoic acid, insulin (100 mU/L), penicillin G (5 x 10^5 U/L) and foetal calf serum (10% v/v, heat inactivated)) were added and the suspension was centrifuged for 5 min at 100g. The supernatant was removed and the cell pellet was resuspended in 20 ml N-DMEM, which was then diluted with N-DMEM to give the required cell density. Cells were plated in 96-well (100 μl per well) and 12-well (1.5 ml per well) plates at a density of $25x10^6$ cells per plate or 6-well plates ($5.5x10^6$ cells in 2 ml per well,) or 35 mm dishes ($5.5x10^6$ cells in 2 ml per dish) or 90 mm dishes ($25x10^6$ cells in 10ml per dish) pre-coated with a 1% (w/v) solution of poly-L-lysine (this was left on plasticware for 2 h at 37°C or overnight at room temperature and then washed off with double the amount of water before plating out). Cytosine arabinoside (final concentration 20 μM) was added 48h after plating to prevent glial proliferation; under these conditions, a 95% neuronal culture is obtained. Experiments were performed on cells after 2, 5 or 7 days in culture. The concentration of Glu in this growth medium is 1.05 ± 0.2 μM (Gorman and Griffiths, 1994).

2.2.2 Assessment of cytotoxicity using the 3-[4.5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

This spectrophotometric method is claimed to measure the viability of cells on the basis of mitochondrial dehydrogenase activity (Mosmann, 1983; Balázs *et al.*, 1988a). MTT is yellowish in colour when it is dissolved in HBS-1 (125 mM NaCl; 5 mM KCl; 20 mM $NaHCO_3$; 50 mM HEPES; 5 mM D-glucose; 0.9 mM $CaCl_2$, pH 7.4) without phenol red. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring selectively, yielding blue/purple formazan crystals (Figure 2.1). The crystals are dissolved in acidified isopropanol, and the resulting colour is read spectrophotometrically. A decrease in absorbance compared with control cells provides a quantitative assessment of cell damage.

Cells were cultured as described above in 96-well plates (Nunc) for 2 or 7 days. The growth medium was removed from the cells and they were immediately washed twice with 100 μl

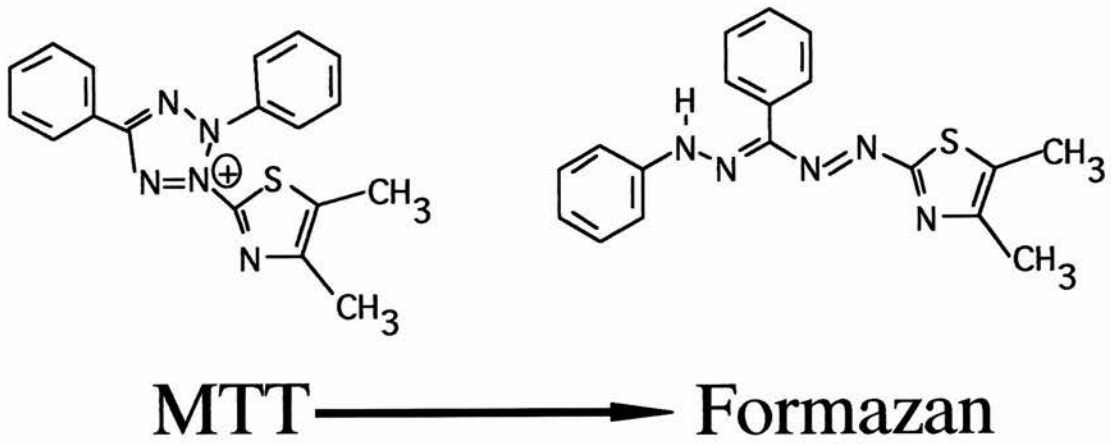


Figure 2.1 Conversion of MTT to formazan, measured in the MTT assay

HBS-1 per well at 37 °C. The cells were then exposed for 15 min with agonist and/or antagonist (in HBS-1) in 50 μl quantities. After this time, the cells were either assayed for cell damage immediately or the test solution was replaced with 100 μl of culture-conditioned medium per well and the cells allowed to recover for 24h at 37°C in a humidified atmosphere of 5% CO_2/95% air.

To assay for cell damage, the cells were washed with 125 μl HBS-1 per well and then 50 μl of MTT solution (made up in HBS-1, final concentration 0.2 mg/ml) was added to each well. The cells were incubated in the dark at 37 °C for 10 min before addition of 125 μl of 10% Triton X-100/0.04M HCl made up in anhydrous isopropanol. The plates were then wrapped in aluminium foil and left overnight at 4 °C to ensure solubilization of the blue/purple formazan crystals. The absorbance was read at 570 nm in a Dynatech MR5000 plate reader and cell viability expressed as a percent of control (untreated, 'medium alone' cells).

2.2.3 Measurement of intracellular-free Ca^{2+} concentration

Fluo-3/AM loading and fluorescence measurements (excitation wavelength, 490 nm; emission wavelength, 538 nm) were performed essentially as described by Kardos *et al.* (1995). Cerebellar granule cells were cultured in 96-well plates (Nunc) for 2 or 7 days. Growth medium was removed from the cells by 'snap-inversion' of the plate onto an absorbent towel, and the cells were washed (100 μl per well) with balanced salt solution (BSS; 125 mM NaCl, 5 mM KCl, 1.0 mM $MgSO_4$, 0.9 mM $CaCl_2$, 50 mM HEPES, 20 mM $NaHCO_3$ and 5 mM D-glucose, pH 7.4) at 37°C. After washing, BSS was removed by 'snap-inversion' and replaced with 30 μl per well BSS (1st lane, control lane) or loading buffer (4.95 ml BSS + 50 μg Fluo-3/AM dissolved in 12.5 μl DMSO + 22 μl Pluronic F-127 solution [25 mg in 75 μl DMSO]) (2nd to 12th lanes, test lanes). The plate was then incubated in the dark for 15 min at room temperature, after which a further 30 μl BSS was added to each well and the plate incubated in the dark for 1h at 37°C. Loading buffer was then decanted from the cells by 'snap-inversion' and the cells were washed twice with 100 μl per well of BSSF (BSS plus 1 mM furosemide) at 37°C. After washing, 50 μl per well of BSSF was added and the fluorescence of the plate (loading control fluorescence) measured in the fluorescence plate reader. The BSSF was then removed from the cells, again by

snap-inversion, and the test solutions (agonist +/- antagonist/inhibitor made up in modified BSS [Mg^{2+}-free]) were added (50 μl per well). The test fluorescence (F) was then measured in the plate reader. Test solutions were removed from the cells and then 50 μl per well of the Ca^{2+} ionophore A23187 (10 μM in BSSF) was added and the plates incubated in the dark at 37°C for 15 min. This allows saturation of the intracellular Fluo-3 with Ca^{2+} ions and thus a maximal fluorescence value (F_{max}) can be measured. $CuSO_4$ (50 μl per well; dissolved in 0.9% NaCl to a final concentration of 5 mM) was then added to quench the maximal fluorescence. After 5 min incubation at 37°C the minimal fluorescence (F_{min}) was measured. The intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ can be calculated using the formula:

$[Ca^{2+}]_i = K_D (F-F_{min})/(F_{max} - F)$, where $K_D = 450$ mM.

2.2.4 Isolation of RNA by guanidinium isothiocyanate/phenol

Total RNA was isolated by a modification of the method of Chomczynski and Sacchi (1987). Cells were cultured in 35 mm Petri dishes (Corning) for 2 or 7 days. At least 17 h before the experiment, the volume of medium was adjusted to 1.5 ml by aspirating and replacing the same medium to enhance the accuracy of the final concentration of the agonist added. After exposure to agonist for the desired time period, the growth medium was removed from the cells and replaced immediately with 500 μl Solution D (6.3M guanidinium isothiocyanate; 39 mM sodium citrate, pH 7.0; 0.8% sarkosyl in 0.1% diethylpyrocarbonate (DEPC)-treated H_2O; to this, 0.72% 2-mercaptoethanol was added immediately before use). Lysates were then homogenised for 20 seconds using a hand-held homogeniser (Camlab Ltd., Cambridge, UK). Two molar sodium acetate, pH 4.0 (50 μl), 500 μl phenol and then 100 μl cholorform:iso-amyl alcohol (49:1) were added, with thorough mixing immediately after each addition. The samples were left on ice for 15 min, then centrifuged at 14000g for 20 min at 4°C. The upper aqueous phase was aspirated and transferred to a clean tube. Isopropanol (500 μl) was added to each sample, again with thorough mixing. The samples were then kept at -20°C for at least 1h, before being centrifuged at 14000g for 20 min at 4°C. The supernatant was aspirated and 150 μl Solution

D then 150 μl isopropanol were added, with thorough mixing after each addition. Samples were stored at -20°C for at least 1h.

The following steps and the next section, "Dot blotting of mRNA extracts", were carried out by personnel at BIBRA International, Carshalton, Surrey, UK.

After centrifugation at 14000g for 20 min, 500 μl of 75% (v/v) ethanol was added to each sample, with mixing. The samples were then centrifuged for 10 min at 14000g and the supernatant was discarded. The pellet was dried on a centrifugal evaporator for 15 min and then re-dissolved in 50 μl DEPC-treated water. Solubilization was effected by incubation in a water bath at 65°C for 10 min, and the samples were stored at -70°C until required.

2.2.5 Dot blotting of mRNA extracts

RNA samples were combined with 3% (v/v) formaldehyde/25% (v/v) formamide/0.06M MOPS, pH 7.0 buffer, incubated at 65°C for 15 min then placed on ice until applied to the membrane. Samples (100 μl) were dot-blotted onto a GeneScreen Plus filter (NEN Dupont [UK] Ltd., Stevenage, UK) using a Dot-blot manifold (Life Technologies, Paisley, UK). The membrane was removed, allowed to dry in a fume hood and then baked at 80°C for 2 h to fix the RNA to the membrane. The membrane was incubated in Pre-hybridisation Solution [5X SSPE (0.75M NaCl, 50 mM $NaH_2PO_4.2H_2O$, 5 mM EDTA); 50% (w/v) deionized formamide; 5X Denhardt's solution (0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidine); 1% SDS; 100μg/ml heat-denatured non-homologous (salmon sperm) DNA] at 42°C for 2-4 h with gentle agitation. The solution was then replaced with fresh Pre-hybridisation Solution (minus the non-homologous DNA). A ^{32}P-labelled probe was heated to 95-100°C for 10 min then chilled on ice to denature it. The specific activity of the probe was 1-5 x 10^9 dpm/μg. This was added to the solution (10 x 10^6 dpm for 20 ml of solution and membrane size 8 x 12 cm) and the membrane was incubated at 42°C overnight. Probes used were p-*fos*, a 600-bp fragment of v-*fos* with very high homology to c-*fos* (Van Beveren *et al.*, 1983), *fos* B2L (contains the whole coding sequence for FosB; Nakabeppu and Nathans, 1991) and pAM-91 (Humphries *et al.*, 1981) which detects β-actin expression.

The membrane was subjected to stringent washing; 2X SSPE at room temperature for 15 min with agitation (twice), 2x SSPE, 2% SDS at 65°C for 45 min with agitation (twice), 0.1X SSPE at room temperature for 15 min with agitation (twice). The membrane was then exposed to X-ray film for up to 21 days. The autoradiographs were analysed by scanning with a Shimadzu dual-wavelength flying spot laser densitometer in order to obtain a semi-quantitative measure of gene expression. Steady-state c-*fos* levels were calculated relative to the expression of a housekeeping gene, β-actin. The β-actin gene has constant expression during the time interval used in this study and so acts a control for the loading of RNA.

2.2.6 Preparation of nuclear extracts for electrophoretic mobility shift assay (EMSA)

Primary cultures of cerebellar granule cells were prepared in 90 mm dishes at a density of $25x10^6$ and grown in culture for 2 or 7 days. If the cells were not to be deprived of serum, they were stimulated by adding agonist directly to the culture medium. For 2 DIV experiments (serum-deprived), the medium was changed to serum-free medium (Dulbecco's modified Eagle's medium supplemented with 25 mM KCl, putrescine cocktail (60 μM putrescine, 0.1 mg/ml apo-transferrin and 30 nM sodium selenite) and 1 nM 3,3',5-triiodo-L-thyronine (T_3)) on day 1, then to serum-free medium at low KCl concentration (5 mM) on day 2, the day of the experiment. The cells were left in this medium for 3 h before stimulation with agonist. Cultures to be used at 7 DIV (serum-deprived) were treated with cytosine arabinoside (final concentration of 20 μM) on day 2 and then the medium was changed to serum-free medium (as above) on day 5. On day 7, the medium was changed to serum-free medium, low KCl for 3 h before the experiment. The agonist of interest was added to the medium of the cells (in triplicate) for the desired time period and then nuclear extracts were prepared using a modified version of the method described by Dignam *et al.* (1983).

Essentially, after exposure to agonist, the cells were washed with PBS and then harvested in up to 1 ml ice-cold PBS. Replicates were pooled and centrifuged at 300g for 10 min at 4°C. The pellet was washed with 5x packed cell volume (PCV) of PBS, resuspended and centrifuged

at 300g for 10 min. The pellet was washed with 5x PCV of Buffer A (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 1 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride (PMSF)), resuspended, left to stand on ice for 10 min and then centrifuged at 300g for 10 min. The pellet was washed again with 2x PCV of Buffer A, resuspended and homogenised using a Dounce glass homogeniser type B (10 strokes). The extract was centrifuged at 300g for 10 min and then at 14000g for 20 min. The pellet was resuspended in Buffer C (20 mM HEPES, pH 7.9; 25% (v/v) glycerol; 0.8M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM PMSF; 1 mM DTT; 0.5% (v/v) aprotinin) and homogenised (4 strokes). The extract was shaken at 4 °C for 30 min and then centrifuged at 14000g for 30 min at 4°C. The supernatant was quick frozen in liquid nitrogen and retained at -70°C for use in EMSA. Protein concentrations were determined using the Bradford method.

2.2.7 Bradford protein assay

Increasing concentrations of BSA in the range 1μg/μl - 15 μg/μl were used to prepare a standard curve. Bradford Reagent (0.1 mg/ml Coomassie brilliant blue G-250; 4.75% (v/v) ethanol; 8.5% (v/v) orthophosphoric acid) was added to each of five BSA concentrations as well as to 10 μl of each of the nuclear extracts, at 1 ml per sample. After 5 min the absorbance was measured at 595 nm. The standard curve was plotted and the amount of protein in each 10 μl sample ascertained. The volume required for 5 μg of protein was calculated.

2.2.8 Oligodeoxynucleotide preparation

The two 18 bp complementary oligodeoxynucleotides 5'-AGCTTGATGAGTCAGCCG-3' and 5'-GATCCGGCTGACTCATCA-3' (Angel *et al.*, 1987) were prepared on a Beckman Oligo 1000 DNA synthesiser by Mr. Ian Armitt, School of Biomedical Sciences, University of St. Andrews. The TRE consensus sequence is underlined.

1 μl of each prepared DNA strand (100 ng/μl) was mixed with 2 μl of 10X T4 polynucleotide kinase (10U/μl), 2 μl of 10X T4 polynucleotide kinase buffer and 4 μl of γ-^{32}P ATP (3000 Ci/mmol) and incubated for 30-45 min at 37 °C. The mixture was then boiled for 3-5 min and left to cool to room temperature to allow annealing. Glycerol (10 μl) was added to the

mixture which was run on a 10% non-denaturing polyacrylamide gel (10% (v/v) acrylamide/bisacrylamide mix (30%:1.034%); 0.04% (w/v) ammonium persulphate; 0.14% TEMED; 1x TBE (90 mM Tris-borate; 2 mM EDTA)) at 175 V for 1 hr in order to separate the double-stranded, labelled probe from incorrectly annealed or labelled probe. Chamber buffer used was 1X TBE (90 mM Tris-borate; 2 mM EDTA). The portion of gel containing the labelled double-stranded DNA was detected using X-ray film, cut out and shaken in 300 μl of TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0) overnight at 4°C. The supernatant was extracted, counted and stored at 4°C before use in EMSA.

2.2.9 Electrophoretic mobility shift assay (EMSA)

The basis of EMSA is that as a complex increases in size, its electrophoretic mobility decreases (Fried and Crothers, 1981; Garner and Revzin, 1981). Thus a length of DNA which is complexed to a DNA-binding protein will move through a gel more slowly than the same section of DNA alone (Hassanain *et al.*, 1993). If the DNA/protein complex is further bound to an antibody specific to the protein, the rate at which this complex moves through a gel is reduced even more (Figure 2.2). Since, in this assay, the fragment of DNA has been labelled with radioactivity, it is possible to observe the progress of each complex through the gel after drying and exposure of the gel to X-ray film.

5 μg of protein from each nuclear extract was mixed with 15 μl of 2X binding buffer (44 mM HEPES; 12 mM KCl; 10 mM DTT; 10 mM spermidine; 4% (v/v) Ficoll; 16% (v/v) glycerol), 2 μl of 0.5 μg/μl polydIdC and 20,000 cpm of labelled oligo and incubated for 5 min at room temperature. For supershift analysis, 4 μl of 100 μg/0.1 ml antibody was added to this mixture which was then incubated overnight at 4°C. Antibodies used in EMSA studies are listed in Appendix 1. One lane per gel contained the binding mixture plus cold, mutant probe (an 18-base pair oligonucleotide containing the sequence AGGCTAA).

DNA-protein complexes were resolved by applying samples to a 6% non-denaturing polyacrylamide gel where electrophoresis was carried out at 175V for 1.5 hr at room temperature.

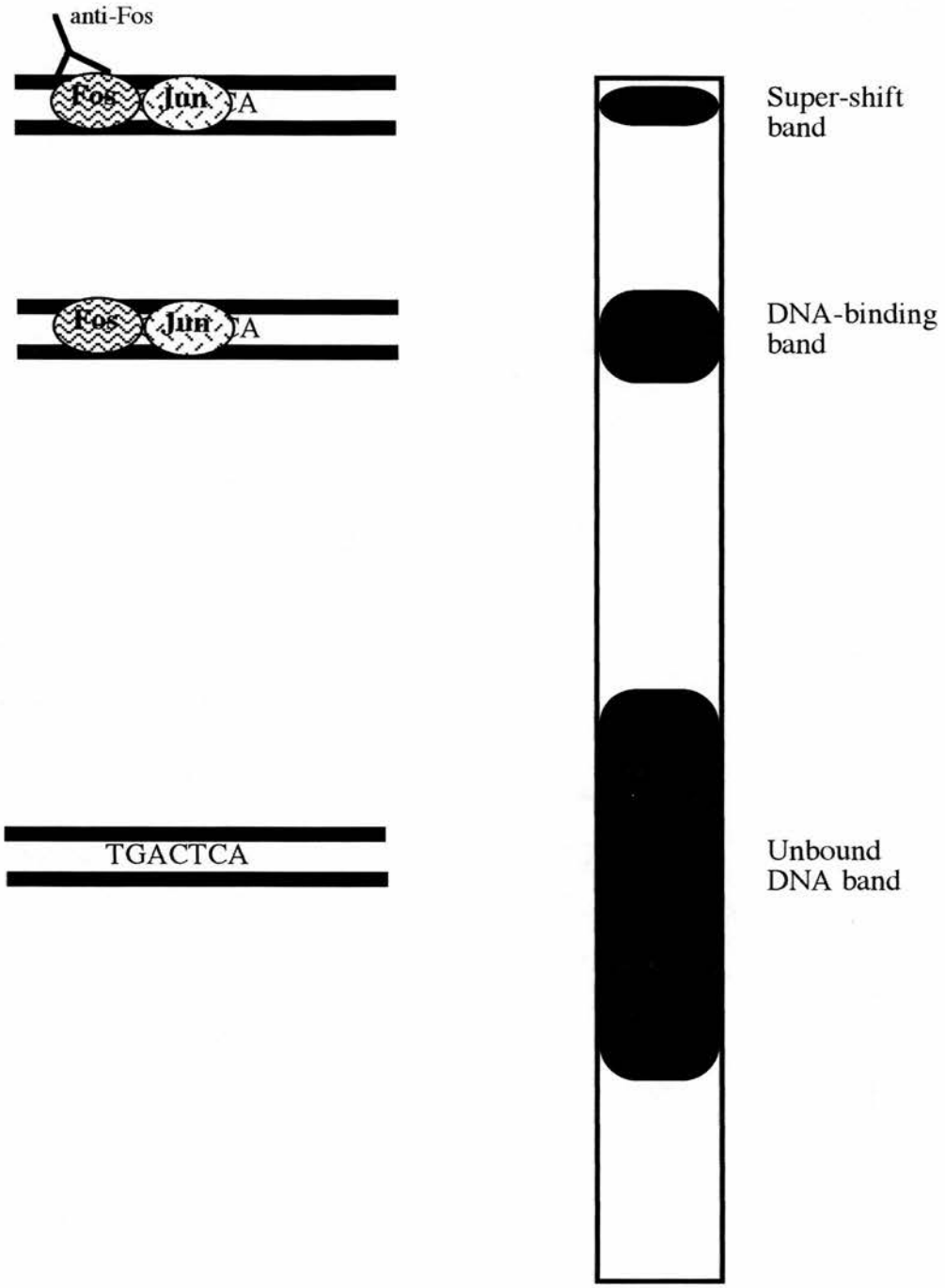


Figure 2.2 Schematic of theory behind the electrophoretic mobility shift assay
Unbound DNA moves through the native gel at the fastest rate. DNA which has been bound by an AP-1 transcription factor complex (Fos/Jun) is retarded due to the increase in molecular weight. Binding of antibody specific to one of the component proteins results in further retardation of the complex, resulting in a 'super-shift'.

Chamber buffer used was 0.5X TBE (45 mM Tris-borate; 1 mM EDTA). A bromophenol blue/glycerol dye mix (4μl) was applied to one lane in order to monitor the progress of the gel. Gels were then dried onto 3M blotting paper (Whatman) and exposed to X-ray film overnight at -70°C.

2.2.10 Western blotting

Primary cultures of cerebellar granule cells were prepared in 6-well plates (Nunc, Corning, Greiner) and grown in culture for 2 or 7 days. This method proved difficult to optimise and thus several slightly different protocols were used during the course of the study (see Appendix 2 for details on development of method). The final protocol was as follows: After exposure to agonist for the required time, cells were washed with 1 ml PBS and then harvested in 150 μl 1X sample buffer (0.0625 M Tris pH 6.8; 2% (w/v) SDS; 10% (v/v) glycerol; 5% (v/v) 2-mercaptoethanol; 0.01 mg/ml bromophenol blue). The viscous lysates were transferred to fresh Eppendorf micro-tubes and sonicated for 20 min in a sonicating waterbath (or for 12 seconds using sonicator probe). The samples were boiled for 5 min, centrifuged at 14000g for 5 min and stored at -70°C until required.

The samples were boiled and centrifuged once more as before and the supernatants loaded onto a 10% SDS-polyacrylamide gel (13% acrylamide/bisacrylamide mix (30%: 1.034%); 490 mM Tris.HCl, pH 8.8; 0.13% (w/v) SDS; 0.08% (w/v) ammonium persulphate; 0.1% (v/v) TEMED). Samples were run for 1-1.5h at 60 mA using a 2103 power supply (Amersham Pharmacia Biotech) with cooling, using a 'Mighty Small' gel rig (Hoeffer). Pre-stained SDS-PAGE Standards, Low Range (Biorad) were run in one lane of the gel to enable recognition of the specific protein of interest. The samples were then transferred to Trans-Blot Transfer Medium (nitrocellulose membrane, 0.45 μm, Biorad) for 1h at 60 mA per gel using a Multiphor II (Amersham Pharmacia Biotech). The membrane was then blocked with a 10% (w/v) solution of milk powder in pH 8.0 buffer (150 mM NaCl; 50 mM Tris pH 8.0; 0.05% (v/v) Tween 20) for 30 min before being incubated in primary antibody solution (1:100 dilution of c-Fos; FosB; c-Jun (Santa Cruz) in 3% (w/v) milk/pH 8.0 buffer) overnight at 4°C. The membrane was washed with

Washing Buffer (150 mM NaCl; 50 mM Tris pH 7.4; 0.05% (v/v) Tween 20) and then incubated for 2 h either in rabbit anti-goat (Dakopatts) (1:400 dilution in 3% (w/v) milk/pH 8.0 buffer) or anti-rabbit HRP-conjugated antibody (Amersham) (1:5000 dilution in 3% (w/v) milk/pH 8.0 buffer). If rabbit anti-goat was used as secondary antibody (due to primary antibody being raised in goat) then anti-rabbit HRP-conjugated antibody was used as a tertiary antibody after washing with Washing Buffer. After the final antibody incubation (secondary or tertiary), the membrane was once again washed in Washing Buffer and then incubated with a 1:1 mixture of ECL reagent 1 (2.5 mM luminol (Fluka); 0.4 mM *p*-coumaric acid (Sigma); 0.1 M Tris pH 8.5) and ECL reagent 2 (0.02% (v/v) H_2O_2; 0.1 M Tris pH 8.5) for 1 minute. The membrane was exposed to X-ray film for between 30 and 120 seconds.

2.2.11 Transfection

Cells were grown initially in 12-well plates (Corning) at a density of $25x10^6$ cells/plate and then, in an attempt to improve the assay conditions, in 6-well plates (Nunc, Corning, Greiner) at a density of $5.5x10^6$ cells per well. Again, the experiment was carried out at two different ages of culture. Immature cells were transfected at 2 days in vitro (DIV) and then exposed to agonist at 3 DIV. Mature cells were transfected at 5 DIV and exposed to agonist at 6 DIV. At 5 DIV, the cells are essentially mature, but have been shown to survive transfection more effectively than cells at 7 DIV (see Figure 3.26).

The plasmid used for the luciferase reporter assay was -361fosluc, kindly donated by the laboratory of Jean-Philippe Loeffler, Institut de Physiologie, URA 1446 du CNRS, Strasbourg, France. This was developed from the parent plasmid AP1wt CONA luc 3A (Imler *et al.*, 1988). The first method of transfection was using the polycation, polyethylenimine (PEI) (Boussif *et al.*, 1995). The cells were transfected with 1 μg of DNA per ml, with 9 equivalents of 0.1 M PEI (25 kD), pH 7.0 being used, i.e. 0.28 μl PEI per 1 μg DNA. DNA and PEI were each mixed with 150 mM NaCl (10% of final volume) separately and then were mixed together and vortexed for 12 min. Neurobasal™ medium (supplemented with 20 mM KCl; 250 μM Gln) was then added to make up the required volume (1 ml per well). The cell medium was aspirated and replaced with

the transfection mixture. The transfection time was 2 h, after which the transfection medium was aspirated and replaced with fresh Neurobasal™ medium (with supplements; 1 ml per well). The cells were allowed to recover overnight before being exposed to agonist.

The second transfection method involved the use of Transfectam™ reagent (Promega). 10 μg of DNA were used per 12-well plate. Both the DNA and the Transfectam™ were mixed separately with serum-free medium, then the two mixtures were combined and added in a 1:5 ratio in serum-free medium to the cells. Again, transfection was carried out for 2 h and the transfection medium replaced with fresh serum-free medium. The guidelines from Promega suggest adding serum-supplemented medium after transfection (Promega, 1996), however, Loeffler *et al.* (1990) reported that they added serum-free medium after transfection. As a consequence of a personal visit to the laboratory of J-Ph. Loeffler in Strasbourg, France, initially the protocol used was that recommended by this laboratory. After at least 16 h, the cells were treated with agonist and/or antagonist for the required time period and then harvested as described below.

In addition, an attempt was made to use the CAT reporter assay (see Section 2.2.13), using the plasmids Fos/CAT (pfc363), Jun/CAT, TRE/tk-CAT, CRE/tk-CAT (Barthel *et al.*, 1996).

2.2.12 Luciferase reporter assay

Cells were exposed (in triplicate) to agonist for the desired time period, then were washed with 1 ml PBS and harvested in 100 μl (150 μl if cells were cultured in 6-well plates) luciferase assay lysis buffer (Promega Luciferase Reporter Assay System with Reporter Lysis Buffer; Cat #E4030) with scraping into Eppendorf micro-tubes. The lysates were vortexed for 15 seconds, centrifuged at 4°C for 2 min, transferred to a fresh Eppendorf tube and stored at -70°C until required.

Aliquots of the lysate (40 μl) were mixed with 100 μl luciferase assay reagent (Promega; Cat #E4030), and the luminescence measured over a 10 second period using a Lumat LB 9501 luminometer (Berthold).

2.2.13 Chloramphenicol acetyltransferase (CAT) reporter assay

Cells were exposed to agonist for the desired time period and the CAT reporter assay carried out basically as described by Gorman *et al.* (1982). Essentially, cells were harvested in 150 μl of 200 mM Tris.HCl, pH 7.4 at 4°C. The cells were then lysed by freezing in liquid nitrogen and thawing three times. The lysates were incubated at 65°C for 8 min before being centrifuged at 13,000 rpm for 8 min at 4°C. The supernatant (100 μl) was added to 20 μl of hot mix (1 mM Acetyl CoA; 4 μl ^{14}C chloramphenicol in 200 mM Tris.HCl, pH 7.4 per sample) and the reaction allowed to proceed for 2h at 37°C. Chloramphenicol and its acetylated derivatives were extracted with 400 μl ethyl acetate and then vortexed for 20s before being microfuged for 1 minute at room temperature. The top layer was removed to a clean tube and evaporated on a speedi-vac for 20 min. Meanwhile, a TLC tank was equilibrated with 100 ml of a 95:5 (v/v) mix of chloroform:methanol. The samples were redissolved in 20 μl ethyl acetate and spotted onto the bottom of a silica gel plate. Thin layer chromatography was allowed to proceed for 20 min before the silica gels were exposed to X-ray film.

2.2.14 Statistical analysis

Statistical significance of data (set at the 95% confidence limit) was analysed by Tukey's test for multiple comparisons (MINITAB release 8; CLE COM Ltd., Birmingham, U.K.) in combination with a one-way ANOVA.

Chapter 3

Evaluation of neurochemical endpoints in mature (7 DIV) mouse cerebellar granule cells

3.1 Introduction

In this chapter, the studies undertaken to explore the actions of murine cerebellar granule cells after 7 days in culture in terms of cytotoxicity, intracellular calcium concentration and cellular immediate-early gene and protein expression are reported. After this period of time in growth medium, these cells are regarded as being mature, and have well-developed neurites which allow communication between cells. Using specified culture conditions, including addition of cytosine arabinoside 48h after plating out to minimise astrocyte proliferation, the neuronal culture is 95% pure, with only 5% astrocyte contamination (Messer, 1977). Development of the central nervous system is primarily a matter of cell proliferation, movement, cell differentiation, cell-cell interaction and programmed cell death. The study of the mechanisms and regulation of signalling by cell-surface receptors for neurotransmitters and modulators, their effects on regulation of neuronal and glial cell gene expression, and the effects of drugs and toxins on these phenomena provides a molecular and mechanistic basis for increasing basic biological knowledge as a foundation for the proposal of new *in vitro* toxicological and pharmacological tests. Use of cultured cerebellar granule cells is a well-established model for glutamatergic neurons, since these cells synthesise, store and release Glu in response to stimulation (Gallo *et al.*, 1982; Drejer *et al.*, 1986; Burgoyne and Cambray-Deakin, 1988; Nicoletti *et al.*, 1988; Schoepp and Conn, 1993). Cerebellar granule cells possess all of the three main types of glutamatergic receptors (NMDA, AMPA/KA and metabotropic) and are therefore a widely-used system for the study of Glu metabolism, and in particular, the mechanism of Glu-mediated toxicity.

The results reported in this chapter are split into three sections. In Section A, studies on the effects of the excitatory amino acid, Glu, on 7 DIV cultured cerebellar granule cells are reported. A consequence of binding of Glu to an ionotropic receptor is depolarisation of the cellular membrane, leading to an increase in the intracellular Ca^{2+} ion concentration, a common mediator of toxicity (Choi, 1992; Verity, 1992). The mechanism of this effect was probed using antagonists of the various glutamatergic receptors as well as the L-type voltage-gated Ca^{2+} channel blocker, nifedipine. The NMDA receptor antagonists APV and TCP work in different ways; TCP is an "open-channel blocker", since its binding site lies deep within the receptor

channel. This means that during Glu-induced excitotoxicity, when the channel is opened for a prolonged period, TCP has a greater chance of reaching its binding site and blocking the channel (Lipton and Rosenberg, 1994). APV, however, binds to the same site as Glu or NMDA, competitively preventing the agonists from binding. TCP is also far more potent than APV, being effective at 1 μM. CNQX is an antagonist of AMPA/KA-type glutamatergic receptors, acting at the AMPA/KA/Glu site.

An understanding of the transcriptional regulation by excitatory amino acids (EAAs) of the expression of early-response genes, in particular, c-*fos* and c-*jun*, appears to be of crucial importance for explaining the molecular mechanisms of neurotoxicity in addition to the more physiological role of these early genes in neural development and long-term adaptive changes in the mature nervous system (Ginty *et al.*, 1992; Gass and Herdegen, 1995; Hughes and Dragunow, 1995; Morgan and Curran, 1995; Herdegen, 1996; Herdegen *et al.*, 1997). Furthermore, it appears that a number of these early-response genes, including c-*fos*, encode transcription factors that might regulate the subsequent transcriptional activity of other late-response genes, some of which might function as survival or toxic factors (Tatter *et al.*, 1995). For example, a close association between NMDA receptor activation and c-*fos* expression in the survival and/or maturation of cerebellar neurons has been demonstrated (Didier *et al.*, 1989; 1992). Brief exposure to low, non-toxic levels of Glu and other EAAs is associated with a rapid, transient expression of steady-state c-*fos* mRNA levels: a calcium-dependent process that is mediated predominantly by the activation of the NMDA receptor (Szekely *et al.*, 1989; Grayson *et al.*, 1990). In contrast, sustained and/or elevated c-*fos* expression is associated with neuronal apoptosis and developmental failures in vivo (Holt *et al.*, 1986; Rüther *et al.*, 1987; Edwards *et al.*, 1988; Smeyne *et al.*, 1993; Chen *et al.*, 1995b) and also in a number of animal models in seizures and neurodegenerative disease (Smeyne *et al.*, 1993; Pennypacker *et al.*, 1994). The Glu-mediated induction of cellular immediate-early genes (cIEGs) has been reported and linked to the toxicity observed in the presence of high levels ($\geq$ 50 μM) of Glu (Gorman *et al.*, 1995).

The concept of regulation of gene transcription by a particular group of early-induced gene-encoded proteins, activated by a stimulus, is one which has applications across the spectrum

of cellular diversity in the mammalian system. Its discovery opened up many new possibilities of mechanisms of regulation of protein expression, which before was thought to be limited to post-translational processing. The Fos-Jun dimer, after being formed in the cytosol in response to a signal, is translocated to the nucleus due to nuclear localisation signals (NLS; Shimokawa and Miura, 1996) in order to affect the transcription of target genes. Thus it is desirable to use an assay which is specific for nuclear proteins, rather than a whole cell preparation. In this way, a more accurate picture of stimulus-specific functional AP-1 dimer production can be obtained. The nuclear extract preparation method used was developed from that originally reported by Dignam *et al.* (1983), and requires very gentle handling of the cell lysate in order to prevent damage to the nuclear membrane. Once prepared and assayed for protein concentration, these nuclear extracts can be added to a mixture containing a binding buffer, a radioactively-labelled DNA probe, and an antibody specific to the protein of interest, for use in the electrophoretic mobility shift assay.

Since the electrophoretic mobility shift assay is only a semi-quantitative assay, it was thought appropriate to use Western blotting to attempt to quantify the expression of the proteins involved in forming the AP-1 transcription factor complex. In addition, an attempt was made to assay for functionality of the gene expression using transient transfection of the cells with plasmids containing the c-*fos* gene attached to a reporter gene (chloramphenicol acetyltransferase (CAT) or luciferase). Whilst this final set of experiments was inconclusive, it is included as part of the work undertaken, and this area is one which could be expanded in further work.

The work reported in Section B involved exposure of 7 DIV cultured cerebellar granule cells to varying concentrations of K^+ ions. When large amounts of these ions are present in the extracellular medium, the membrane becomes depolarised and the intracellular concentration of Ca^{2+} ions increases. This effect, however, is mediated by a different mechanism than that caused by extracellular Glu. The K^+ ions move across the cell membrane via non-inactivating channels, causing a voltage change to occur inside the cell. This voltage change activates voltage-gated Ca^{2+} channels, thus increasing the intracellular Ca^{2+} ion concentration.

In addition to the studies described above, an investigation into one of the specific cascade pathways by which the effects of Glu receptor binding causes regulation of immediate-early gene

induction was carried out. The phospholipase A_2 pathway, which produces arachidonic acid (which in turn is metabolised to epoxides, leukotrienes, prostaglandins and thromboxanes), was considered to be an appropriate pathway to study due to the involvement of arachidonic acid in cytotoxicity (Carine and Hudig, 1984; Okuda *et al.*, 1994). There is also evidence for the induction of immediate-early genes as an end-point to the PLA_2 pathway (see Figure 1.8; Bazán, 1989; Lerea and McNamara, 1993). These studies are reported in Section C.

3.2 Results

Section A: Glu-mediated effects on 7 DIV cultured cerebellar granule cells

3.2.1 Measurement of excitatory amino acid-induced toxicity

The morphological effects of Glu-mediated toxicity are shown in Figure 3.1B. When compared to control cells (Figure 3.1A), cells which have been treated with 100 μM Glu for 24h show widespread damage, with the appearance of dead cells, debris and breakdown of intercellular connections (Figure 3.1B). This damage can be prevented by coadministration of the NMDA receptor antagonist N-[1-(2-thienyl)cyclohexyl]-piperidine (TCP; Figure 3.1C), but not the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Figure 3.1D) or the L-type voltage-gated calcium channel blocker nifedipine (Figure 3.1E). Exposure of these cells to 55 mM KCl for 24h causes no change to the morphology (Figure 3.1F). Cerebellar granule cells at 7 days *in vitro* (DIV) were exposed to increasing concentrations (1-1000 μM) of Glu for 4 or 24h in culture-conditioned medium before being assayed for cell viability using the MTT assay. Figure 3.2 shows the concentration-dependent decrease in absorbance, corresponding to a decrease in cell viability. The IC_{50} was estimated to be approximately 50 μM Glu after either exposure time. The maximum reduction in absorbance after 4h indicated approximately 75% toxicity (Figure 3.2A), whereas that measured after a 24h exposure indicated approximately 90% toxicity (Figure 3.2B); it appears that the remaining 10% cell viability could be related to the 5% astrocyte population in these cultures (Juurlink and Hertz, 1993). Alternatively, it may be due to an enzymatic reduction of MTT by non-mitochondrial dehydrogenases (Jensen *et al.*, 1998b). There are several ways to assess cell viability, and another widely used method is the lactate dehydrogenase (LDH) assay. In this assay, the leakage of the cytosolic enzyme LDH into the medium is measured and used as a measure of the amount of necrotic cell death. The results of this assay under the same conditions (Griffiths *et al.*, 1997) correlate well with the results of the MTT assay presented in Figure 3.2B.

In order to confirm the receptor-specificity of the observed Glu-mediated toxicity, the cells were stimulated with 250 μM Glu, alone and in conjunction with Glu receptor antagonists; 2-

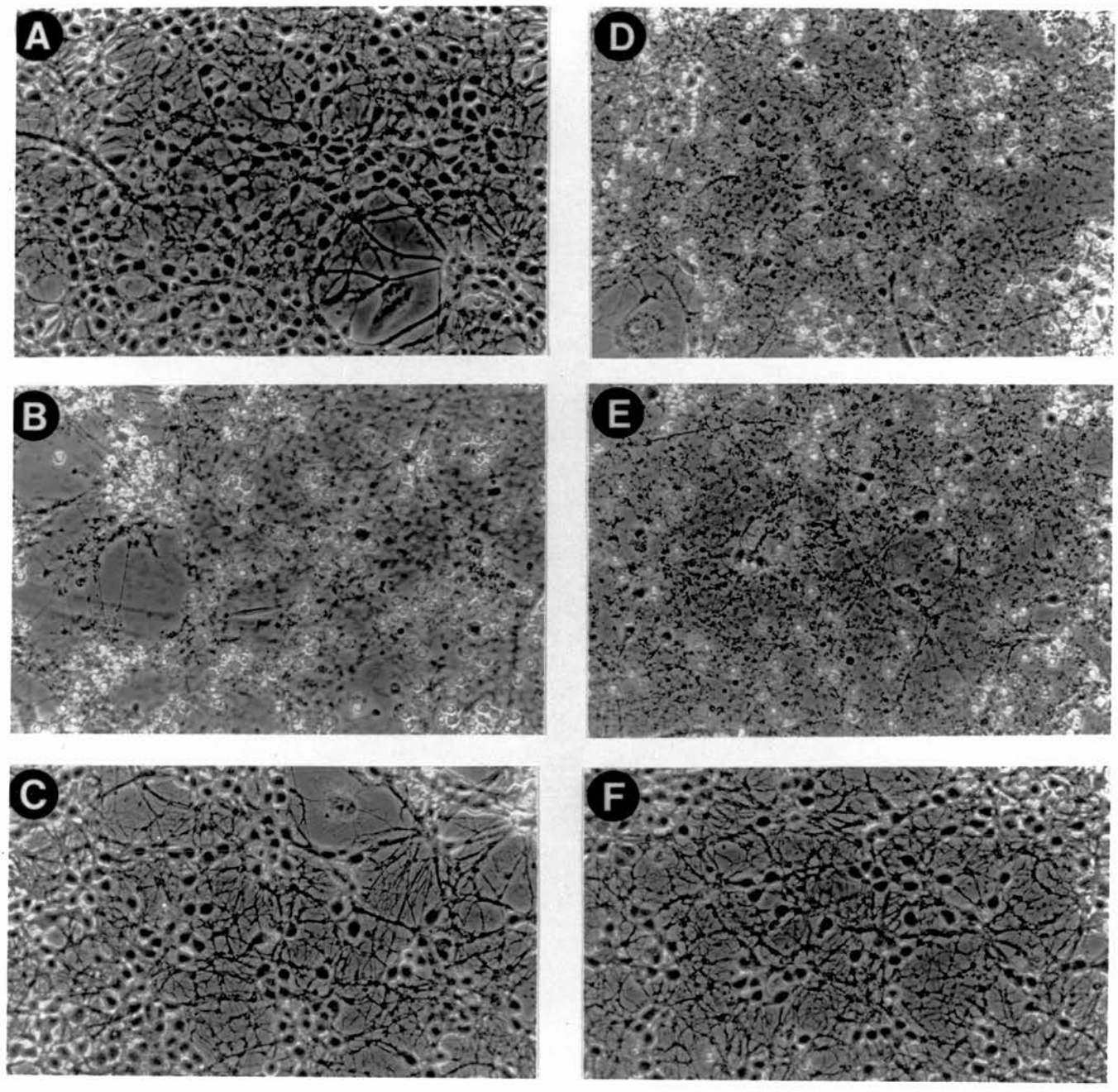


Figure 3.1 Photomicrographs of 7 DIV murine cerebellar granule cells.
Cells were cultured for 7 days as described in Materials and Methods before treatment for 24h with conditioned medium alone (A), 100 μM Glu (B), 100 μM Glu and 1 μM TCP (C), 100 μM Glu and 10 μM CNQX (D), 100 μM Glu and 5 μM nifedipine (E) and 55 mM KCl (F). All photomicrographs were taken at the same magnification.

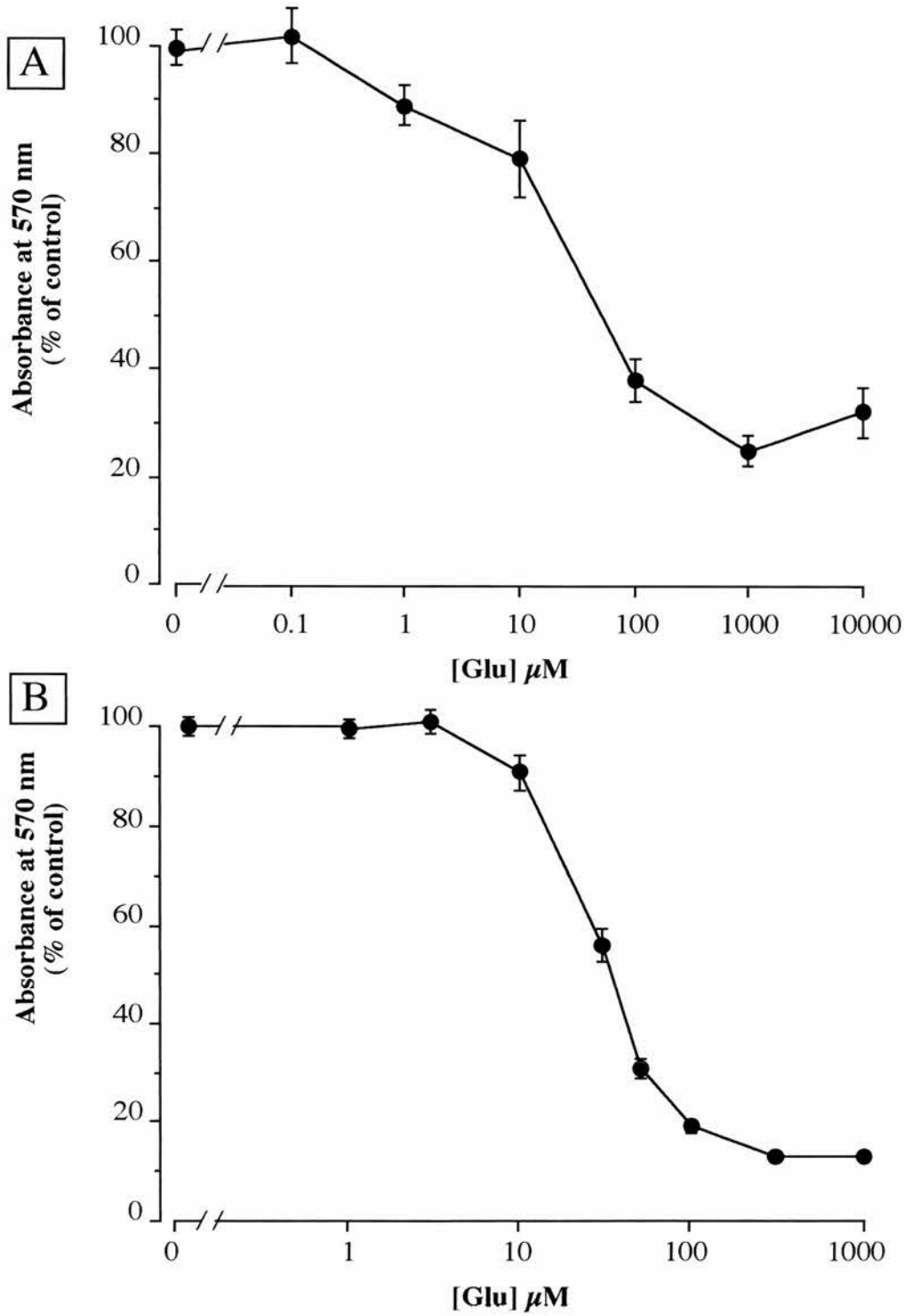


Figure 3.2 Concentration-dependent effect of L-glutamate (Glu) on cytotoxicity in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 7 days before use. Cells were exposed for 4h or 24h in culture-conditioned medium to increasing concentrations of Glu before being assayed for cell viability by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining. The absorbance at 570 nm was expressed as a percentage of control (untreated) cells. Data represent mean ± SEM values (n = 8-16). The absorbance values for control cells were 0.272 ± 0.008.

amino-5-phosphonovaleric acid (APV), TCP and CNQX. Figure 3.3 shows that the Glu-mediated toxicity was blocked almost entirely by APV and TCP, but not at all by CNQX. This correlates with the photomicrographs shown in Figure 3.1 B-D, and suggests that the Glu-induced toxicity imposed on murine cerebellar granule cells is mediated predominantly via NMDA receptor activation.

3.2.2 Glu-induced excitotoxicity is associated with a rise in intracellular calcium levels

Cerebellar granule cells at 7 DIV were loaded with Fluo-3A/M, washed, exposed to increasing concentrations of Glu (1-1000 μM) and assayed immediately for intracellular calcium concentration. Figure 3.4 shows the concentration-dependent effect of Glu, which correlates with the toxicity concentration-response curve shown in Figure 3.2. Thus as the concentration of extracellular Glu increases, the amount of calcium influx increases, and this coincides with a decrease in cell viability. Cells were subsequently exposed to 250 μM Glu alone or coadministered with the L-type voltage-gated Ca^{2+} channel blocker, nifedipine (5 μM). Figure 3.5 shows that the increased level of intracellular calcium ions was partially blocked by nifedipine, suggesting that a route of Ca^{2+} ion influx in response to both toxic and non-toxic concentrations of Glu is via the L-type voltage-gated Ca^{2+} channel.

3.2.3 Toxic and non-toxic levels of Glu stimulate differing mRNA induction profiles of the immediate-early genes c-*fos*, c-*jun* and *fos*B

Cerebellar granule cells were treated with low and high levels of Glu and the mRNA induction profiles of immediate-early genes were detected. In Figure 3.6, the induction of c-*fos* (A), c-*jun* (B) and *fos*B (C) mRNA is shown over a time-course of 6h with constant stimulation of the cells with either 10 or 250 μM Glu. In the case of c-*fos* (A), following a non-toxic (10 μM) dose of Glu, the induction of mRNA peaked after only 1h, and was transient, falling back to basal

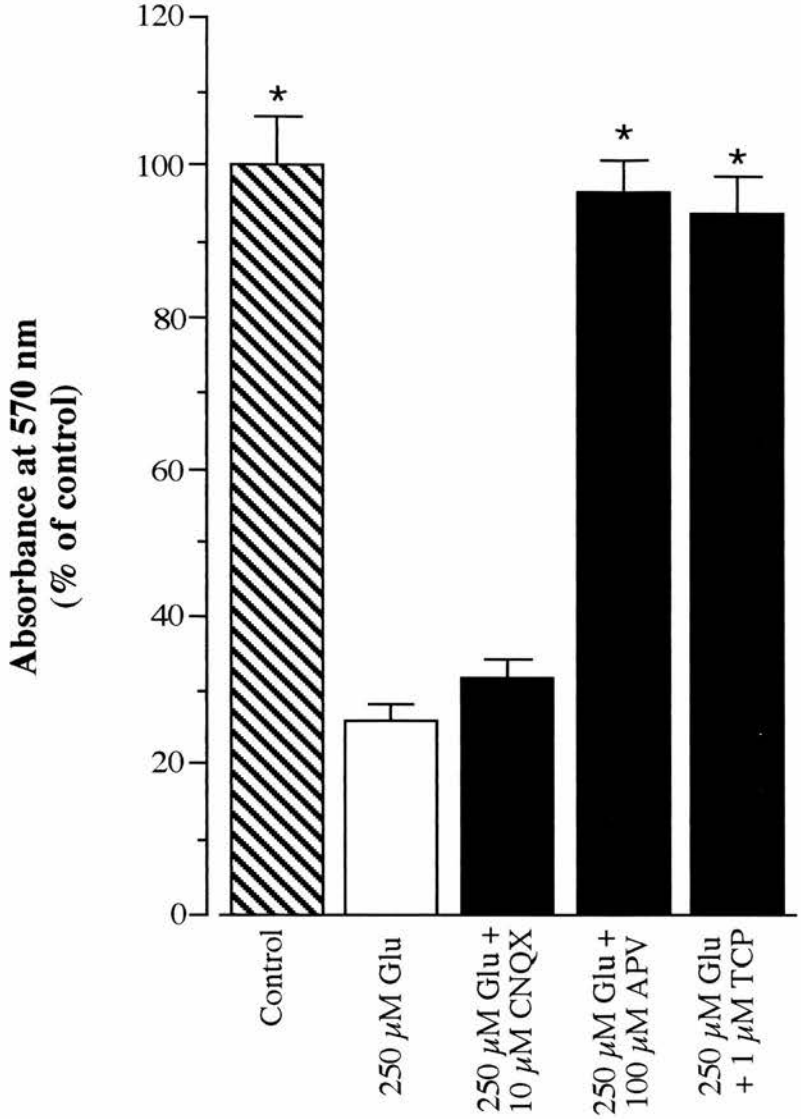


Figure 3.3 Pharmacology of Glu-stimulated cytotoxicity in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 7 days before use. Granule cells were pre-exposed for 15 min in culture-conditioned medium to ionotropic receptor antagonists as indicated prior to exposure to 250 μM Glu for 4h. Cell viability analysis was carried out using MTT staining. The absorbance at 570 nm was expressed as a percentage of control (untreated) cells. Data represent mean ± SEM values (n = 8). Asterisks indicate statistical difference from 250 μM Glu alone.

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione;
APV: 2-amino-5-phosphonovaleric acid;
TCP: N-[1-(2-thienyl)cyclohexyl]-piperidine.

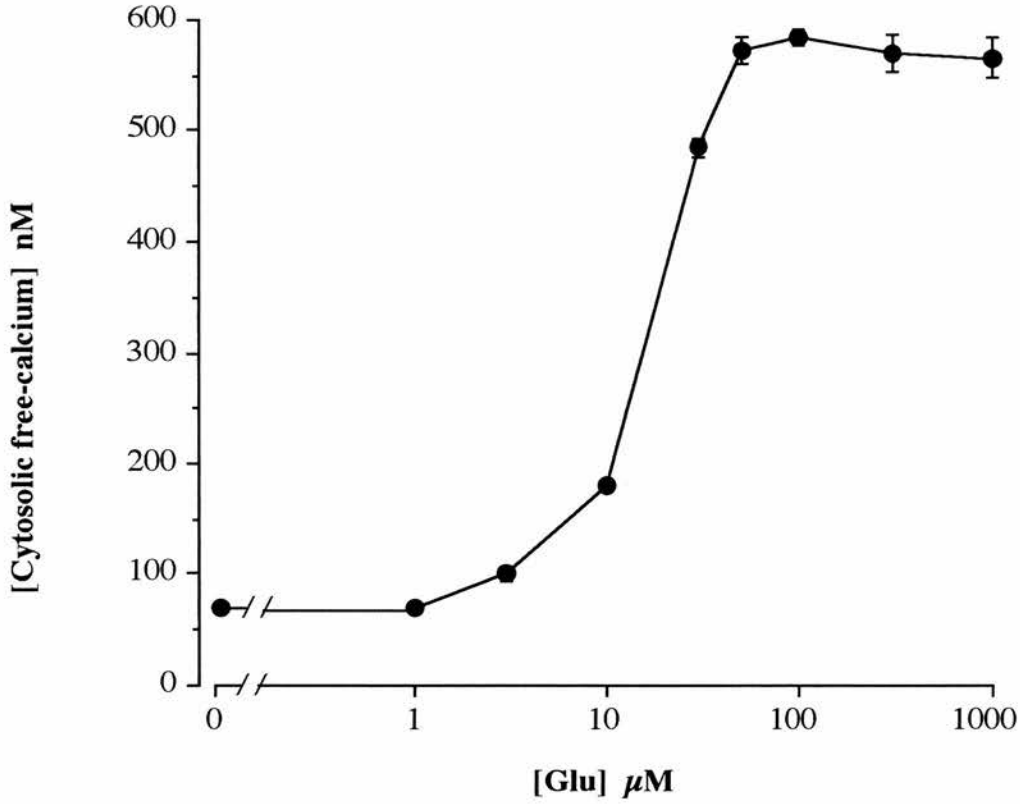


Figure 3.4 Concentration-dependent effect of L-glutamate (Glu) on stimulated increases in intracellular-free calcium levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 7 days before use. Granule cells were washed with balanced salt solution, loaded with Fluo-3/AM and exposed to increasing concentrations of Glu as described in Materials and Methods. Data are the mean ± SEM values (n = 8-16).

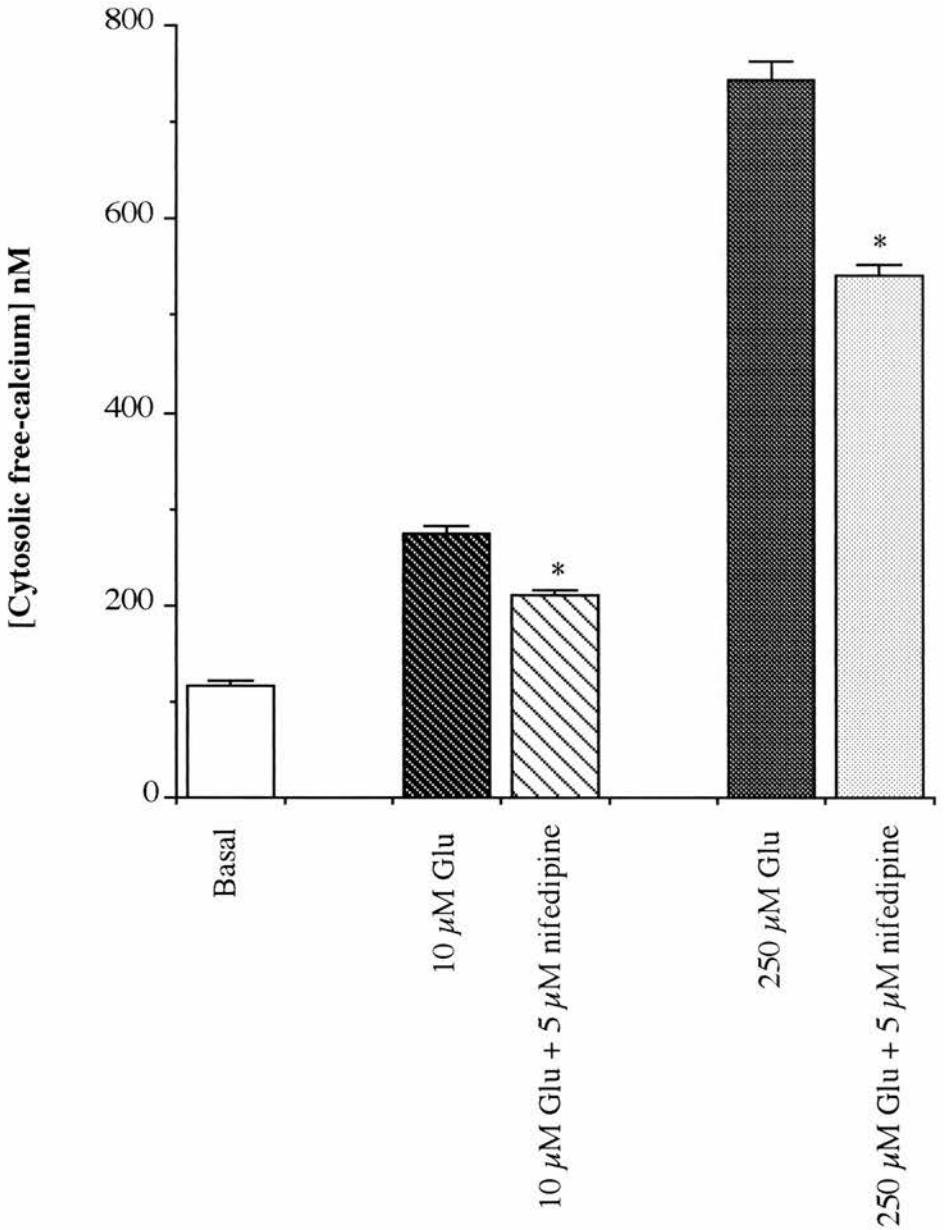


Figure 3.5 Effect of the dihydropyridine L-type calcium channel blocker, nifedipine, on increases in intracellular-free calcium levels stimulated by non-toxic and toxic concentrations of Glu in cultured cerebellar granule cells. Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 7 days before use. Granule cells were washed with balanced salt solution, loaded with Fluo-3/AM and exposed to either a non-toxic (10 μM) or toxic (250 μM) concentration of Glu alone or coadministered with 5 μM nifedipine. Data are the mean ± SEM values (n = 8-16). Asterisks indicate statistical difference from Glu alone in each case.

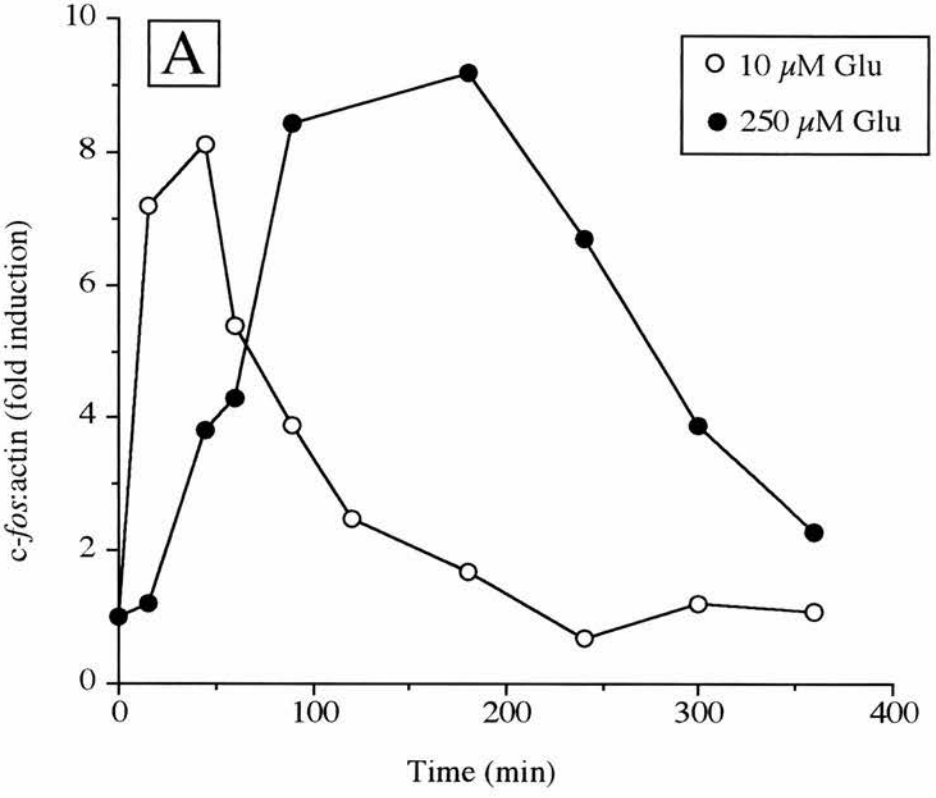
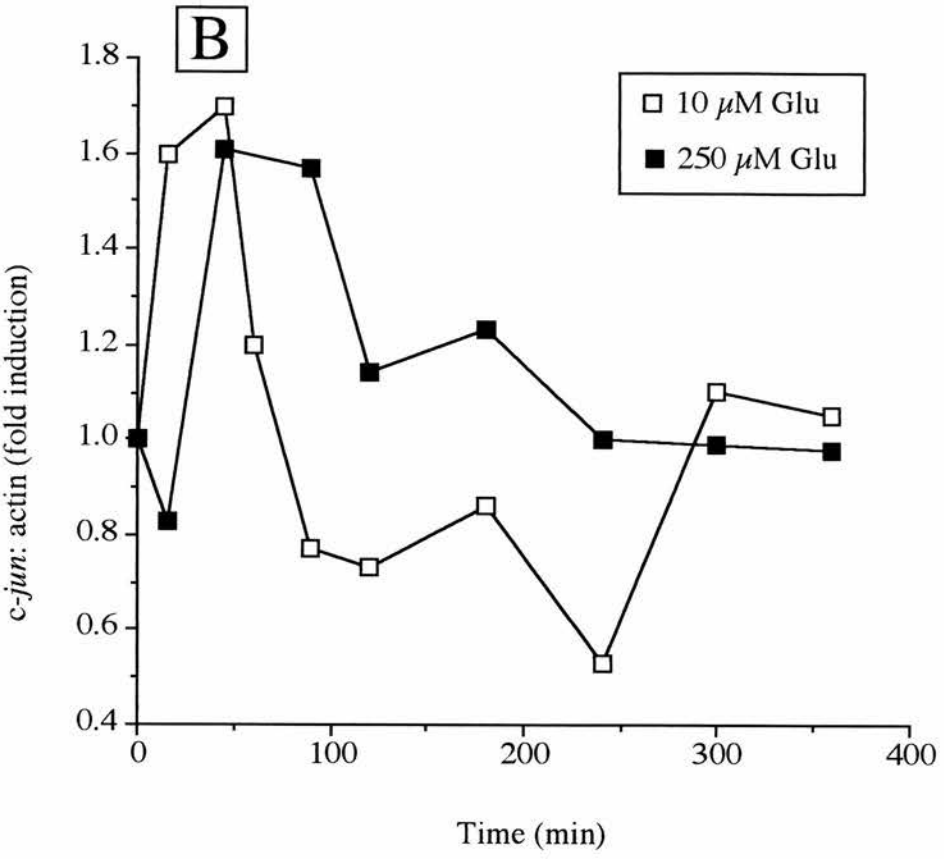
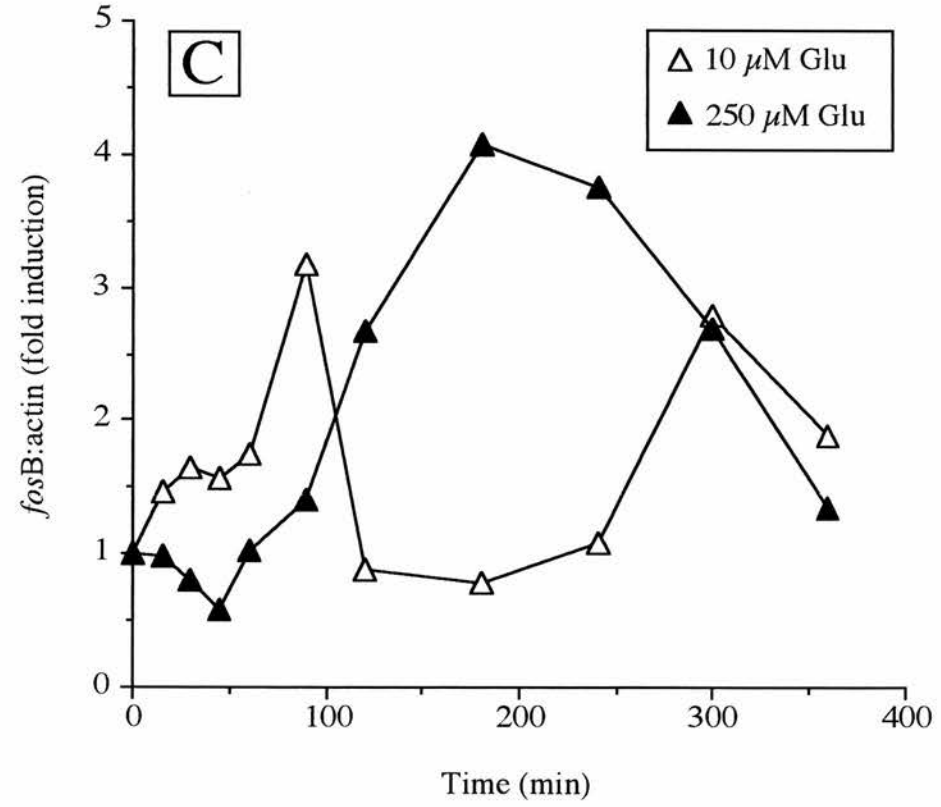


Figure 3.6 Time-course of Glu-stimulated, steady-state proto-oncogene mRNA levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old post-natal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure to 10 μM or 250 μM Glu for up to 6h. Total RNA was prepared from the cultures, and the amount of *c-fos* (A), *c-jun* (B) and *fosB* (C) mRNA was quantified. The results refer to clEG mRNA expression relative to that of β-actin. The β-actin gene has a constant expression during the time interval used and thus acts as a control for the loading of RNA.


B
10 μM Glu
250 μM Glu
c-jun: actin (fold induction)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0
100
200
300
400
Time (min)


C
10 μM Glu
250 μM Glu
fosB:actin (fold induction)
5
4
3
2
1
0
0
100
200
300
400
Time (min)

levels by 4h. In contrast, after exposure to toxic (250 μM) levels of Glu, c-*fos* mRNA induction was delayed (peaking at ~3h), elevated when compared to that induced by 10 μM Glu, and sustained for a longer period of time (basal levels only achieved by 6h). The differences between the high and low Glu-induced induction peaks of c-*jun* mRNA (B) were less marked, but the level of mRNA produced in response to 10 μM Glu reverted to basal levels by 2h, compared with 4h after exposure to 250 μM Glu. *fos*B mRNA was induced by exposure to both 10 μM and 250 μM Glu, the former eliciting a transient response which returned to basal within 2h. However, a second peak was observed at 5h in response to non-toxic Glu. The *fos*B mRNA profile in response to 250 μM Glu was delayed and elevated, as in the case of c-*fos*, peaking at 3h and returning to basal by 6h. Thus there seems to be some effect of toxicity on the induction profiles of these immediate-early genes. In additional experiments, cells were stimulated with 250 μM Glu alone or coadministered with either TCP (1 μM) or nifedipine (5 μM), and the steady-state levels of c-*fos* mRNA were evaluated. Both of these compounds prevented the 'elevated, sustained' profile characteristic of toxicity and displayed the 'transient' profile seen after exposure of the cells to non-toxic levels of Glu (Figure 3.7).

3.2.4 The composition of the AP-1 transcription factor complex changes with Glu concentration and culture conditions

In the following paragraphs, the results of the studies carried out on the Glu-mediated composition of the AP-1 transcription factor complex in 7 DIV cultures of cerebellar granule cells are reported. In all cases, a DNA-binding band was observed, which constitutes a complex of the TRE and an AP-1 dimer present in the nuclear extract. However, in order to analyse the composition of these AP-1 dimers, antibodies to the Fos and Jun families were added to the binding mixture. The presence of a particular protein in the AP-1 complex is indicated by a 'super-shift', i.e. a band which has been retarded to a greater extent than the DNA-binding band. This band represents a complex of the TRE, an AP-1 dimer and an antibody specific for one of the AP-1 dimer components (see Figure 2.2). The position of this super-shift band varies depending on the size of the protein bound to the antibody.

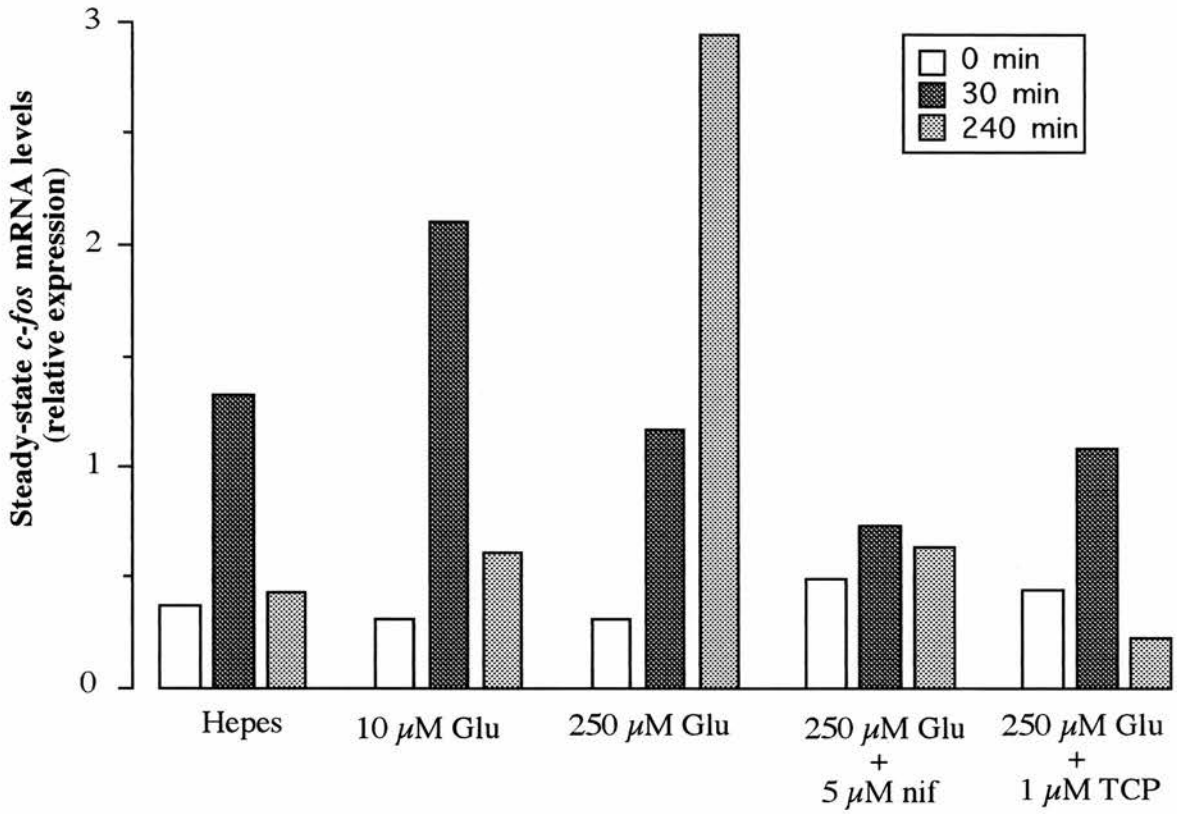


Figure 3.7 Pharmacology and temporal profiles of Glu-stimulated, steady-state *c-fos* mRNA levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure for different times (0, 30 or 240 min) to 10 μM and 250 μM Glu alone or coadministered with various pharmacological antagonists, as indicated. After exposure, total RNA was prepared from the cultures, and the amount of *c-fos* and β-actin mRNA was quantified. The results represent the average of at least duplicate experiments and refer to *c-fos* mRNA expression relative to that of β-actin. The β-actin gene has a constant expression during the time interval used in this study; thus it acts as a control for the loading of RNA.

TCP: N-[1-(2-thienyl)cyclohexyl]piperidine; nif: nifedipine.

Initially, it was thought that by exposing the cells to Glu for different times and analysing the amount of each protein present at each timepoint, a quantitative evaluation of the composition of the AP-1 transcription factor complex over a period of time could be obtained, allowing comparison with mRNA timecourse data. However, the electrophoretic mobility shift assay is not regarded as a quantitative assay, and thus only suggestions can be made about the change in composition over the length of the assay.

In order to confirm the specificity of each newly-synthesised probe, one lane of the gel, in addition to binding buffer, nuclear extract and probe, contained an excess of non-labelled probe. Thus the absence of a DNA-binding band in this lane indicated that the TRE probe used was of the correct sequence. In another lane, an excess amount of a mutant, non-labelled TRE probe was added to the binding mixture; the presence of a DNA-binding band in this lane showed that the binding to the labelled TRE probe was specific.

3.2.4.1 Cells maintained in serum-free medium

After stimulation with a non-toxic dose (10 μM) of Glu in serum-free medium, nuclear extracts from 7 DIV cells were incubated in binding buffer containing TRE probe without or with the addition of antibodies specific for each of the Fos and Jun family member proteins. Only Fra-2 and JunD appeared to be part of the AP-1 complex (Figure 3.8). The intensity of the Fra-2 supershifted band increased with time, and did not decrease to basal within the confines of the experiment (4 h). The negative results (i.e. where a super-shift was not observed in the presence of antibody) for each EMSA experiment are shown in Appendix 3.

A large proportion of the AP-1 complex population involved JunD under non-toxic conditions (10 μM Glu); a comparison of the intensity of the AP-1 binding band in the set of lanes with no antibody present (1-6) and the set containing anti-JunD (14-19) shows that most of the DNA-binding band was retarded to the supershift position.

Under toxic conditions (250 μM Glu), however, FosB, JunB, c-Jun and JunD all appeared as part of the AP-1 complex (Figure 3.9). This allows the possibility of a large number of different dimeric complexes, comprising homodimers and heterodimers of the Jun family

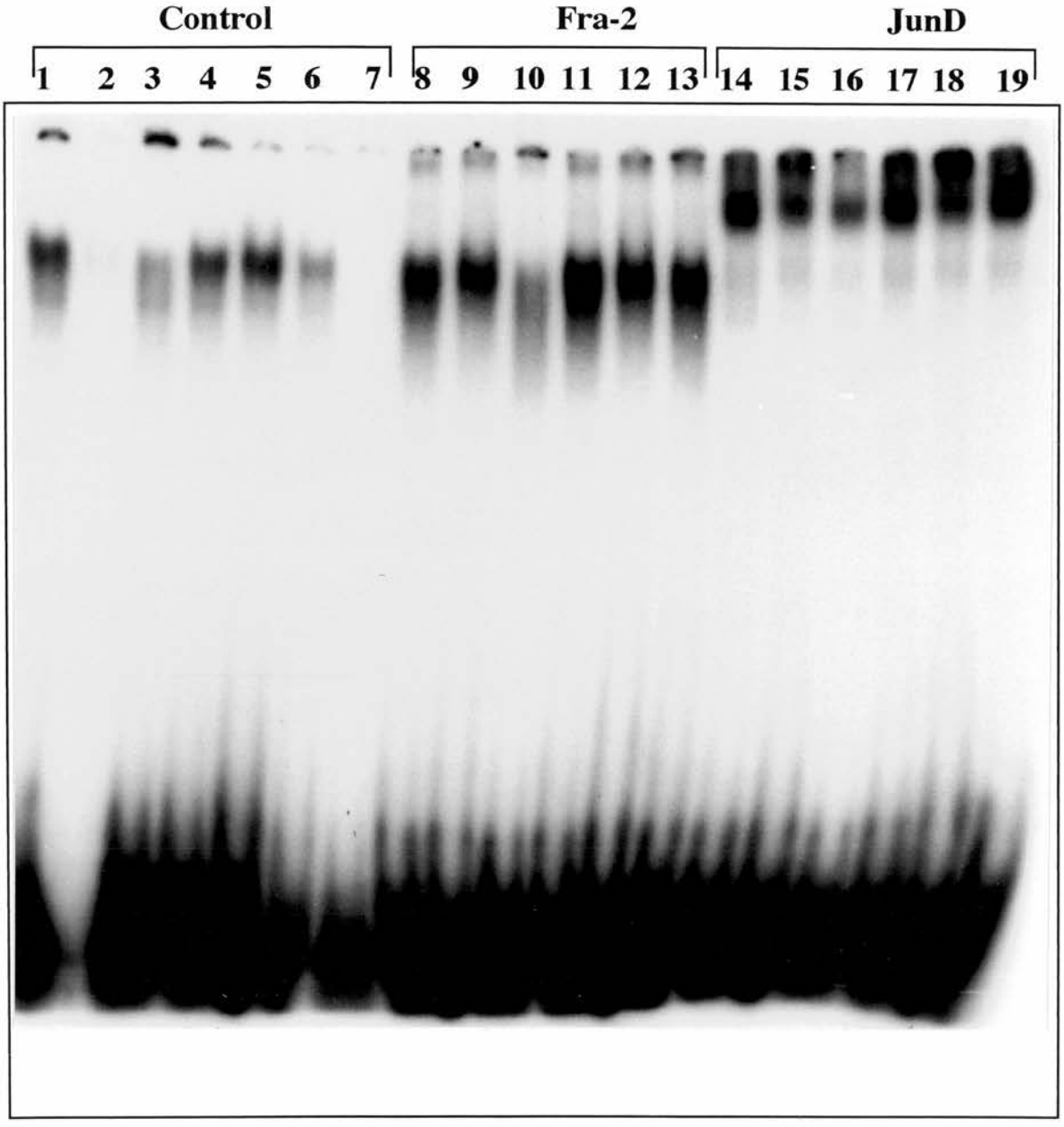


Figure 3.8A Electrophoretic mobility shift assay to evaluate the composition of the AP-1 transcription factor complex following stimulation by a non-toxic concentration of Glu in the absence of serum.
Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella. Granule cells were maintained in culture for 5 days. On day 5, the culture medium was exchanged for a serum-free medium (25 mM KCl) as defined in Materials and Methods. On day 7 (the day of the experiment) the medium was again changed, to a serum-free medium (5 mM KCl) as defined in Materials and Methods, before exposure to 10 μM Glu for 0 min (lanes 1, 8, 14); 15 min (lanes 2, 9, 15); 30 min (lanes 3, 10, 16); 60 min (lanes 4, 11, 17); 90 min (lanes 5, 12, 18) or 240 min (lanes 6, 13, 19). Nuclear extracts were prepared and aliquots incubated with a radioactively-labelled DNA fragment corresponding to the TRE, without (Control; lanes 1-6) or with antibody specific to Fra-2 (lanes 8-13) or JunD (lanes 14-19). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film. Lane 7: 45 min exposure, excess cold TRE added as negative control.

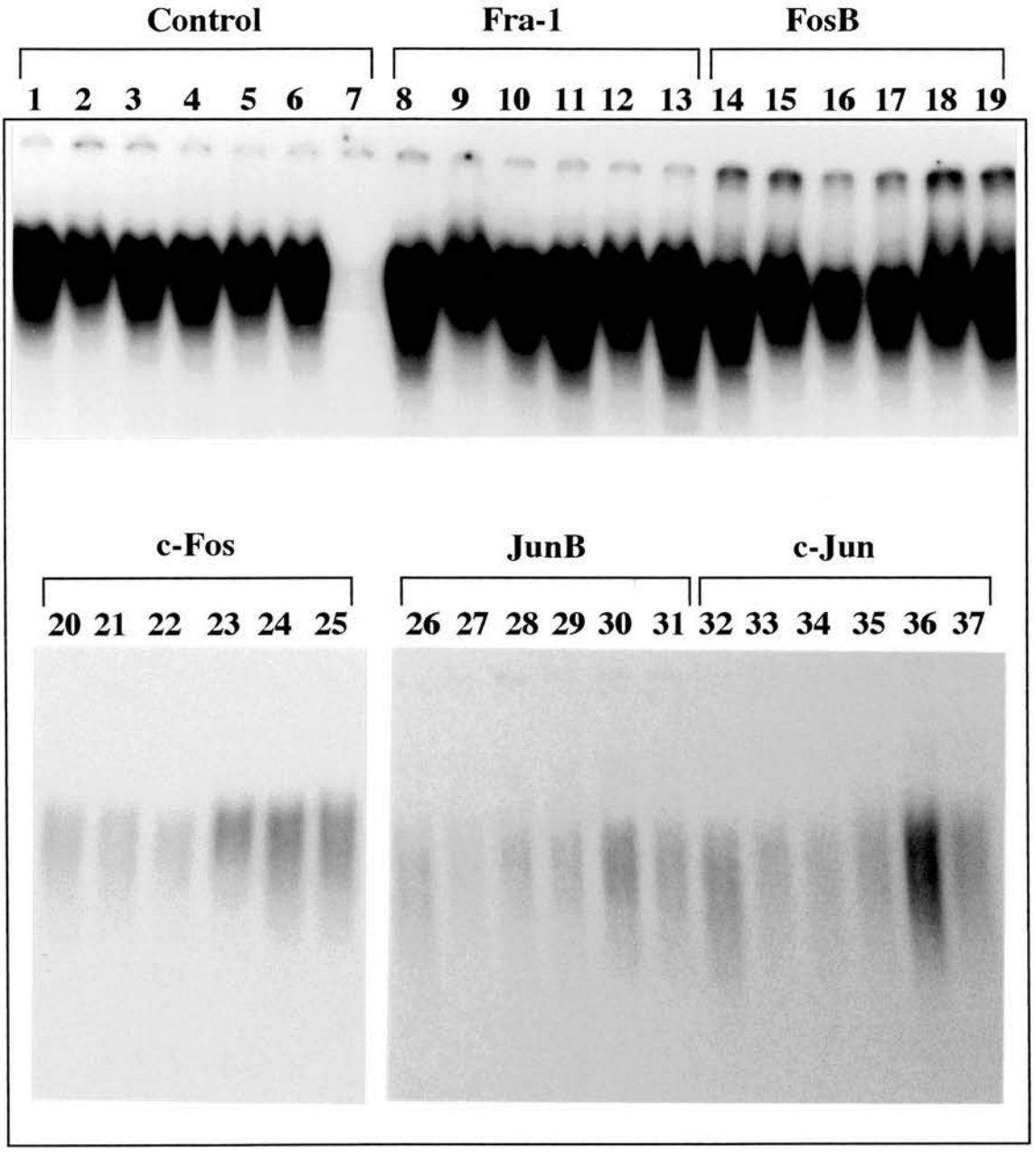


Figure 3.8B Electrophoretic mobility shift assay to evaluate the composition of the AP-1 transcription factor complex following stimulation by a non-toxic concentration of Glu in the absence of serum.
Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella. Granule cells were maintained in culture for 5 days. On day 5, the culture medium was exchanged for a serum-free medium (25 mM KCl) as defined in Materials and Methods. On day 7 (the day of the experiment) the medium was again changed, to a serum-free medium (5 mM KCl) as defined in Materials and Methods, before exposure to 10 μM Glu for 0 min (lanes 1, 8, 14, 20, 26, 32); 15 min (lanes 2, 9, 15, 21, 27, 33); 30 min (lanes 3, 10, 16, 22, 28, 34); 60 min (lanes 4, 11, 17, 23, 29, 35); 90 min (lanes 5, 12, 18, 24, 30, 36) or 240 min (lanes 6, 13, 19, 25, 31, 37). Nuclear extracts were prepared and aliquots incubated with a radioactively-labelled DNA fragment corresponding to the TRE, without (Control; lanes 1-6) or with antibody specific to Fra-1 (lanes 8-13), FosB (lanes 14-19), c-Fos (lanes 20-25), JunB (lanes 26-31) or c-Jun (lanes 33-37). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film. Lane 7: 45 min exposure, excess cold TRE added as negative control.

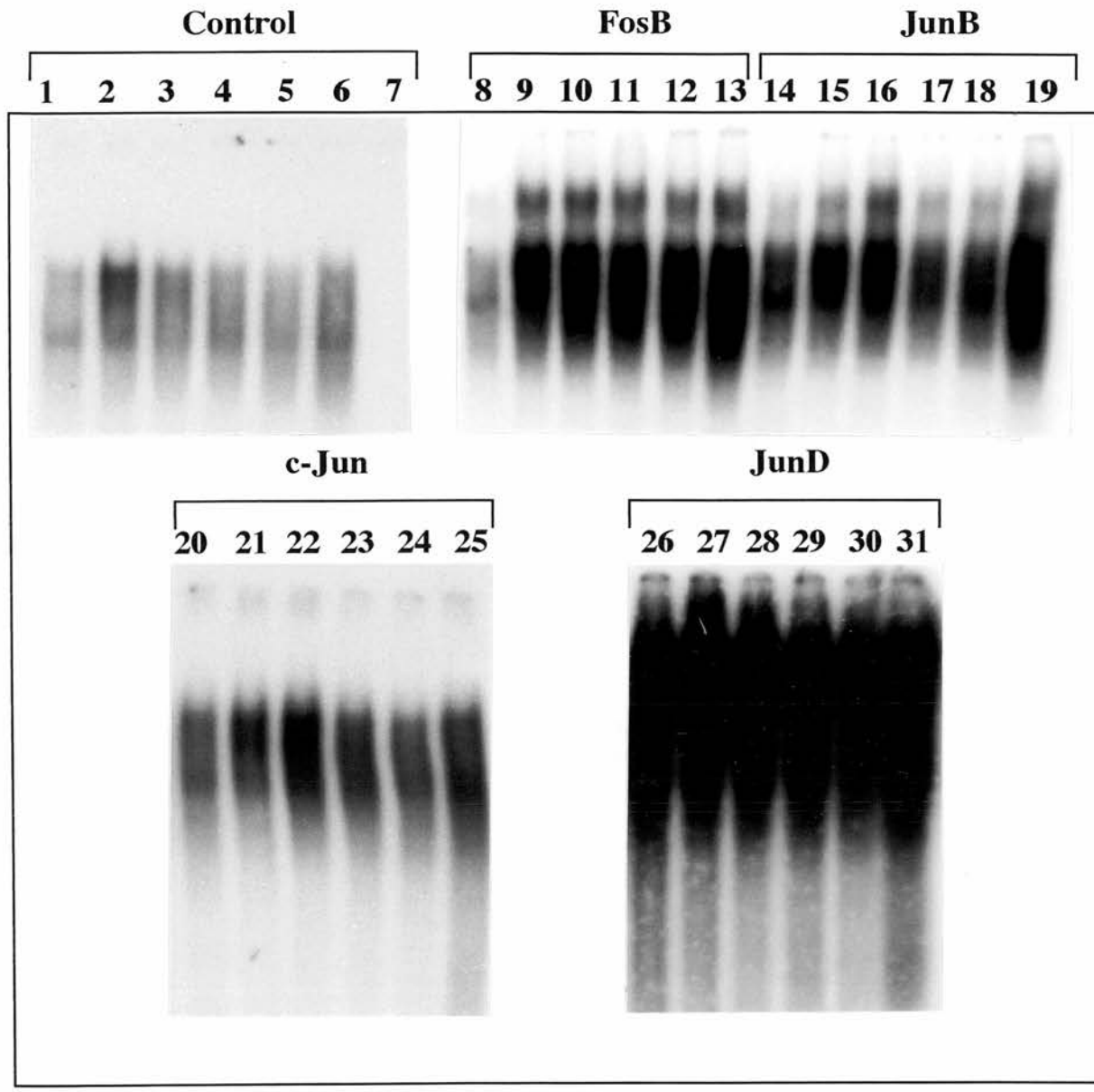


Figure 3.9 Electrophoretic mobility shift assay to evaluate the composition of the AP-1 transcription factor complex following stimulation by a toxic concentration of Glu in the absence of serum.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella. Granule cells were maintained in culture for 5 days. On day 5, the culture medium was exchanged for a serum-free medium (25 mM KCl) as defined in Materials and Methods. On day 7, the day of the experiment, the medium was changed, to a serum-free medium (5 mM KCl) as defined in Materials and Methods, before exposure to 250 μM Glu for 0 min (lanes 1, 8, 14, 20, 26); 15 min (lanes 2, 9, 15, 21, 27); 30 min (lanes 3, 10, 16, 22, 28); 45 min (lanes 4, 11, 17, 23, 29); 60 min (lanes 5, 12, 18, 24, 30) or 90 min (lanes 6, 13, 19, 25, 31). Nuclear extracts were prepared and aliquots incubated with a radioactively-labelled DNA fragment corresponding to the TRE, without (Control; lanes 1-6) or with the addition of antibodies specific for FosB (lanes 8-13), JunB, (lanes 14-19), c-Jun (lanes 20-25) or JunD (lanes 26-31). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film. Lane 7: 90 min exposure, excess cold TRE added as a negative control.

members as well as heterodimers of FosB and each of the Jun proteins in response to toxic levels of Glu.

3.2.4.2 Cells maintained in serum-containing medium

When cells were grown in serum-containing medium and then exposed, in serum-containing culture-conditioned medium, with a non-toxic (10 μM) concentration of Glu for varying times (0-240 min), the antibodies which caused a supershift to occur were c-Fos, Fra-2 and JunD (Figure 3.10).

After stimulation of these cells with toxic (250 μM) levels of Glu, c-Fos, FosB, Fra-2 and JunD were part of the AP-1 transcription factor complexes formed (Figure 3.11). The intensity of the Fra-2 expression band increased with time, and exhibited evidence of late induction (Figure 3.11); an observation similar to that reported in Kovács (1998).

In the case of c-Fos expression, induced by either non-toxic or toxic concentrations of Glu, the intensity of the supershifted band at time zero (lane 10, Figures 3.10 and 3.11) was far less than those of the time points following stimulation with Glu. It is thought to be possible to switch on transcription of c-*fos* by handling of the cells, and whilst every care was taken to avoid it, this could enhance the stimulation of very low levels of c-Fos in the time zero lanes.

The expression of FosB in cells exposed to toxic levels of Glu was not, as in the case of c-Fos, apparent at all time points. The super-shift band appeared at the 15 min time-point and remained until the 60 min time-point; however, no super-shift was observed at either the 90 min or the 240 min time-point.

The effect of NMDA receptor antagonism on Glu-induced AP-1 transcription factor complex composition under toxic conditions was investigated by exposing cells to a combination of 250 μM Glu and 1 μM TCP, a highly potent antagonist of the NMDA receptor. The presence of TCP in conjunction with Glu in the medium prevented the participation of FosB and c-Fos in the AP-1 complex (Figure 3.12; c.f. Figure 3.11).

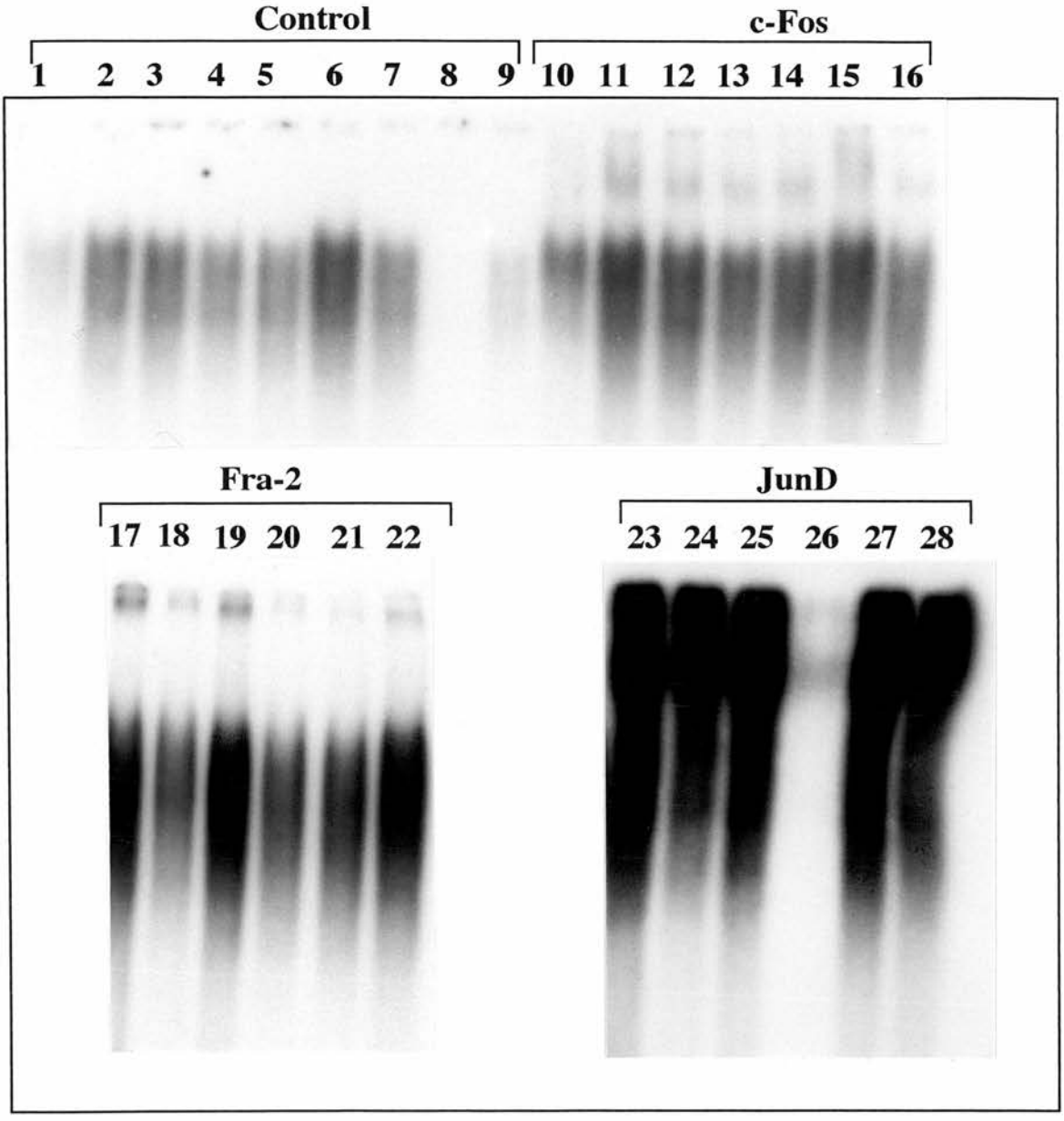


Figure 3.10 Electrophoretic mobility shift assay to evaluate the composition of the AP-1 transcription factor complex following stimulation with a non-toxic concentration of Glu in the presence of serum.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure to 10 μM Glu for 0 min (lanes 1, 10, 17, 23); 15 min (lanes 2, 11, 18, 24); 30 min (lanes 3, 12, 19, 25); 45 min (lanes 4, 13); 60 min (lanes 5, 14, 20, 26); 90 min (lanes 6, 15, 21, 27) or 240 min (lanes 7, 16, 22, 28). Nuclear extracts were prepared and aliquots incubated with a radioactively-labelled DNA fragment corresponding to the TRE, without (control; lanes 1-7) or with the addition of antibodies specific for c-Fos (lanes 10-16), Fra-2 (lanes 17-22) or JunD (lanes 23-28). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film. Lane 8: 45 min exposure, excess cold TRE added as a negative control. Lane 9: 45 min exposure, excess cold, mutant TRE added as a positive control.

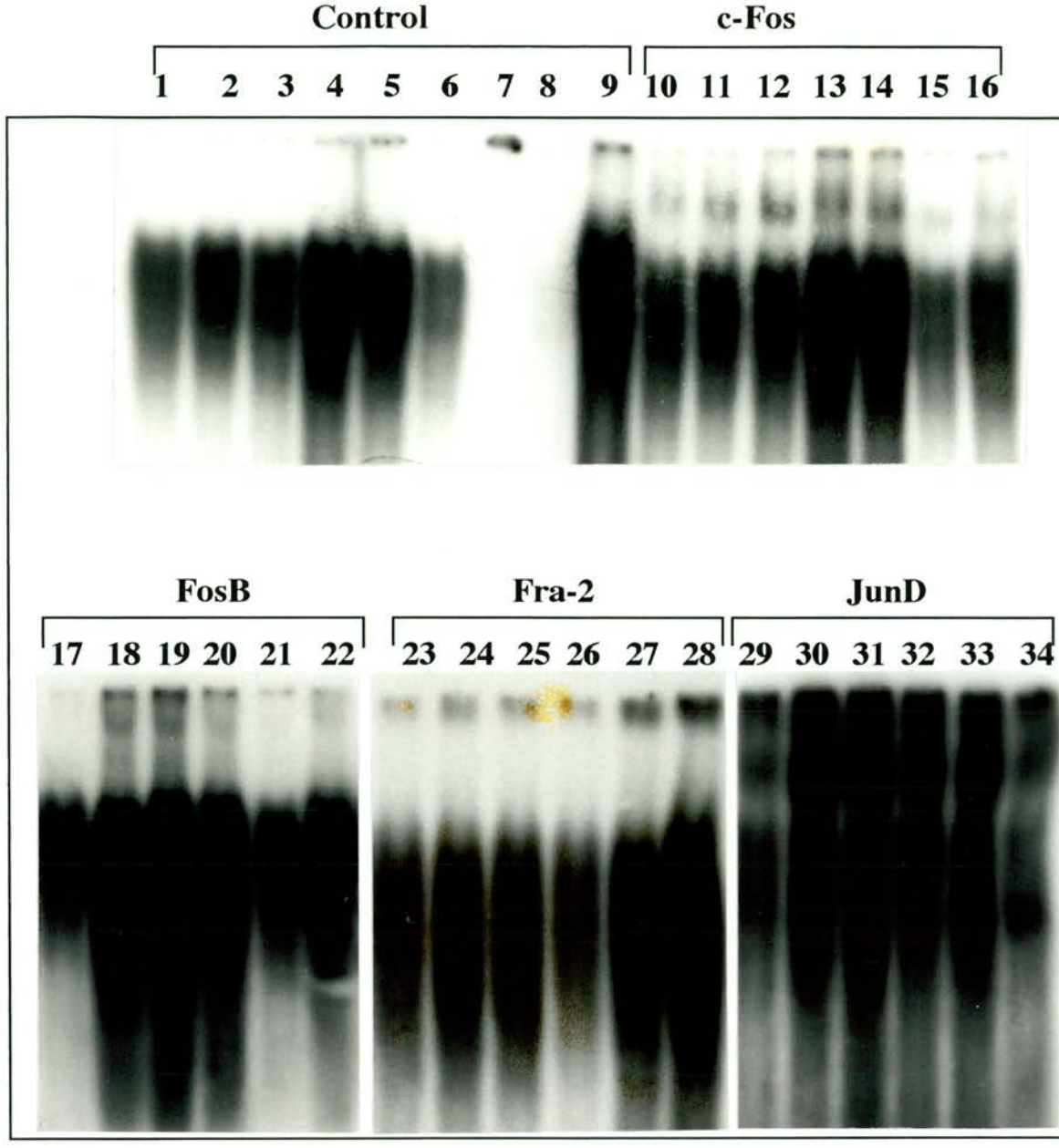


Figure 3.11 Electrophoretic mobility shift assay to evaluate the composition of the AP-1 transcription factor complex following stimulation with a toxic concentration of Glu in the presence of serum.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure to 250 μM Glu for 0 min (lanes, 1, 10, 17, 23, 29); 15 min (lanes 2, 11, 18, 24, 30); 30 min (lanes 3, 12, 19, 25, 31); 45 min (lanes 4, 13); 60 min (lanes 5, 14, 20, 26, 32); 90 min (lanes 6, 15, 21, 27, 33) or 240 min (lanes 7, 16, 22, 28, 34). Nuclear extracts were prepared and aliquots incubated with a radioactively-labelled DNA fragment corresponding to the TRE, without (control; lanes 1-7) or with the addition of antibodies specific for c-Fos (lanes 10-16), FosB (lanes 17-22), Fra-2 (lanes 23-28) or JunD (29-34). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film. Lane 8: 60 min exposure, excess cold TRE added as a negative control. Lane 9: 60 min exposure, excess cold, mutant TRE added as a positive control.

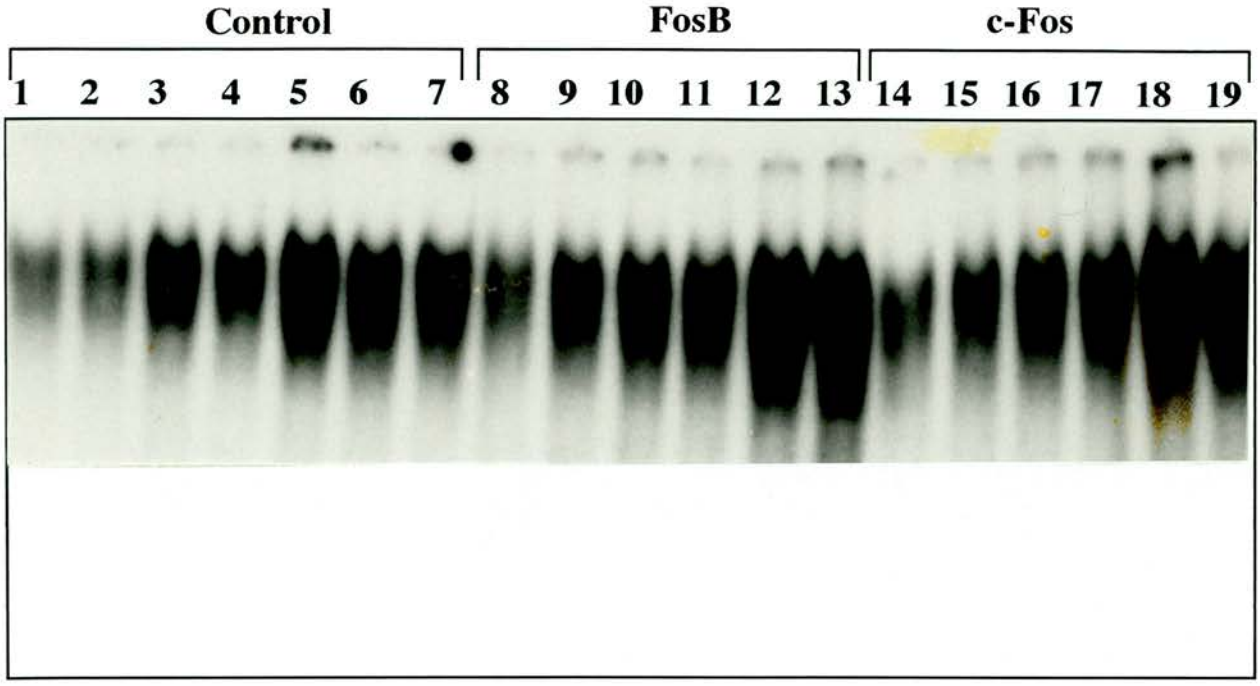


Figure 3.12 Electrophoretic mobility shift assay of the composition of the AP-1 transcription factor complex following stimulation with a toxic concentration of Glu in the presence of the NMDA receptor antagonist, TCP.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure to 250 μM Glu coadministered with 1 μM TCP for 0 min (lanes 1, 8, 14); 15 min (lanes 2, 9, 15); 30 min (lanes 3, 10, 16); 60 min (lanes 4, 11, 17); 90 min (lanes 5, 12, 18) or 240 min (lanes 6, 13, 19). Nuclear extracts were prepared and aliquots incubated with a radioactively-labelled DNA fragment corresponding to the TRE, without (control; lanes 1-7) or with the addition of antibodies specific for FosB (lanes 8-13) or c-Fos (lanes 14-19). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film. Lane 7: 240 min exposure, excess cold, mutant TRE added as a positive control.

3.2.5 Toxic and non-toxic levels of Glu stimulate differential c-Fos protein expression profiles, as detected by Western blotting

Cultured cerebellar granule cells (7 DIV) were stimulated for 1, 2 and 4h with either 10 μM or 250 μM Glu, and the expression patterns for c-Fos and FosB proteins analysed using Western blotting (Figure 3.13). The positive control used was 5 μM forskolin, which is known to switch on c-Fos expression by activation of adenylate cyclase and stimulating accumulation of cAMP (Barthel *et al.*, 1996). Low levels of c-Fos were expressed even in the absence of stimulation (Figure 3.13A, Lane 1), and after exposure to 10 μM Glu; however, the levels were greatly increased by the addition of 250 μM Glu for 2-4h (the end-point of this expression was not reached in the experiment). FosB was only detected after the cells had been exposed to 250 μM Glu for 2-4h (Figure 3.13B); no protein band was observed in the control lanes or following stimulation with 10 μM Glu. Again, the expression did not decrease within the time-course of the experiment. Forskolin caused expression of c-Fos, as expected, and to a lesser extent, FosB.

3.2.6 Transient transfection studies on the effect of Glu on fos-luc3A induction

The functional nature of c-Fos protein expression was investigated using transient transfection of cultured cerebellar granule cells. The cells were cultured for 5 days before being transfected with -361fosluc plasmid, which contained the fos gene linked to a reporter gene, coding for the enzyme luciferase. After the transfection process the cells were treated with non-toxic (10 μM) and toxic (250 μM) levels of Glu, with 5 μM forskolin acting as a positive control. Non-toxic levels of Glu (10 μM) caused an increase in the expression of luciferase (and therefore c-Fos) significantly above basal, as did forskolin (Figure 3.14). However, protein expression after the administration of 250 μM Glu remained at basal levels.

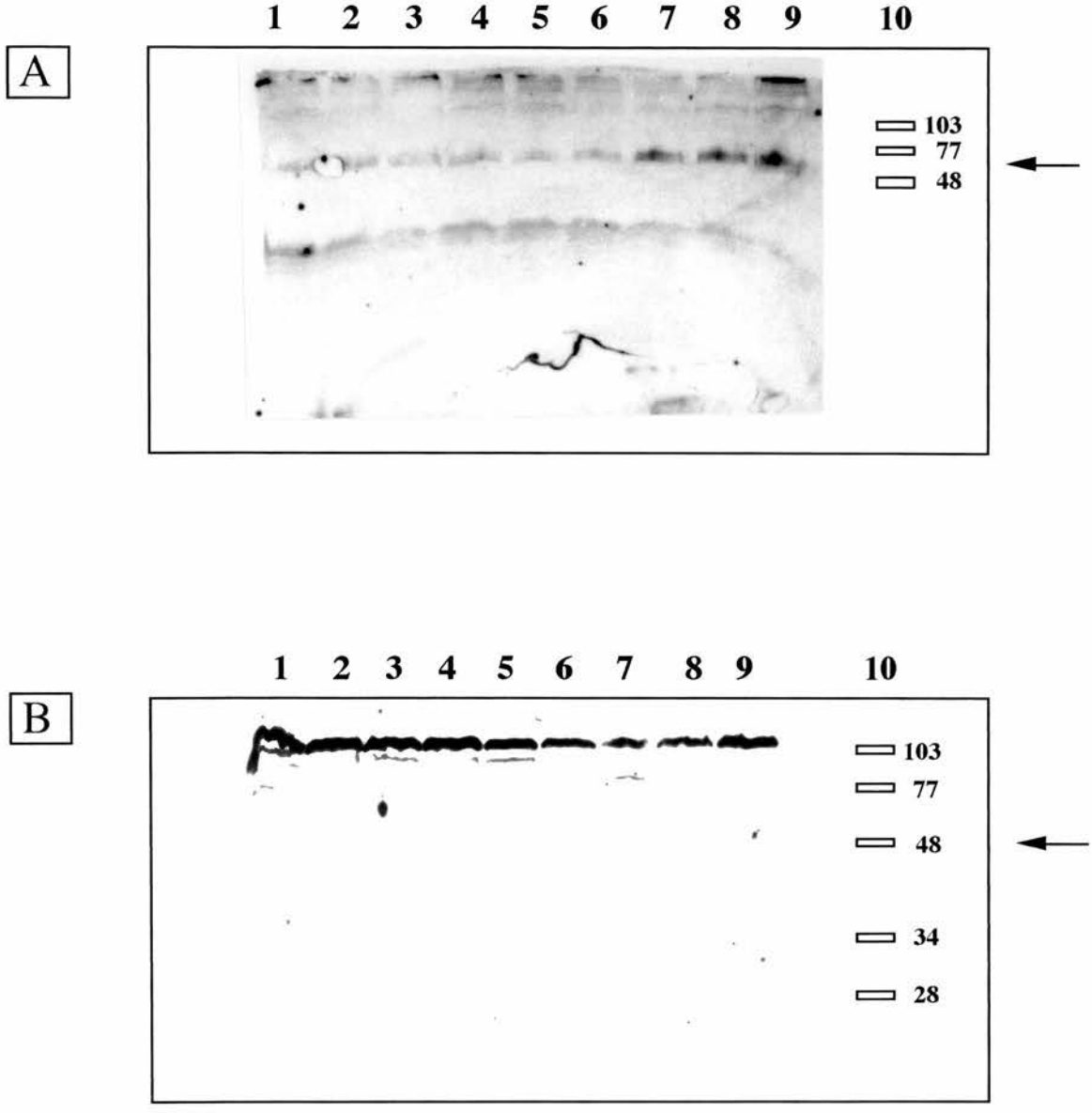


Figure 3.13 Expression of c-Fos and FosB protein in cerebellar granule cells in response to Glu exposure.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure to 10 μM or 250 μM Glu for up to 4h. 5 μM forskolin was used as a positive control. Cellular extracts were prepared and subjected to SDS-PAGE on a 10% gel. The proteins were then blotted onto nitrocellulose and probed using specific antibodies for c-Fos (A) and FosB (B) protein. The presence of protein was visualised by enhanced chemiluminescence. Lanes 1 and 5: Control, no exposure. Lane 2: 10 μM Glu, 1h exposure. Lane 3: 10 μM Glu, 2h exposure. Lane 4: 10 μM Glu, 4h exposure. Lane 6: 250 μM Glu, 1h exposure. Lane 7: 250 μM Glu, 2h exposure. Lane 8: 250 μM Glu, 4h exposure. Lane 9: 5 μM forskolin, 4h exposure. Lane 10: Molecular weight markers in kilodaltons. Arrow indicates position of c-Fos (A) or FosB (B) protein band.

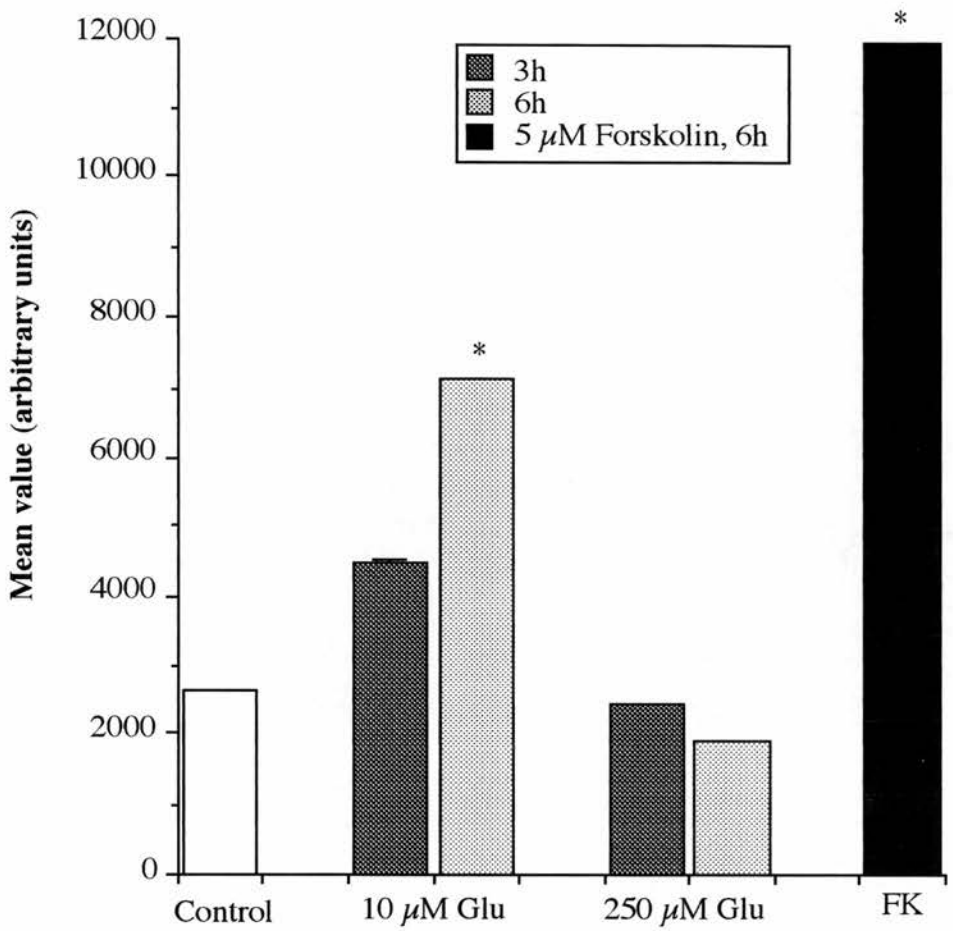


Figure 3.14 Analysis of c-Fos protein expression using the luciferase reporter gene assay.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 5 days before use. Cells were transfected using Transfectam with -361fosluc plasmid in serum-free defined medium and then assayed for luciferase protein expression using a luminometer. Data represent mean ± SEM values (n = 3). Asterisks indicate significant difference from control value.

Section B: Investigation of the effect of low and high levels of K^+ ions on cerebellar granule cells

3.2.7 Introduction

In Section 3.2.4 above, it was shown that FosB protein only comprises part of the AP-1 transcription factor complex (as assayed by electrophoretic mobility shift assay) in 7 DIV cells following stimulation with a toxic concentration (250 μM) of Glu. It was thought that the presence of FosB in the AP-1 complex could be a marker of a toxic event. It was thus decided to probe the mechanism further by stimulating cells with high levels of K^+ ions, influx of which causes depolarisation of the membrane and increase in intracellular Ca^{2+} ion concentration. Thus this is an alternative method for studying the effects of increased intracellular Ca^{2+} on the composition of the AP-1 transcription factor complex in 7 DIV cerebellar granule cells.

3.2.7.1 Potassium ions are not toxic to cerebellar granule cells but do cause a large increase in the intracellular calcium concentration

Cells were grown for 7 days in culture as before, and then exposed to increasing concentrations (24.5-55 mM) of KCl for 24h prior to being analysed using the MTT assay (the concentration of KCl in the growth medium used was 24.5 mM). Figure 3.15 shows that even at the highest concentration of K^+ ions used (55 mM), there was no significant reduction in absorbance, indicating no apparent toxicity. This correlates with the observation that no change in morphology occurred following exposure to 55 mM KCl (Figure 3.1F). However, when cells were exposed to increasing concentrations of KCl (24.5-55 mM) and immediately assayed for intracellular calcium using the Fluo-3 assay, the amount of calcium in the cytoplasm increased with the concentration of KCl by ~6-fold over basal (Figure 3.16). In order to ascertain the mechanism of K^+ ion-induced calcium ion influx, cells were treated with 55 mM KCl in conjunction with various receptor antagonists and the voltage-gated Ca^{2+} channel blocker, nifedipine. Figure 3.17 shows that none of the glutamatergic ionotropic receptor antagonists (APV (500 μM), TCP (1 μM), CNQX(10 μM) and NBQX (100 μM)) were able to block the influx of calcium ions. However, the L-type voltage-gated calcium channel blocker, nifedipine,

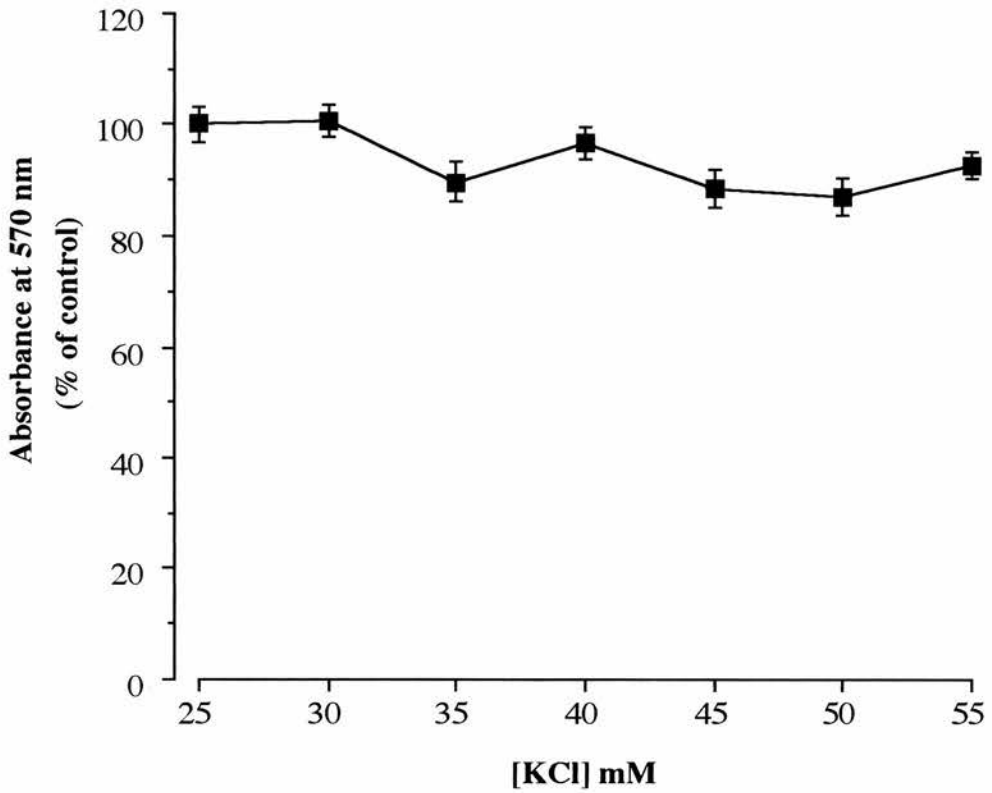


Figure 3.15 Survival of cerebellar granule cells in primary culture after exposure to increasing concentrations of KCl.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 7 days before use. Granule cells were exposed (24h) to increasing concentrations (25-55 mM) of KCl prior to assessment of cell viability by MTT staining. The absorbance at 570 nm was expressed as a percentage of control cells, corresponding to 25 mM KCl, at which cells were maintained in culture. Data represent mean ± SEM values (n = 8-16). The absorbance values for control cells were 0.223 ± 0.007.

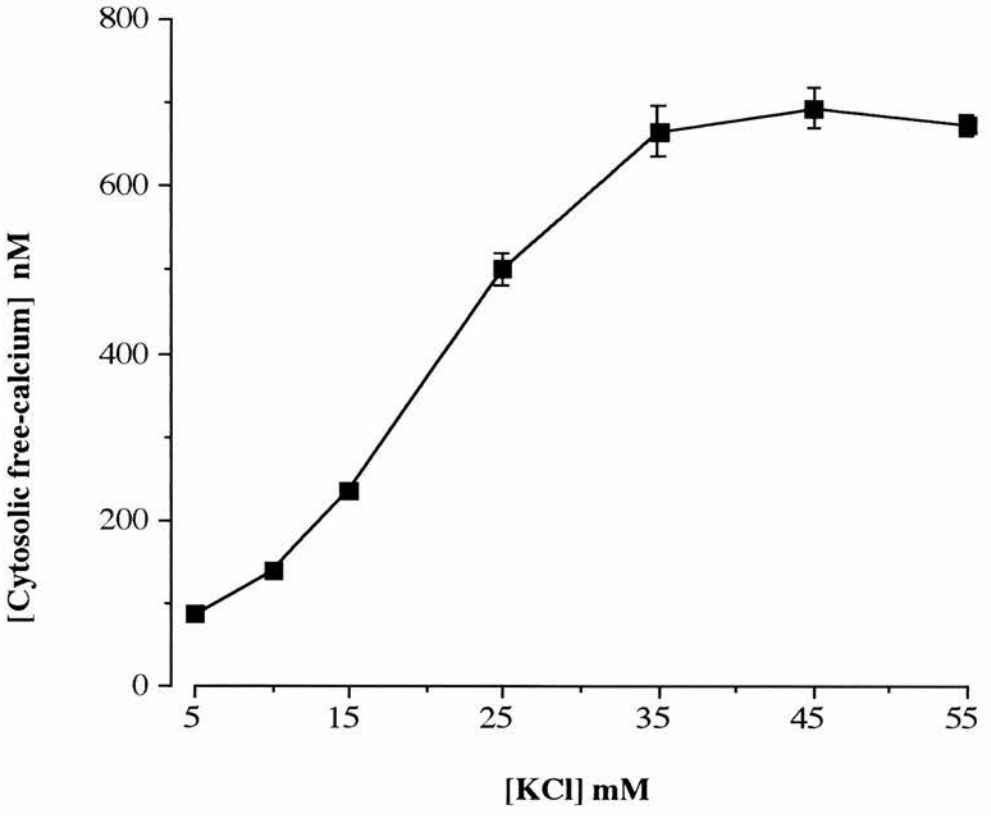


Figure 3.16 Concentration-dependent effect of KCl on stimulated increases in intracellular-free calcium levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 7 days before use. Granule cells were washed with balanced salt solution, loaded with Fluo-3/AM and exposed to increasing [KCl], as indicated. Data are the mean ± SEM values (n = 8-16).

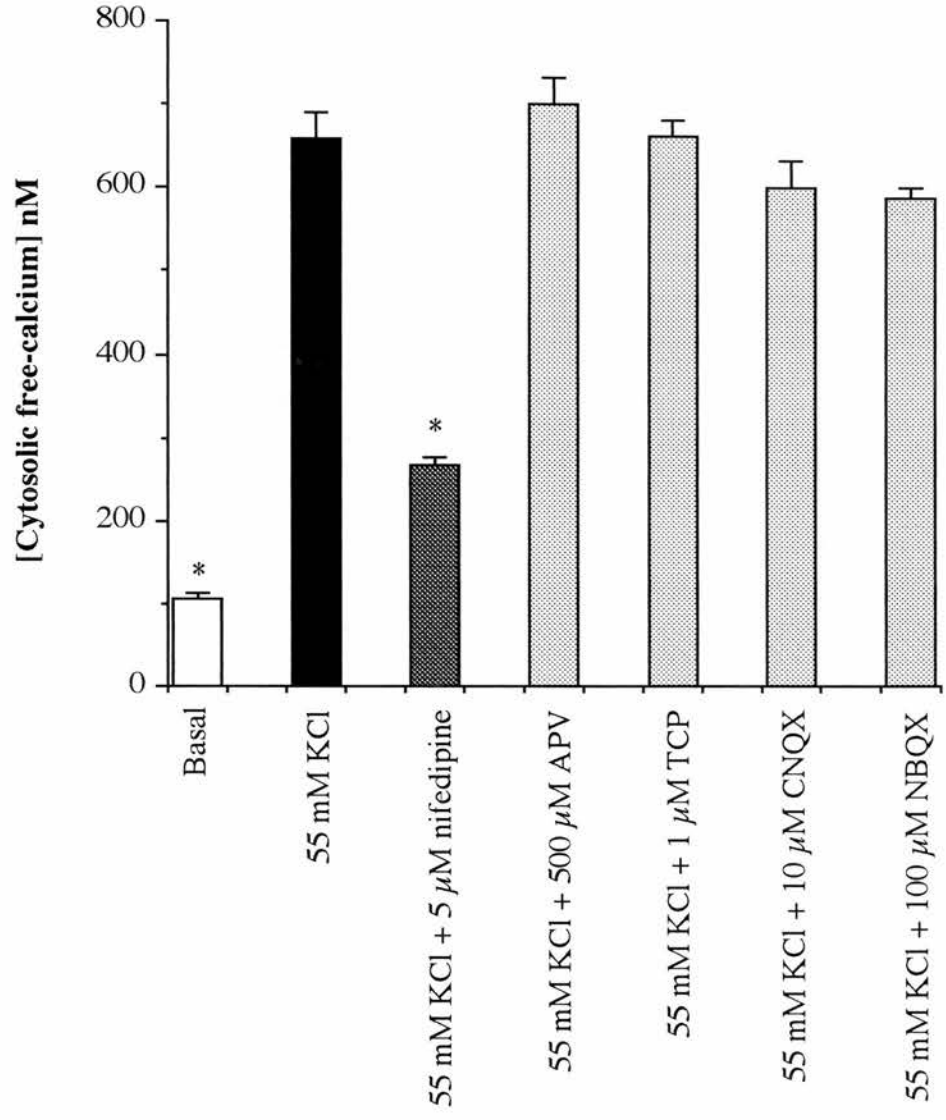


Figure 3.17 Pharmacology of KCl-stimulated increases in intracellular-free calcium levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 7 days before use. Granule cells were washed with balanced salt solution, loaded with Fluo-3/AM and exposed to 55 mM KCl alone or coadministered with pharmacological agents, as indicated. Data are the mean ± SEM values (n = 8-16). Asterisk indicates statistical difference from 55 mM KCl alone.

was able to dramatically decrease by ~75% the amount of calcium influx in response to membrane depolarisation by K^+ ions. Thus it can be concluded that K^+ ions mediate Ca^{2+} ion influx via activation of L-type calcium channels, and not via the NMDA receptor, which is the main route of Glu-mediated Ca^{2+} ion influx.

3.2.7.2 KCl-mediated induction profiles of the immediate-early genes, c-fos and fosB are concentration-dependent

Cerebellar granule cells were grown in culture for 7 days and then treated with 5 mM and 55 mM KCl for up to 6h before being assayed for c-*fos* and *fos*B mRNA induction. Figure 3.18 shows that for both genes, 55 mM KCl caused a steady increase in mRNA levels over the time period of the experiment, which did not level off by 6h. In contrast, low levels of KCl (5 mM) appeared unable to increase the mRNA induction of either gene above basal. Figure 3.19 shows the effect of NMDA receptor antagonists APV and TCP and the L-type voltage-gated Ca^{2+} channel blocker nifedipine on the mRNA induction profiles of c-*fos*. Induction of the delayed, elevated profile in response to 55 mM KCl was prevented by nifedipine (5 μM), but not by APV (500 μM) or TCP (1 μM).

3.2.7.3 FosB protein is produced in response to K^+ ions but is not functional as part of the AP-1 transcription factor complex

In response to 55 mM KCl, 7 day old cells grown in the presence of serum expressed c-Fos as part of the AP-1 transcription factor complex, but not FosB, as analysed by EMSA (Figure 3.20). Paradoxically, when the expression of c-Fos and FosB protein was analysed using Western blotting, it was found that both c-Fos and FosB proteins were expressed after stimulation with 55 mM KCl (Figure 3.21). Similar to its expression in response to Glu, c-Fos was expressed at low levels even in the control lane, but after 2-4h exposure to 55 mM KCl, the expression was increased (A). FosB was expressed only after stimulation with 55 mM KCl for 2-4h (B). Again, in both these cases, the expression did not return to basal within the confines of the experiment.

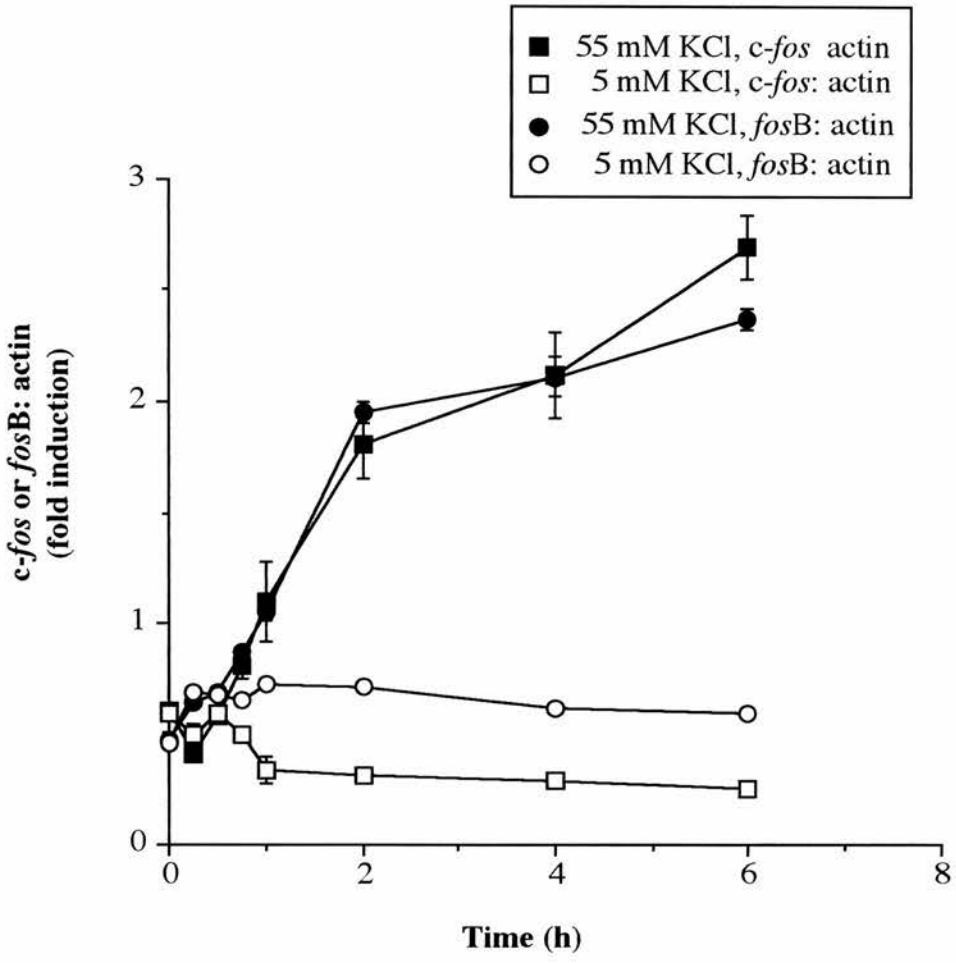


Figure 3.18 Time-course of KCl-stimulated, steady-state c-*fos* and *fos*B mRNA levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before continuous exposure to 5 mM or 55 mM KCl for up to 6h. Total RNA was prepared from the cultures, and the amount of c-*fos* and *fos*B mRNA was quantified. The results refer to cIEG mRNA expression relative to that of β-actin. The β-actin gene has a constant expression during the time interval used and thus acts as a control for the loading of RNA.

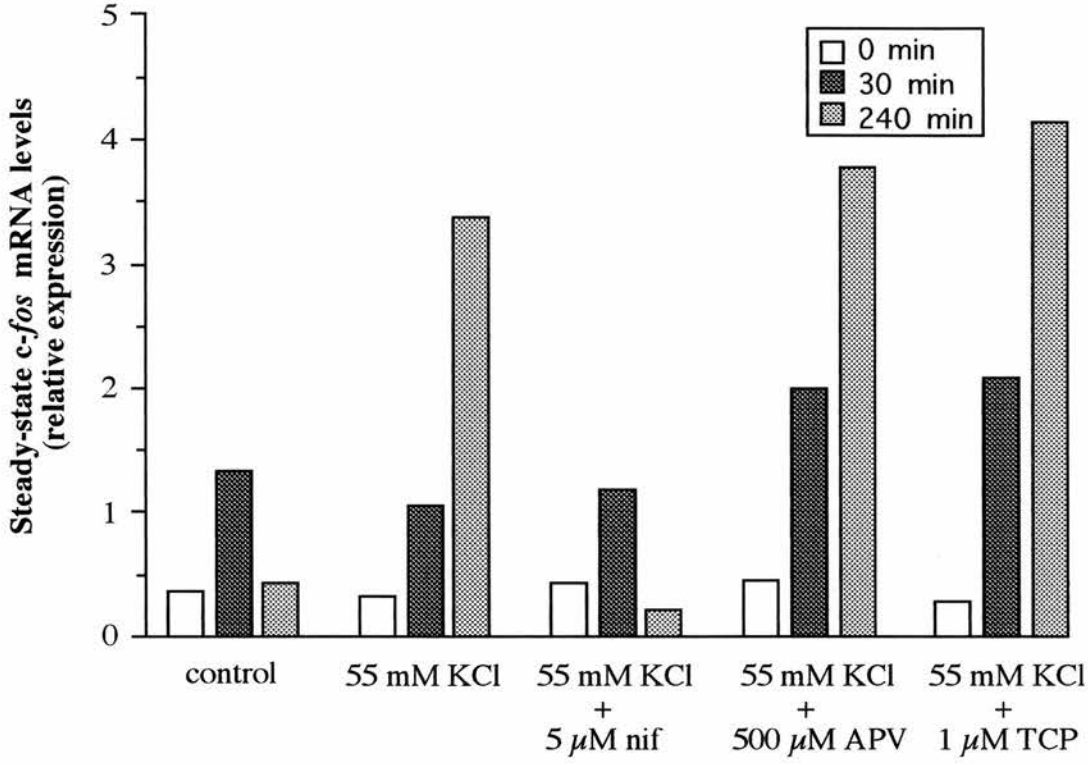


Figure 3.19 Pharmacology and temporal profiles of KCl-stimulated, steady-state *c-fos* mRNA levels in cultured cerebellar granule cells.
Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure for different times (0, 30 or 240 min) to 55 mM KCl alone or coadministered with various pharmacological antagonists, as indicated. After exposure, total RNA was prepared from the cultures, and the amount of *c-fos* and β-actin mRNA was quantified. The results represent the average of at least duplicate experiments and refer to *c-fos* mRNA expression relative to that of β-actin. The β-actin gene has a constant expression during the time interval used in this study; thus it acts as a control for the loading of RNA.
nif: nifedipine; APV: D,L (±)-2-amino-5-phosphonopentanoic acid; TCP: N-[1-(2-thienyl)cyclohexyl]-piperidine.

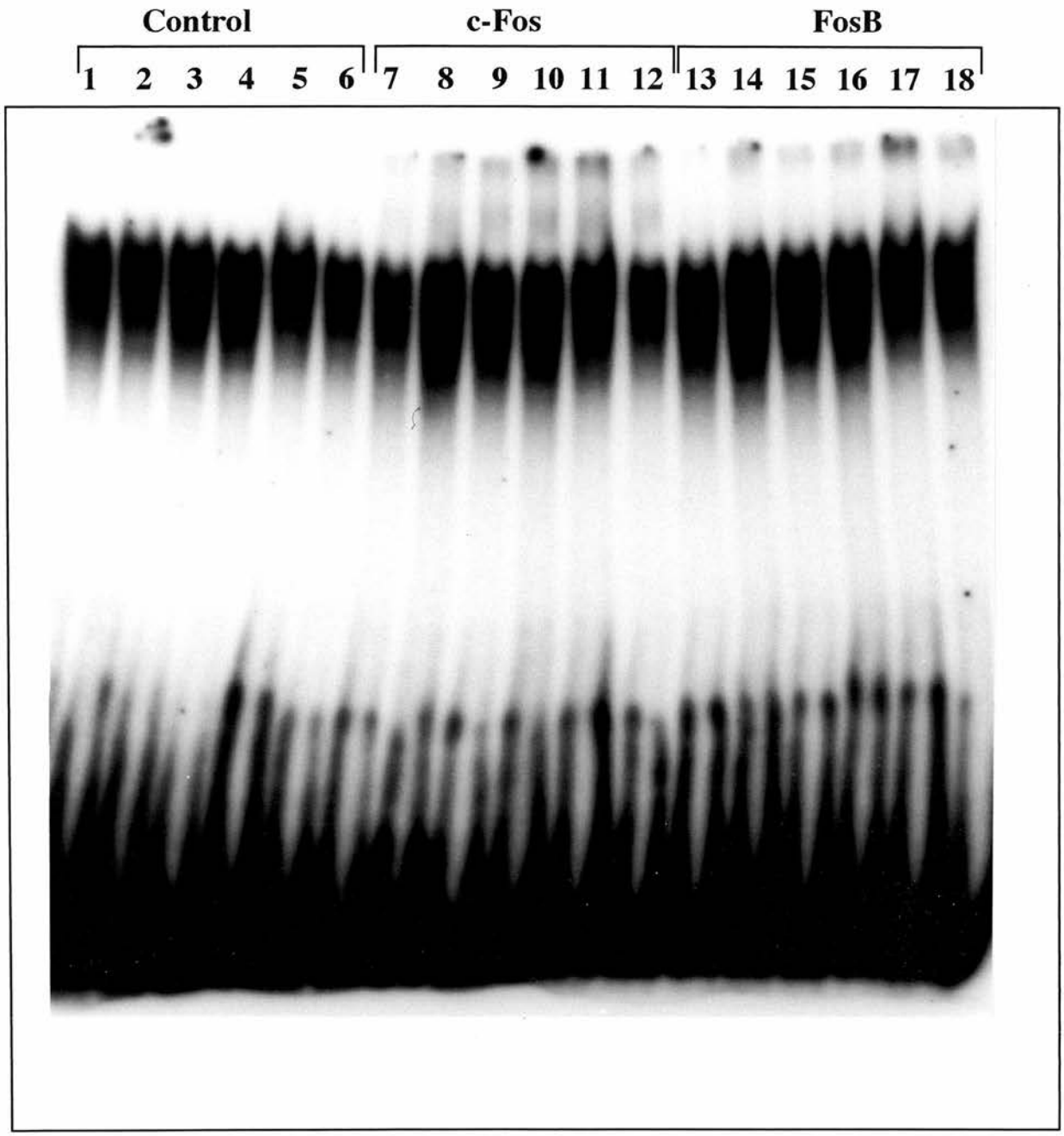


Figure 3.20 Electrophoretic mobility shift assay to evaluate the composition of the AP-1 transcription factor complex in cerebellar granule cells following stimulation with potassium ions. Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure to 55 mM KCl for 0 min (lanes 1, 7, 13); 30 min (lanes 2, 8, 14); 60 min (lanes 3, 9, 15); 90 min (lanes 4, 10, 16) or 240 min (lanes 5, 11, 17). As a positive control, cells were exposed to 250 μM Glu for 4h (lanes 6, 12, 18). Nuclear extracts were prepared and incubated with a radioactively-labelled DNA fragment corresponding to the TRE, without (control; lanes 1-6) or with the addition of antibodies specific for c-Fos (lanes 7-12) or FosB (lanes 13-18). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film.

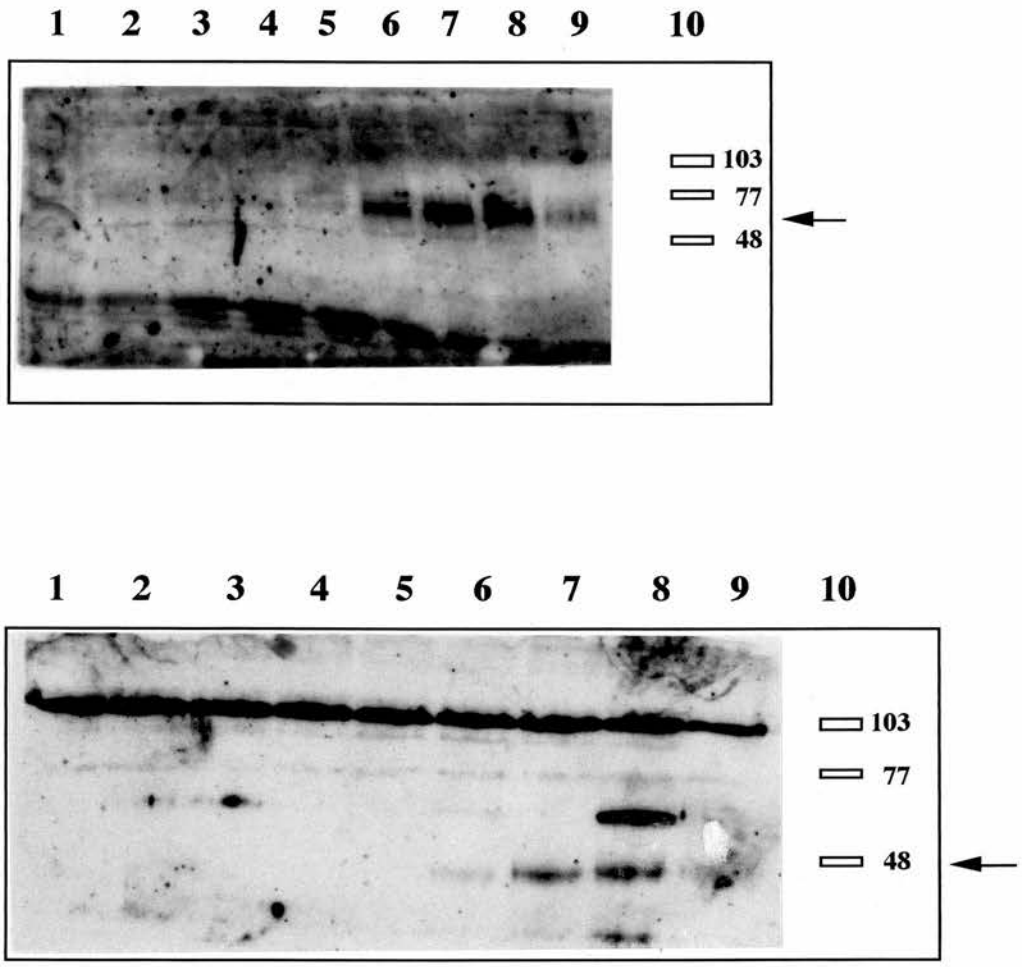


Figure 3.21 Expression of c-Fos and FosB protein in cerebellar granule cells in response to KCl exposure.
Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure to 55 mM KCl for up to 4h. 250 μM Glu was used as a positive control. Cellular extracts were prepared and subjected to SDS-PAGE on a 10% gel. The proteins were then blotted onto nitrocellulose and probed using specific antibodies for c-Fos (A) and FosB (B) protein. The presence of protein was visualised by enhanced chemiluminescence. Lanes 1 and 5: Control, no exposure. Lane 2: 25 mM KCl, 1h exposure. Lane 3: 25 mM KCl, 2h exposure. Lane 4: 25 mM KCl, 4h exposure. Lane 6: 55 mM KCl, 1h exposure. Lane 7: 55 mM KCl, 2h exposure. Lane 8: 55 mM KCl, 4h exposure. Lane 9: 250 μM Glu, 4h exposure. Lane 10: Molecular weight markers in kilodaltons. Arrow indicates position of c-Fos (A) or FosB (B) protein band.

Section C: An investigation into the role of the arachidonic acid pathway (catalysed by phospholipase A_2) in Glu-mediated cIEG induction

3.2.8 Evaluation of the effects of PLA_2 pathway inhibitors on Glu-mediated cytotoxicity, Ca^{2+} ion influx and immediate-early gene induction and product expression

When cerebellar granule cells at 7 DIV were stimulated for a 4h period with 250 μM Glu, the level of toxicity monitored was 65% (Figure 3.22A). However, after a 24h exposure period, toxicity was increased to 80-85% (Figure 3.22B). Cells were stimulated with Glu in combination with each of five compounds which inhibit enzymes involved in the phospholipase A_2 pathway. Aspirin (100 μM) proved to be effective in preventing this toxicity at both timepoints (4 and 24h); however, 1 μM indomethacin appears to be a slow-acting inhibitor and only made a significant difference after 24h, reducing the toxicity to 30%. Aristolochic acid, quinacrine and NDGA had no significant effect on the cell viability after Glu exposure. The site of inhibitory action for both indomethacin and aspirin is prevention of the conversion of arachidonic acid to prostaglandins and thromboxanes, catalysed by cyclooxygenase.

When cells were exposed to Glu alone or coadministered with various inhibitors of the PLA_2 pathway before being assayed for intracellular free calcium ion concentration, there was no effect by any of the enzyme inhibitors (Figure 3.23). The effect of quinacrine was not tested in this experiment since the compound forms a fluorescent solution and therefore it was impossible to assay using this method.

When the effect of these inhibitors on Glu-induced c-*fos* mRNA induction was measured, and the $Q^{240/30}$ ratios calculated, it was found that although the ratio for 250 μM Glu alone was 1.22, i.e. less than that required for an 'excitotoxic' event (Malcolm *et al.*, 1997), aspirin and quinacrine were able to bring the ratio to <1, which is deemed to be a non-excitotoxic level (Figure 3.24). However, the values obtained were not convincing enough to derive any significant conclusion from this result.

Glu-mediated c-Fos protein expression and the effect of the enzyme inhibitors used above on this expression was then analysed using Western blotting (Figure 3.25). The results shown in

this figure are fairly inconclusive due to the quality of the autoradiogram, but it is possible to report that c-Fos protein is expressed in response to both high and low concentrations of Glu. After stimulation with 10 μM Glu, only a small amount of c-Fos expression was detected. However, in the presence of aristolochic acid or indomethacin (to a lesser extent), the density of the c-Fos band was increased. NDGA had no effect on the amount of c-Fos expression.

3.2.9 Effect of experimentation on cell viability

Further to these studies, it was thought appropriate to use the MTT assay to ascertain whether any of the procedures carried out during this study caused significant cell death, which would potentially invalidate the results obtained. Some of the studies using the electrophoretic mobility shift assay (EMSA) involved depriving cells of serum and potassium ions before treatment with agonist. It has been reported that, under these conditions, cells undergo apoptosis (D'Mello *et al.*, 1993; Miller and Johnson, 1996). Neurotrophins (BDNF and NT-4/5) have been reported to protect cerebellar granule neurons against apoptosis at 1-4 DIV. Subsequently, these neurotrophins lose efficacy and NT-3 is the main neurotrophin involved in preventing neuron loss (Skaper *et al.*, 1998; for review see Tatter *et al.*, 1995). The survival effect appears to involve increases in intracellular Ca^{2+} concentration (Zirrgiebel *et al.*, 1995). Indeed, Copani *et al.* (1995) report that NMDA is able to protect against low $[K^+]$-induced apoptosis by increasing the intracellular Ca^{2+} ion concentration. It is also claimed that the functional activity of NMDA receptors increases with age. Thus it may be the case that immature cerebellar granule cells are not susceptible to apoptosis due to the low activity of their NMDA receptors (Resink *et al.*, 1992). However, KCl removal also results in decreased mitochondrial activity. When coupled with treatment with Glu, this can lead to necrosis in cultured cerebellar granule cells (Chang and Wang, 1997).

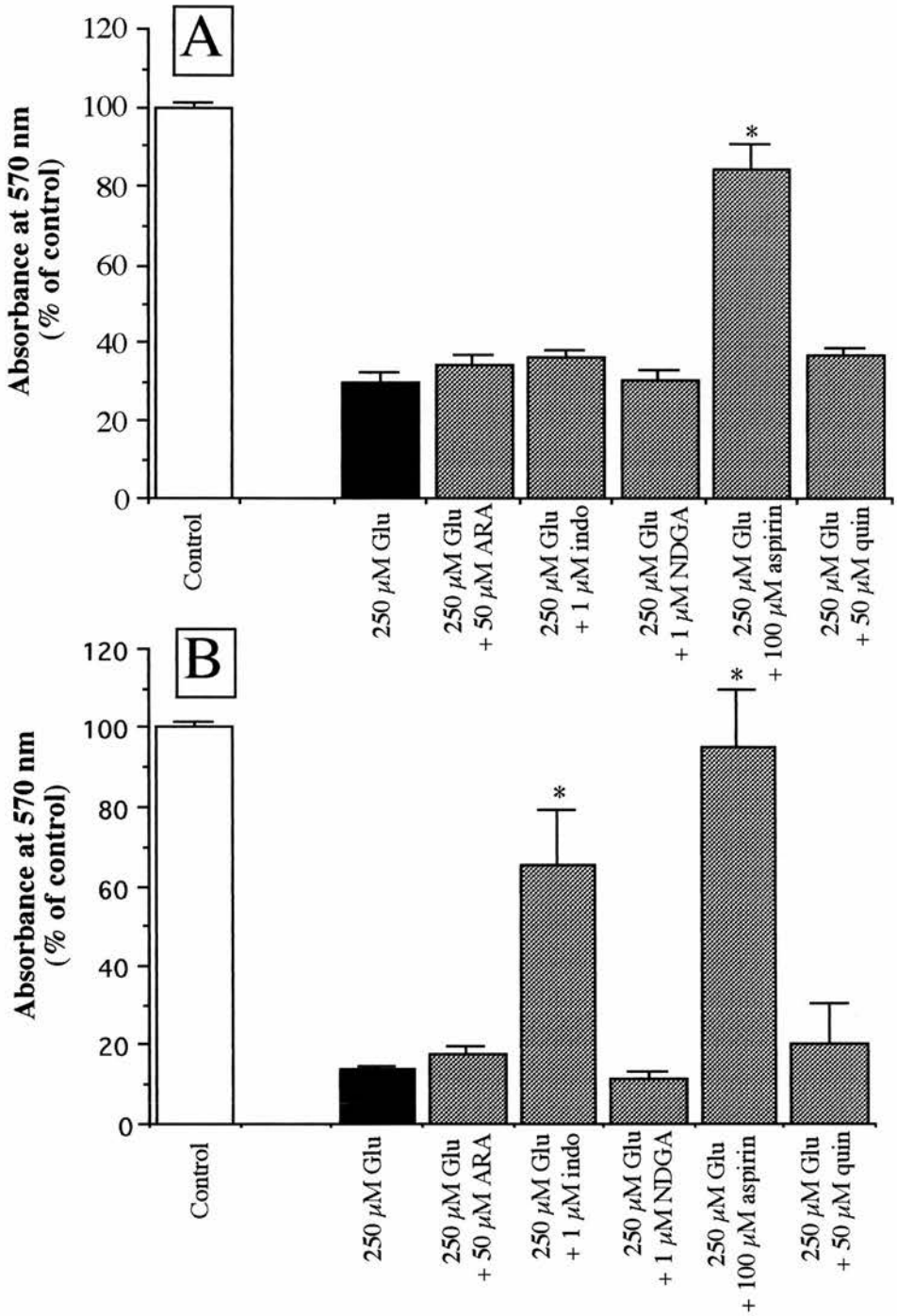


Figure 3.22 Effect of inhibitors of the PLA2 pathway inhibitors on Glu-stimulated cytotoxicity in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 7 days before use. Granule cells were exposed for 4h (**A**) or 24h (**B**) in culture-conditioned medium to 250 μM Glu alone or coadministered with various inhibitors of the phospholipase A2 pathway, as indicated, prior to analysis of cell viability using MTT staining. The absorbance at 570 nm was expressed as a percentage of control (untreated) cells. Data represent mean ± SEM values (n = 8-16). The absorbance values for control cells were 0.193 ± 0.011 (**A**) and 0.209 ± 0.008 (**B**). Asterisks indicate statistical difference from 250 μM Glu alone.

ARA: aristolochic acid; indo: indomethacin; NDGA: norhydroguarietic acid; quin: quinacrine.

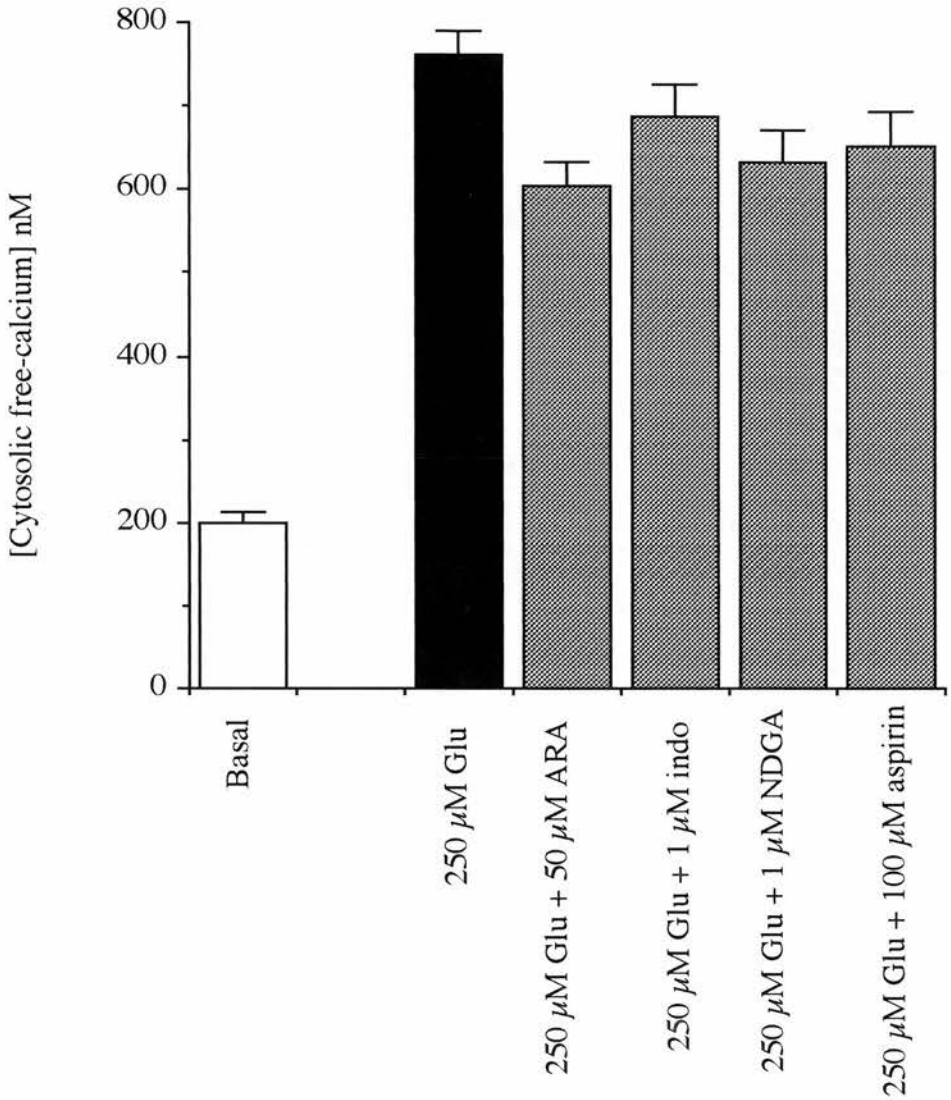


Figure 3.23 Effect of inhibitors of the PLA2 pathway on Glu-stimulated increases in intracellular-free calcium levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 7 days before use. Granule cells were washed with balanced salt solution, loaded with Fluo-3/AM and exposed to 250 μM Glu alone or coadministered with inhibitors of the phospholipase A2 pathway, as indicated. Data are the mean ± SEM values (n = 8-16).

ARA: aristolochic acid; indo: indomethacin; NDGA: norhydroguariaretic acid.

Exposure regime	$Q^{240/30}$ mean (actual values)
Control (HEPES)	0.62 (0.67, 0.56)
250 μM Glu	1.22 (1.20, 1.23)
250 μM Glu + 100 μM aspirin	0.88 (0.84, 0.93)
250 μM Glu + 50 μM aristolochic acid	1.45 (1.52, 1.38)
250 μM Glu + 1 μM indomethacin	1.12 (0.98, 1.26)
250 μM Glu + 1 μM NDGA	1.18 (1.13, 1.24)
250 μM Glu + 50 μM quinacrine	0.93 (0.88, 0.98)

Figure 3.24 Analysis of effect of PLA_2 pathway inhibitors on Glu-stimulated, steady-state c-*fos* mRNA levels in cultured cerebellar granule cells using the $Q^{240/30}$ ratio.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before being exposed for different times (30 or 240 min) to 250 μM Glu alone or coadministered with an inhibitor of the PLA_2 pathway, as indicated. After exposure, total RNA was prepared from the cultures, and the amount of c-*fos* and β-actin mRNA was quantified. The levels of c-*fos* mRNA at each of the timepoints were expressed relative to those of β-actin. The results shown indicate the ratio of c-*fos*/β-actin at 240 min to that at 30 min.

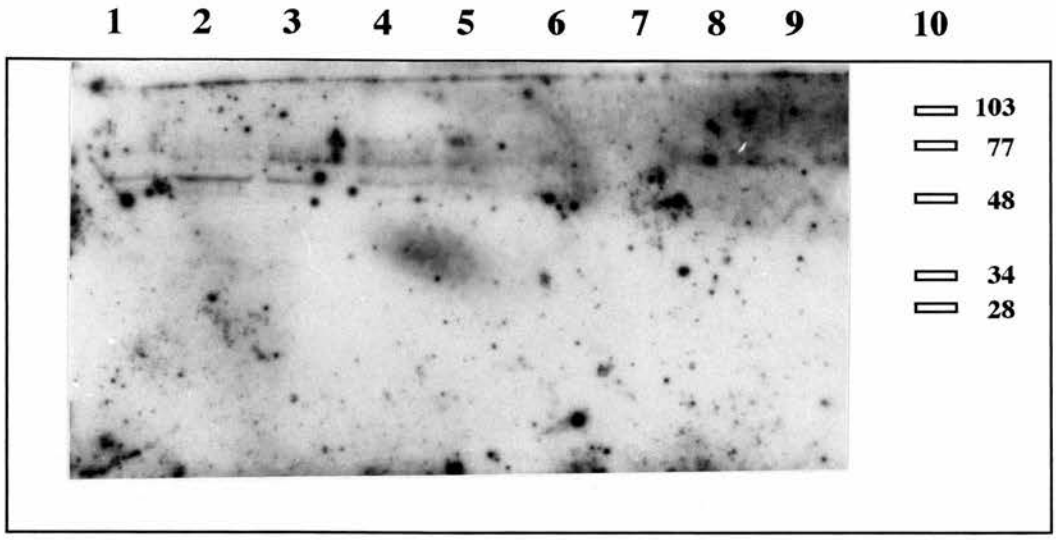


Figure 3.25 Effect of PLA2 pathway inhibitors on the expression of c-Fos protein in cerebellar granule cells in response to Glu exposure.
Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 7 days before exposure for 4h to 10 μM and 250 μM Glu alone or coadministered with various inhibitors of enzymes involved in the PLA2 pathway. Cellular extracts were prepared and subjected to SDS-PAGE on a 10% gel. The proteins were then blotted onto nitrocellulose and probed using specific antibodies for c-Fos protein. The presence of protein was visualised by enhanced chemiluminescence. Lane 1: Control, no exposure. Lane 2: 10 μM Glu alone. Lane 3: 10 μM Glu + 50 μM aristolochic acid. Lane 4: 10 μM Glu + 1 μM indomethacin. Lane 5: 10 μM Glu + 1 μM NDGA. Lane 6: 250 μM Glu alone. Lane 7: 250 μM Glu + 50 μM aristolochic acid. Lane 8: 250 μM Glu + 1 μM indomethacin. Lane 9: 250 μM Glu + 1 μM NDGA. Lane 10: Molecular weight markers in kilodaltons.

However, the length of time that the cells were in serum-deprived medium containing 5 mM K^+ was short enough that no morphological damage was apparent, and when an MTT assay was performed to compare cells in N-DMEM and those in serum-deprived medium containing 5 mM K^+, the decrease in the absorbance values was not significant (Figure 3.26; 10% decrease in absorbance).

In Section 3.2.6, the results of the transfection studies are described. In response to the results, it was thought that an investigation of the viability of the cells after transfection at various stages of culture using the MTT assay would be useful. Figure 3.27 shows that there was a significant decline in the absorbance of cells transfected at 6-8 days in culture when compared with those cells transfected at 4-6 days in culture. Those cells which were transfected later on in the culture period (i.e. 6-8 DIV) seemed to be less robust and more susceptible to cell damage.

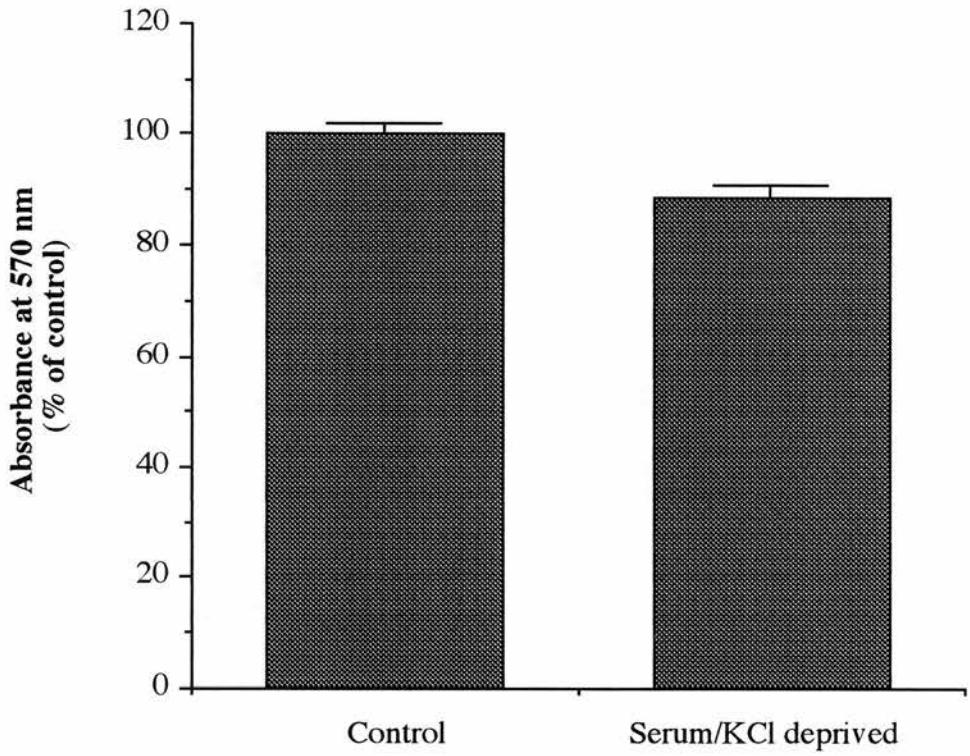


Figure 3.26 Effect of serum and potassium ion deprivation on developing cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 5 days. On day 5, growth medium was replaced with serum-free medium (25 mM KCl) as defined in Materials and Methods. On day 6, medium was replaced with serum-free medium (5 mM KCl) as defined in Materials and Methods. On day 7, cell viability was analysed using MTT staining. The absorbance at 570 nm was expressed as a percentage of control (untreated) cells. Data represent mean ± SEM values (n = 16). The absorbance values for control cells were 0.263 ± 0.005. Statistical analysis showed that there was no significant difference between control cells and treated cells.

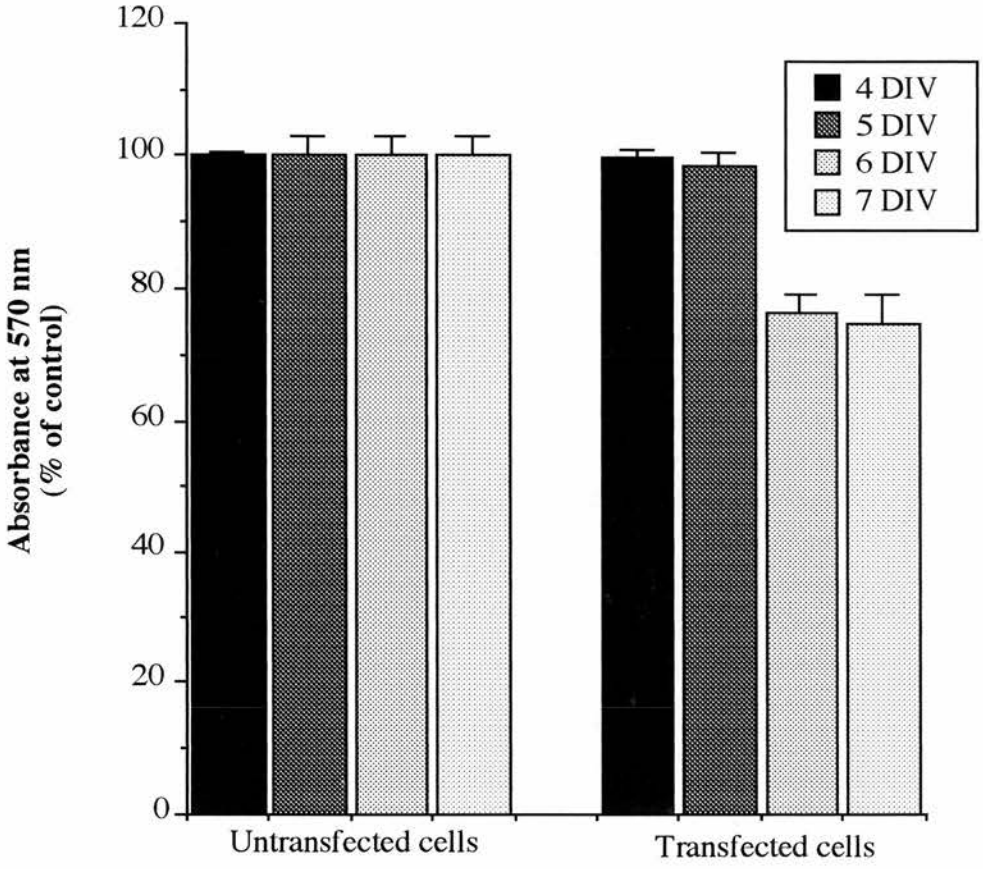


Figure 3.27 Effect of transfection on different ages of cerebellar granule cells in culture

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella, and were maintained in culture for 4, 5, 6 or 7 days, as indicated. Cells were transfected with -351fosluc plasmid in serum-free defined medium and then assayed for cell viability using MTT staining as described in Materials and Methods. The absorbance at 570 nm was expressed as a percentage of control (untreated) cells. Data represent mean ± SEM values (n = 8).

3.3 Discussion

3.3.1 Excitotoxicity and intracellular [Ca^{2+}] levels causing differential mRNA temporal profiles are mediated by differing routes of Ca^{2+} entry

Excessive Ca^{2+} influx via hyperactivated NMDA receptor ion channels is strongly implicated as the initial event in apoptotic or necrotic neuronal cell death following activation of a range of calcium-dependent enzymes that, subsequently, mobilises a variety of intracellular cascades, leading ultimately to the overproduction/activity of free radicals and degradative enzymes (Kure *et al.*, 1991; Nicotera *et al.*, 1992; Bonfoco *et al.*, 1995; Nicotera *et al.*, 1996). In keeping with this role for Ca^{2+}, it was observed that excitotoxic levels of Glu (>50 μM) caused an immediate and marked increase in $[Ca^{2+}]_i$ levels relative to those mediated by non-toxic levels (<10 μM) of Glu (Figure 3.4). The non-toxic nature of vehicle and antagonists was ascertained (data not shown). It is well established that transient exposure (5-30 min) to high concentrations of Glu is sufficient to induce neuronal death in mature cerebellar granule cells (Dessi *et al.*, 1994; Ankarcrona *et al.*, 1995; Malcolm *et al.*, 1996a, b). Continuous exposure of cells to toxic levels of Glu (250 μM) for up to 30 min has been shown to stimulate a marked increase in $[Ca^{2+}]_i$ compared with basal levels and also compared with $[Ca^{2+}]_i$ levels induced by low, non-toxic concentrations (10 μM) of Glu (Griffiths *et al.*, 1998).

However, it would appear from the present findings that an elevation in $[Ca^{2+}]_i$ levels, per se, may not be linked directly to toxicity, because exposure to 55 mM K^+ ions for 24h was not toxic to granule cells (Figure 3.15), even though this high concentration of K^+ ions stimulated increases in $[Ca^{2+}]_i$ levels similar to those seen in the presence of excitotoxic Glu concentrations (c.f. Figures 3.4, 3.16). Further support for this suggestion comes from the observation that the dihydropyridine L-type calcium channel blocker, nifedipine, inhibited increases in $[Ca^{2+}]_i$ induced by 250 μM Glu to levels lower than those observed with 55 mM K^+ ions alone (Figure 3.5), yet failed to protect against Glu-induced excitotoxicity (Griffiths *et al.*, 1998). Although the absolute level of $[Ca^{2+}]_i$ is an important parameter for the onset of toxicity, the present findings suggest that the route of Ca^{2+} entry is one of the major determining factors. Thus, an approximately 6-fold increase (over basal levels) in $[Ca^{2+}]_i$ mediated solely by Ca^{2+} influx via

voltage-gated L-type calcium channels does not appear to be associated with a toxic event (Figure 3.16), whereas a similar increase in $[Ca^{2+}]_i$ levels arising from calcium entry following NMDA receptor activation is converted into an excitotoxic event (Figure 3.4). It should be emphasised that, in this latter situation, at least 30% of the increase in $[Ca^{2+}]_i$ is due to influx of Ca^{2+} through L-type channels (i.e. the nifedipine-sensitive component) and not directly via NMDA receptor-associated Ca^{2+} channels (Griffiths *et al.*, 1998), thus supporting an earlier conclusion that the quantity of Ca^{2+} influx is unlikely to be the sole determinant of an excitotoxic event. NMDA receptor involvement in Glu-mediated Ca^{2+} influx has been demonstrated in a number of cell preparations (Murphy *et al.*, 1987; Schousboe *et al.*, 1992).

The inhibitory effects of nifedipine on Glu-induced increases in $[Ca^{2+}]_i$ can be explained on the basis of the findings by Courtney *et al.* (1990), who showed that, in cultured cerebellar granule cells, there exist three modes of elevation in intracellular calcium levels following activation of Glu receptors. The first is a direct entry of calcium via NMDA receptors that is characterised by the retention of a calcium signal in the presence of nifedipine (after voltage-inactivation of nifedipine-insensitive calcium channels), a finding that is supported by the results shown in Figure 3.5. These findings (Figure 3.5) also serve to support the explanation for a second mode of calcium elevation that is indirect and that occurs by calcium influx through voltage-sensitive calcium channels as a consequence of kainate receptor-mediated depolarisation of the plasma membrane (Courtney *et al.*, 1990). Such calcium entry is characterised by high sensitivity to nifedipine, and, as such, this component of influx would be inhibited when nifedipine is present (c.f. Figure 3.5). Finally, Courtney *et al.* (1990) observed a small, transient elevation of calcium in the presence of (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-ACPD), thus implicating a role for metabotropic Glu receptors. However, the method utilised in the present study to measure $[Ca^{2+}]_i$ places a time resolution on the detection, such that this small component of influx would not be detected. The use of nifedipine to inhibit the non-inactivating L-type calcium channels, thus, allows observation of the effects of calcium entry through the NMDA receptor-associated calcium channel in the absence of secondary influx through voltage-gated calcium channels.

The mode of calcium entry also appears to be an important parameter in the regulation of neuronal gene expression, including that of the proto-oncogene, c-*fos*. Not only are a number of intracellular calcium-dependent enzyme systems coupled to the induction of c-*fos* mRNA (Tsuda, 1996; Bito *et al.*, 1997), but the route of calcium entry appears to dictate the selective activation of different sets of genes (Bading *et al.*, 1993; Gallin and Greenberg, 1995; Ghosh and Greenberg, 1995; Ginty, 1997). For example, calcium entry via voltage-gated calcium channels and subsequent activation of various Ca^{2+}/cAMP-response element (CRE) binding protein (CREB) kinases are reported to be more effective in activating Ca^{2+}/CRE-mediated c-*fos* transcription in hippocampal neurons than calcium that enters via activated NMDA receptor ion channels; whereas calcium entry through either voltage-gated calcium channels or NMDA receptor ion channels can trigger serum-response element (SRE)-mediated transcription of c-*fos* (Bading *et al.*, 1993). It was reasonable to consider, therefore, that the altered temporal profile in c-*fos* mRNA observed under excitotoxic conditions (Figure 3.7; Gorman *et al.*, 1995; Griffiths *et al.*, 1997) could be explained directly by excessive Ca^{2+} influx via the NMDA receptor. In this hypothesis, such excessive Ca^{2+} influx would result initially in an (over)activation of c-*fos* mRNA transcription. Following translation, the Fos protein functioning as part of the heterodimeric activator protein-1 (AP-1) transcription factor complex (Morgan and Curran, 1995; Herdegen, 1996) would regulate the expression of a late-response gene that possibly encodes a protein, which might exert an adverse effect on neuronal function (for example, see Tatter *et al.*, 1995).

The results presented in this chapter show clearly, however, that, following exposure of cells to toxic levels of Glu, the influx of calcium via the NMDA receptor-associated Ca^{2+} channel is an unlikely explanation for the observed changes in the temporal profile of steady state c-*fos* mRNA levels. Rather, such changes appear to be driven solely by calcium influx via L-type voltage-gated calcium channels. In support of this explanation, exposure of cerebellar granule neurons to 55 mM K^+ ions, although a non-toxic dose (Figure 3.15) with the ability to mediate the flux of similar amounts of calcium compared with toxic levels of Glu (c.f. Figure 3.4, Figure 3.16), was seen to cause a change in the temporal profile of steady-state c-*fos* mRNA levels to a delayed, elevated status (Figure 3.19). In addition, nifedipine, which selectively blocks L-type

voltage-gated calcium channels, prevented the delayed, elevated appearance of the K^+- induced c-*fos* mRNA, although APV and TCP did not (Figure 3.19); whereas nifedipine, by blocking only partially the Glu-induced increases in $[Ca^{2+}]_i$ (Figure 3.5), prevented the delayed, elevated profile of c-*fos* mRNA associated with a toxic dose of Glu (Figure 3.7). This ability of nifedipine to prevent the appearance of a delayed, elevated c-*fos* mRNA profile in granule cells exposed to toxic levels (250 μM) of Glu (Figure 3.7), therefore, provides strong support for the conclusion that the route of calcium entry, specifically, influx via dihydropyridine-sensitive, non-inactivating L-type channels, is responsible for the altered temporal profile in steady-state c-*fos* mRNA measurements.

The coadministration of 100 μM Glu with 10 μM CNQX in Figure 3.1D might be expected to prevent relief of the Mg^{2+} block on the NMDA receptor, and thus cause an inhibition in Glu-mediated toxicity; however, the cells were cultured under slightly depolarising conditions, thus allowing chronic relief of the NMDA receptor Mg^{2+} block.

3.3.2 Differential expression of Fos and Jun family proteins as part of the AP-1 transcription factor complex in response to Glu or K^+ ion stimulation

The electrophoretic mobility shift assay (EMSA) is a useful tool in molecular biology which allows observations concerning DNA-binding proteins. Introduction of specific antibodies into the binding mixture gives added information allowing the composition of the DNA-binding protein to be evaluated. The reported experiments were performed in an attempt to gain more information about the composition of the AP-1 transcription factor complex under a number of different circumstances. Firstly, cerebellar granule cells were exposed to the stimulus either in the serum-containing growth medium or a serum-free medium; the stimulus being either 10 μM Glu (a non-toxic dose) or 250 μM Glu (a toxic dose). The nuclear extracts from these cells were then analysed to ascertain the Fos and Jun family members present under the various conditions. The fact that when a supershift occurred, not all of the binding activity could be attributed to the particular protein probed can be explained at least in part by the fact that c-Jun, JunB, c-Fos and Fra-1 all form heterodimers with members of the activating transcription factor (ATF) family of

transcription factors (Hai and Curran, 1991), and therefore the information gained by the studies reported herein is not exhaustive. As seen in Figure 3.8A, a large amount of the AP-1 binding can be attributable to JunD. Since JunD is thought to be produced constitutively in these cells, this presumably means that only a small increase in the expression of the cIEG-encoded proteins occurs as a result of stimulation with excitatory amino acid. However, the regulation of this event is complex (positive auto-regulation and negative feedback of cIEG transcription, cIEG-encoded protein transcriptional activity and DNA-binding ability by cIEG-encoded proteins has been reported (Angel *et al.*, 1988a; Sassone-Corsi *et al.*, 1988a,b; Chiu *et al.*, 1989; Schütte *et al.*, 1989; Boyle *et al.*, 1991; Ham *et al.*, 1995)), and the cascade nature of the pathways involved means that even this small effect can cause significant changes in the induction and expression of late genes and, by application, the viability of the cell.

The peptide sequences used to prepare the antibodies used in these studies are described in Appendix 1. The DNA-binding domain of all Fos and Jun family members are found in the C-terminal part of the protein (Nakabeppu and Nathans, 1989; Figure 1.4). In most cases, the antibody used recognised an epitope in the central region or the N-terminal region of the protein; however, the antibodies to JunD and Fra-2 used recognised part of the C-terminal. Since this area of the protein also contains the DNA-binding domain, it is possible that the removal of the DNA-binding band in the presence of anti-JunD in Figure 3.8A is due to the fact that the epitope recognised by this antibody comprised part of the DNA-binding domain.

Taking all the results of this section of the study together, several interesting findings are apparent. Firstly, c-Fos protein was found to be part of the AP-1 complex in the presence of serum under both circumstances (toxic and non-toxic conditions), but largely absent from the complex in the absence of serum. This could lead to speculation that c-Fos is indeed serum-inducible, as other workers have reported (Treisman, 1985; Stumpo and Blackshear, 1986). In addition, c-Fos is found to be a component of the AP-1 transcription factor complex after cellular stimulation with 55 mM KCl (Figure 3.20), which is known to be non-toxic (Figure 3.15). Thus a role for Ca^{2+} ion influx in the formation of AP-1 dimers, and thereby the transcriptional regulation of late target genes, has been suggested (Finkbeiner and Greenberg, 1998).

Coadministration of TCP with 250 μM Glu prevented the appearance of the supershift in the presence of anti-c-Fos antibodies, indicating that the NMDA receptor-mediated Ca^{2+} influx is a contributing factor in the formation of AP-1 transcription factor complexes containing c-Fos protein as a component of the heterodimer.

JunD has been reported to have constitutive expression and incorporation into the AP-1 complex (Hope *et al.*, 1994; Hou *et al.*, 1997). The results shown here support that theory (Figures 3.8A-3.11). However, the dimerisation of JunD with different Fos or Jun family members will cause differential effects; indeed, a role for FosB/JunD dimers in the response to electroconvulsive seizures had been suggested (Hiroi *et al.*, 1998). Fra-1 and Fra-2, suggested to have a late onset role (Kovács, 1998), were part of the AP-1 complex only under non-toxic conditions. There have been some trophic roles suggested for Fos family member protein expression, particularly during development (Ferhat *et al.*, 1993; Simpson and Morris, 1994); however, more work must be done to elucidate the role of the Fos-related antigens. In contrast, JunB was only detected in the complex under toxic conditions, in the absence of serum. This supports the theory that serum has a role in the induction and expression of immediate-early genes. JunB is thought to have an inhibitory function in the transactivational activity of IEGs (Chiu *et al.*, 1989), which may be connected with a trophic role for certain dimers containing this protein.

A supershift was only obtained with anti-FosB under toxic conditions where 7 DIV cerebellar granule cells were exposed to 250 μM Glu. When cerebellar granule cells were exposed to 55 mM KCl, no supershift was observed in the presence of anti-FosB. However, a supershift was observed under these conditions with anti-c-Fos in the binding mixture. This finding suggests that FosB may only be a component of the AP-1 transcription factor complex in response to a toxic event, rather than solely due to a high intracellular Ca^{2+} level. Conversely, the production of c-Fos appears not to be dependent on toxicity, but rather on secondary messages (Ca^{2+}, arachidonic acid) which may or may not be activated by a toxic event. In support of this theory, coadministration of TCP, which prevents Glu-mediated toxicity by blocking the NMDA receptor ion channel, was able to prevent the FosB supershift appearing in response to stimulation

by 250 μM Glu. However, when protein expression studies were carried out using Western blotting, FosB protein was present after exposure of 7 DIV cells to high, but non-toxic, KCl levels. One explanation for this could be that the FosB protein detected in Western blotting is present in the cytoplasm, but is not part of the AP-1 transcription factor complex in response to KCl. This result also asserts the complexity of the regulatory processes involved in cIEG induction.

When the induction of c-*fos* and *fos*B mRNA in response to 55 mM KCl was analysed, it was found that both genes exhibited similar temporal patterns of induction over a period of 6h (Figure 3.18). However, the importance of this result is that both c-*fos* and *fos*B mRNA are induced in response to 55 mM KCl, while only c-Fos protein (and not FosB) appears as part of the transcription factor complex as detected by EMSA (Figure 3.20).

3.3.3 Effect of serum on expression of cIEGs

The ability of serum stimulation to cause induction and expression of c-*fos* and its product, c-Fos, is well documented (Müller *et al.*, 1984; Treisman, 1985; Treisman, 1986; Mohun *et al.*, 1987; Gilman, 1988; Chen *et al.*, 1996). However, in the case of this study, the cells were grown in the presence of serum, and Glu was subsequently added as a stimulus after several days in culture. This methodology contrasts with a number of studies, where the cells are deprived of serum and serum is added as a stimulus.

In the present study, the presence of c-Fos as a component of the AP-1 transcription factor, as indicated by a super shift of the DNA/protein complex in the presence of anti-c-Fos, was apparent at all timepoints, including zero time, when the cells were prepared and maintained in culture in the presence of serum for 7 days before being stimulated with 250 μM Glu (Figure 3.11) However, when the cells were deprived of serum for at least 24h before the same experiment, no supershift occurred at zero time but there was strong evidence for c-Fos being expressed between 15 min and 4 h after exposure to 250 μM Glu (Figure 3.9). Thus any supershift in the latter case was due to the effects of Glu, and not of serum, and thus the results

presented in Figures 3.9 and 3.11 show that both Glu and serum play a part in the expression of this protein and its incorporation into the AP-1 transcription factor.

3.3.4 Transfection studies

The transfection studies reported herein were carried out in an attempt to obtain information on the functionality of the c-Fos protein which was detected using other techniques. Transient transfection of primary cultured neurons is accepted as being a difficult technique, and the results obtained in the time available were unsatisfactory. It was found that, whilst 10 μM Glu exposure caused significant expression of the reporter gene, exposure of the cells to 250 μM Glu failed to elicit a response above basal. Exposure of the cells to forskolin, however, elicited a positive response.

One hypothesis for these results would be that, since 250 μM Glu causes excitotoxicity in these cells very quickly following exposure (Malcolm *et al.*, 1996a, b), significant cell death had occurred before expression of protein had been achieved to any great extent. This would explain the fact that non-toxic levels of Glu (10 μM) were able to elicit a response from the cells, since we have seen in the EMSA studies that c-Fos protein is produced in response to 10 μM Glu in these cells.

3.3.5 Effect of phospholipase A_2 pathway enzymes on Glu-induced toxicity, mRNA induction and protein expression

The significance of studying the pathway that involves production of arachidonic acid by phospholipase A_2 has been outlined in the introduction to this chapter. Briefly, arachidonic acid is subsequently metabolised to prostaglandins, thromboxanes, epoxides and leukotrienes by a battery of enzymes, and prostaglandins are able to stimulate induction of cIEG expression by activation of PKA.

When cells were exposed (4h) to 250 μM Glu in conjunction with a number of PLA_2 pathway enzyme inhibitors, only the presence of aspirin decreased the extent of the Glu-mediated toxicity. However, after exposure for 24h, both aspirin and indomethacin significantly decreased

the toxic effect (Figure 3.22). The site of inhibitory action for both indomethacin and aspirin is prevention of the conversion of arachidonic acid to prostaglandins and thromboxanes, catalysed by cyclooxygenase. These inhibitory compounds appear to have different potencies in different systems and cell preparations (Chang *et al.*, 1984). For example, in rat cerebellar slices, 100 μM indomethacin is able to prevent NMDA-induced toxicity, whereas aspirin has no effect up to 1 mM (Lehmann, 1987). Aspirin is able to prevent production of prostaglandins, eicosanoids responsible for enhancing the inflammatory response, by acetylation of cyclooxygenase. However, prostaglandins, in addition to their involvement in the inflammatory response, cause the production of cAMP from ATP via adenylate cyclase and thus, via PKA, cause regulation of the immediate-early genes c-*fos* and c-*jun*. Since the prevention of the production of prostaglandins by aspirin and indomethacin blocks the toxicity caused by Glu, it is reasonable to speculate that induction of Fos family members may be involved in the toxic response of cells to Glu.

In accordance with these results, the $Q^{240/30}$ index value of c-*fos* mRNA induction was changed from >1 to <1 when high levels of Glu were used to stimulate cells in conjunction with aspirin. In addition, the use of quinacrine as an inhibitor of the action of PLA_2 itself on phospholipids caused a decrease of the $Q^{240/30}$ index value to 0.93, but due to the fluorescent nature of this compound in solution it proved difficult to confirm this result using the MTT and intracellular calcium assays. Quinacrine has been reported to protect against tumour necrosis factor-induced toxicity in transformed cells (Suffys *et al.*, 1987). Complete abolition of NMDA-mediated c-*fos* mRNA induction was effected by the presence of 1 μM indomethacin in cultured dentate gyrus cells, whilst a 55% reduction was achieved by the presence of 100 μM aspirin (Lerea and McNamara, 1993). These workers also reported a decrease in the induction of c-*fos* mRNA by coadministration of NMDA with 50 μM quinacrine or 50 μM aristolochic acid, but no such effect was demonstrated in the presence of 1 μM NDGA. These inhibitors were effective only on NMDA-mediated mRNA induction, and had no effect on c-*fos* mRNA induced by stimulation with KA.

There was no significant difference between the intracellular Ca^{2+} ion concentration after exposure to 250 μM Glu alone or in conjunction with any of the inhibitors. This is as expected, since the site of action of each inhibitor appears after the influx of Ca^{2+} ions which occurs in response to receptor binding by Glu. NMDA-mediated increases in $[Ca^{2+}]_i$ in cultured dentate gyrus cells were found to be unaffected by any of these inhibitors (Lerea and McNamara, 1993).

The protein expression analysis by Western blotting shown here is incomplete; however, the positive effect of both aristolochic acid and indomethacin on the expression of c-Fos after stimulation with 10 μM Glu is interesting. This may be explained by the activation of some kind of feedback mechanism, where in the case of the pathway involving PLA_2 and arachidonic acid being blocked, c-Fos expression is stimulated by another route, perhaps involving PKA. Further work could be done to analyse the effects of aspirin and quinacrine on the expression of c-Fos, and indeed other Fos and Jun family member proteins in response to excitation by Glu.

Chapter 4

Evaluation of neurochemical endpoints in immature (2 DIV) mouse cerebellar granule cells

4.1 Introduction

In the previous chapter, the effects of Glu and K^+ ions on 7 DIV mouse cerebellar granule cells in terms of cytotoxicity, intracellular calcium ion concentration and cellular immediate-early gene induction and protein expression were reported and discussed. Differentiation of these cells can be observed after 3-4 days in culture, in culture medium containing 25 mM KCl. This concentration of KCl causes depolarisation of the cell membrane and thus allows Ca^{2+} ion influx, which is necessary for survival of these cells in culture in serum-containing medium (Gallo *et al.*, 1987; Balázs *et al.*, 1988b). The differentiation is complete by 8 DIV, and cells begin to spontaneously die after 13 DIV (Lin *et al.*, 1997). Thus, at 2 DIV, these cells can be used to study the mechanisms which take place in immature neurons. At an early and immature stage in development of cultured cerebellar granule cells, neurotransmitters, e.g. Glu, are thought to have a role in growth regulation, by affecting proliferation, cell death and formation of synapses (Sugioka *et al.*, 1998). Indeed, chronic exposure of primary cultures of cerebellar granule cells to NMDA promotes survival even in the absence of high, depolarising levels of K^+ ions (Balázs *et al.*, 1988a, c; 1989). This trophic role for Glu and its analogues indicates the need for exquisite levels of control of flux of ions and other active molecules in and out of these cells.

Cerebellar granule cells at 2 DIV would be expected to be less susceptible to the toxic effects of EAA-mediated receptor stimulation than those at 7 DIV, due to the evidence of the trophic actions of NMDA cited above. It has been reported that cerebellar granule cells are not susceptible to Glu-mediated cytotoxicity at either 3 or 5 DIV, but that by 8 DIV, Glu and its analogues are able to cause significant cell death (Frandsen and Schousboe, 1990). However, experiments in this laboratory have found that Glu causes cytotoxicity at 3 DIV onwards. During the development of cerebellar granule cells from 4 DIV to 20 DIV (i.e. the period in which the role of Glu is thought by some to change from trophic to toxic), the amount of NMDA-mediated Ca^{2+} ion influx does not change significantly (E. Tremblay, personal communication). Thus a large influx of Ca^{2+} ions is not alone sufficient to account for NMDA-mediated toxicity. Developmental changes in other factors, e.g. immediate-early gene induction, may be involved in the formation of a toxic response to Glu and its analogues. Additionally, there is some speculation that the

receptor complexes are either not fully formed or not completely functional (Choi *et al.*, 1987), or indeed that there is not a sufficient number of receptors present to allow a toxic effect (Garthwaite and Garthwaite, 1989). Each of these scenarios would obviously have an effect on the efficacy of binding and thus Glu-mediated toxicity. Indeed, in cortical neurons, expression of Ca^{2+}-permeable AMPA receptors only occurs late on in culture, thus preventing influx of Ca^{2+} ions via this route in immature cortical cultures (Jensen *et al.*, 1998b). In cultured hippocampal neurons, different Ca^{2+} channels which release glutamate are employed at different stages of development (Verderio *et al.*, 1995). Wahl and colleagues have reported that in cultured cortical neurons, neurotransmitter receptor development occurs in a similar fashion to their development *in vivo*, thus allowing the possibility of immature receptors in younger cells in *in vitro* preparations (Wahl *et al.*, 1993). The expression of voltage-dependent Ca^{2+} channels also changes throughout development *in vivo* and *in vitro* (Rossi *et al.*, 1994; Verderio *et al.*, 1995; Parri and Lansman, 1996; Harrold *et al.*, 1997), and in fact, L-type Ca^{2+} channels are especially important in Ca^{2+} ion regulation during development of granule cells in culture (Evans and Pocock, 1999). There is, however, in the literature, evidence of immature cultured cells experiencing EAA-mediated cytotoxicity. Cortical neurons are susceptible to KA-induced toxicity, the extent of which increases with age, but is nevertheless significant at 2 DIV (Jensen *et al.*, 1999).

During development of these cerebellar granule cells in culture, in addition to receptor formation being completed over a period of time, the presence of Glu transporters changes (Furuta *et al.*, 1997). These sodium-dependent transporters are essential for the removal of excess neurotransmitter from the synaptic cleft, in order to prevent neurotoxicity. However, it appears that there is a definite pattern to the function of these transporters in developing neurons. EAAC1, a neuronally-located transporter, is prevalent in newborn brain and then its expression decreases as the cells approach maturity. The main cerebellar neuronal Glu transporter, EAAT4, is expressed more in mature cells than in immature cells. Thus there is a distinct coordination which occurs to regulate the concentration of extracellular Glu during development (Furuta *et al.*, 1997).

This chapter reports on:

i) evaluation of the cytotoxic potential of both Glu and KCl in 2 DIV cerebellar granule neurons, the pharmacology of this effect being probed using selective receptor antagonists and channel blockers;

ii) measurement of the levels and elucidation of the routes of Ca^{2+} ion influx induced by these two compounds;

iii) induction of the immediate-early genes c-*fos*, c-*jun* and *fos*B and measurement of steady-state levels of mRNA, demonstration of gene product protein expression and their incorporation into the AP-1 transcription factor complex.

In order to allow comparison of the results obtained in this chapter with those in Chapter 3, the same concentrations of Glu and KCl were used. In this chapter, 'low' and 'high' Glu represent 10 μM and 250 μM respectively, and 'low' and 'high' KCl represent 5 mM and 55 mM, respectively. The results presented in this chapter are split into 2 sections. Section A reports on the study of the effects of Glu in 2 DIV cultured cerebellar granule cells in terms of cell viability, intracellular calcium ion concentration and proto-oncogene induction and protein expression as part of the AP-1 transcription factor complex, and in Section B the study of the effects of membrane depolarisation by KCl in 2 DIV cerebellar granule cells is presented.

4.2 Results

Section A: Glu-mediated effects on 2 DIV cultured cerebellar granule cells

4.2.1 Cerebellar granule cells are not susceptible to Glu-mediated toxicity after 2 days in culture

Cerebellar granule cells at 2 DIV were exposed for 4h and 24h with increasing concentrations of Glu and then assayed for cell viability using the MTT assay. No significant toxicity was observed at any of the concentrations tested (Figure 4.1), since in all cases the cell viability remained above 85% (4h) or 80% (24h exposure).

4.2.2 Measurement of changes in intracellular calcium levels in response to 'low' and 'high' levels of Glu

Assaying 2 DIV cerebellar granule cells for intracellular-free Ca^{2+} ion concentration immediately after stimulation with increasing concentrations of Glu revealed that the concentration of Ca^{2+} ions increased with the concentration of Glu until it reached a peak at ~500 nM (5-fold of basal) in response to 100 μM Glu (Figure 4.2). The EC_{50} was estimated at ~20 μM Glu. When cells were stimulated with 10 μM and 250 μM Glu alone or coadministered with 10 μM nifedipine, it was found that the intracellular Ca^{2+} ion concentration induced by either concentration of Glu was reduced in the presence of nifedipine (Figure 4.3). Although neither 10 μM nor 250 μM Glu were toxic to 2 DIV cells, these concentrations were used for study in order to be able to carry out comparisons with the results obtained in Chapter 3.

4.2.3 The immediate-early genes c-*fos,* c-*jun and fos*B *show differential induction profiles when stimulated with 'high' and 'low' levels of Glu*

Cerebellar granule cells at 2 DIV were exposed continuously for 6h to either 10 μM or 250 μM Glu, and the levels of c-*fos* , c-*jun* and *fos*B mRNA induced were measured at various time-points within this period (Figure 4.4 A-C, respectively).

In the case of c-*fos* mRNA, 10 μM Glu elicited a transient response, peaking at 8.5-fold

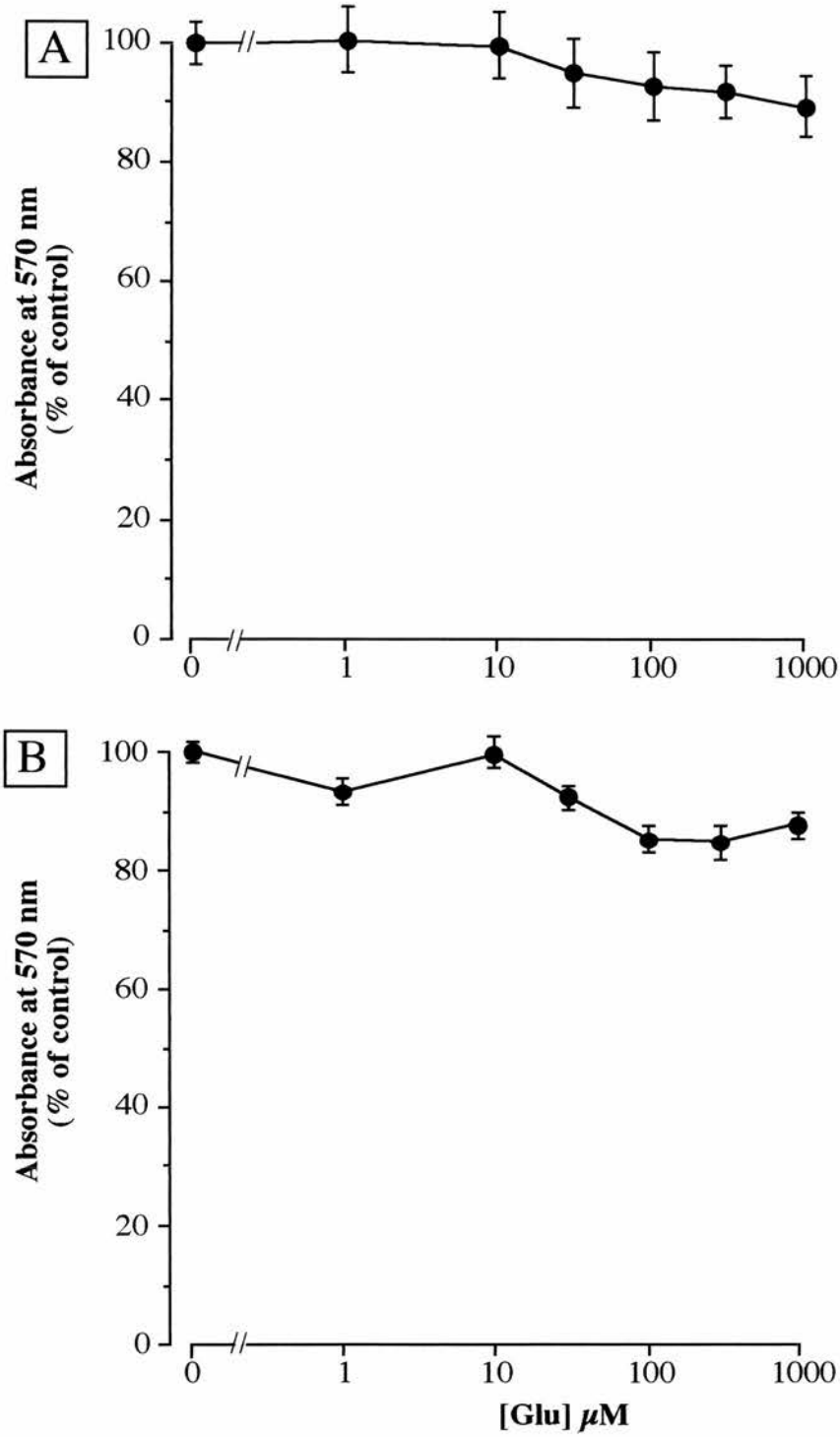


Figure 4.1 Survival of cerebellar granule cells in primary culture after exposure to increasing concentrations of Glu.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella, and were maintained in culture for 2 days before use. Cells were exposed for 4h (A) and 24h (B) in culture-conditioned medium to increasing concentrations of Glu before being assayed for cell viability using MTT staining. The absorbance at 570 nm was expressed as a percentage of control (untreated) cells. Data represent mean ± SEM values (n = 8-16). The absorbance values for control cells were 0.238 ± 0.008 (A) and 0.311 ± 0.008 (B).

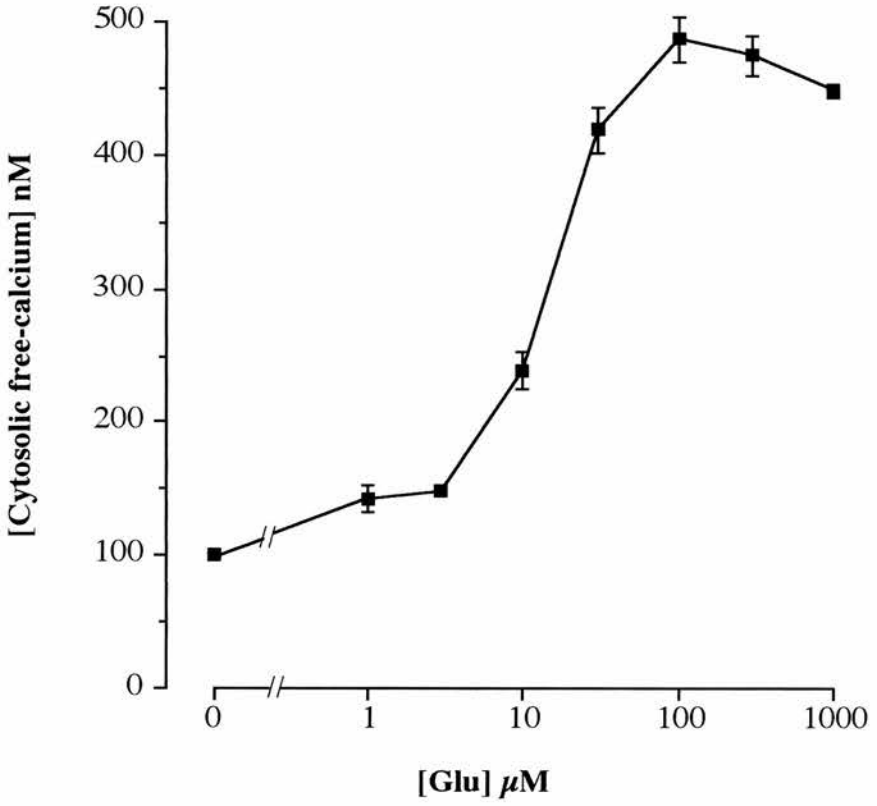


Figure 4.2 Concentration-dependent effect of Glu on stimulated increases in intracellular-free calcium levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella, and were maintained in culture for 2 days before use. Granule cells were washed with balanced salt solution, loaded with Fluo-3/AM and exposed to increasing concentrations of Glu, as indicated. Data are the mean ± SEM values (n = 7-8).

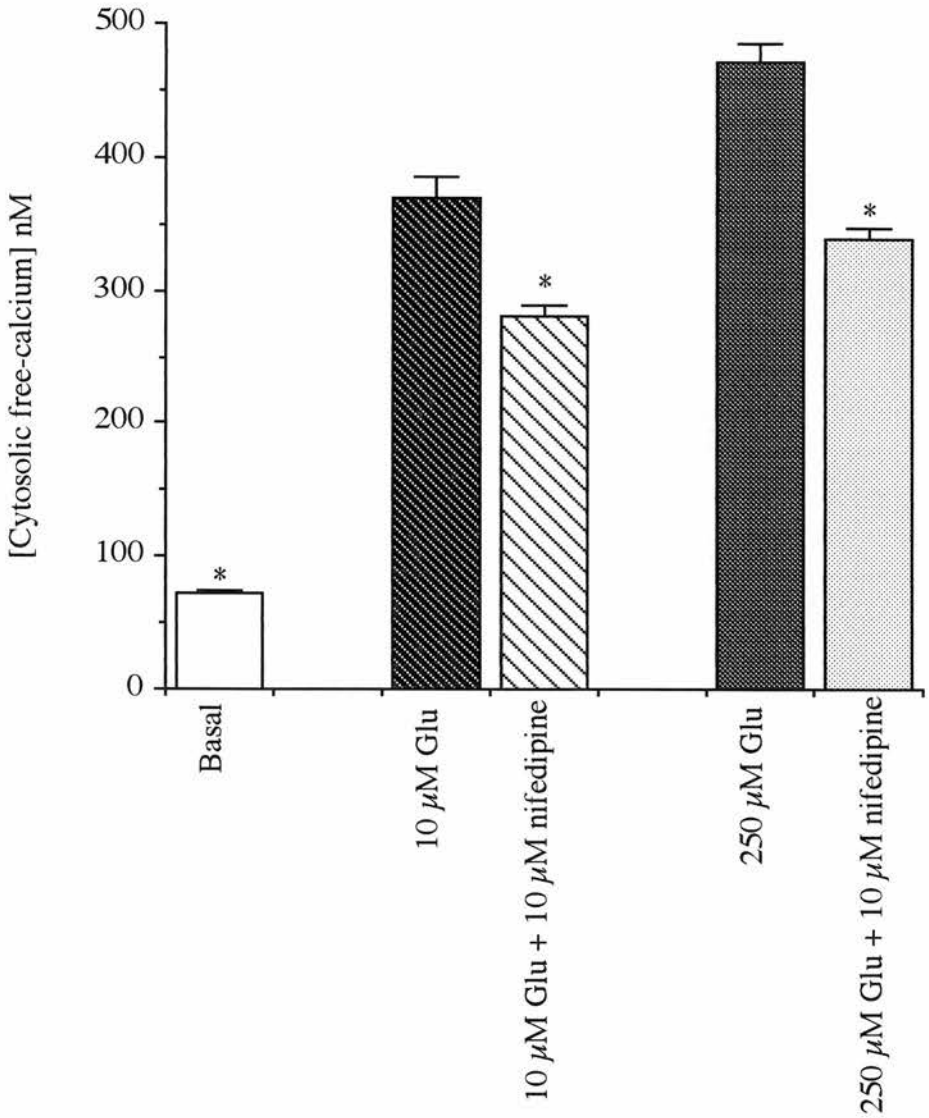


Figure 4.3 Effect of the dihydropyridine L-type calcium channel blocker, nifedipine, on increases in intracellular-free calcium levels stimulated by 'low' and 'high' concentrations of Glu in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 2 days before use. Granule cells were washed with balanced salt solution, loaded with Fluo-3/AM and exposed to either a low (10 μM) or high (250 μM) concentration of Glu alone or coadministered with 10 μM nifedipine. Data are the mean ± SEM values (n = 8-16). Asterisks indicate statistical difference from Glu alone in each case.

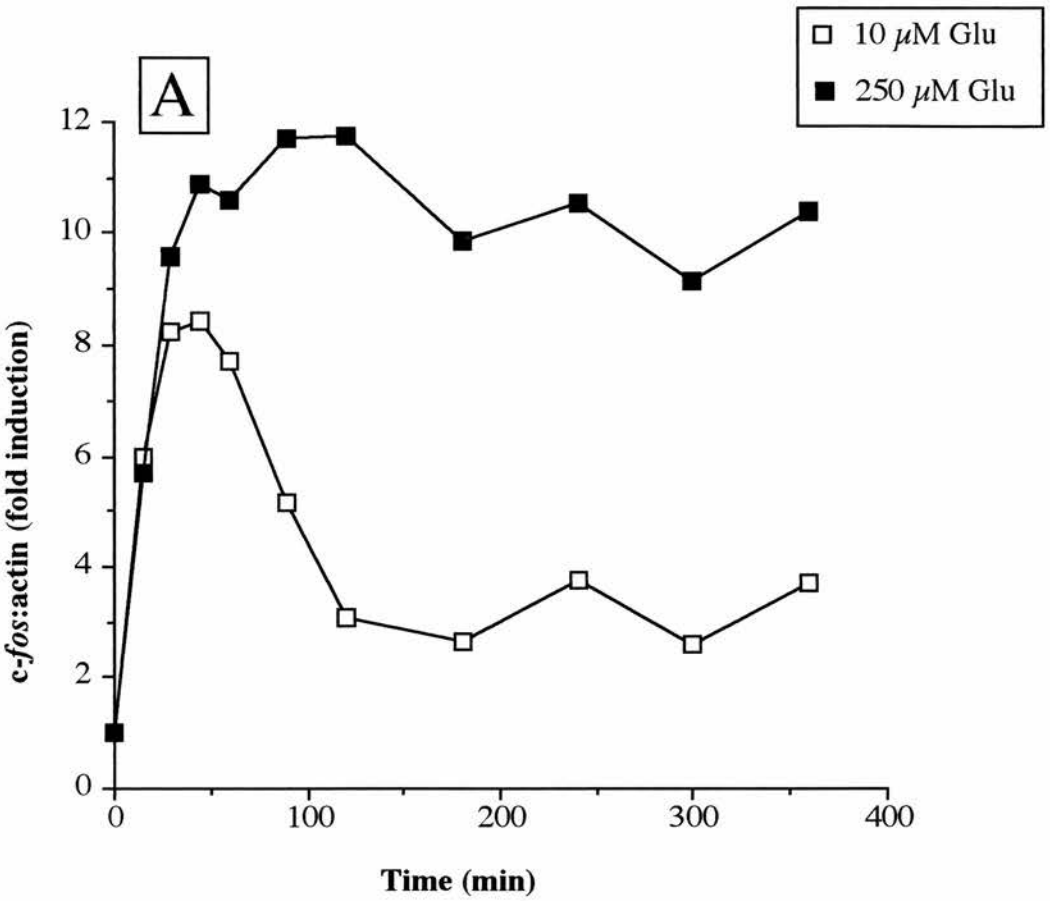
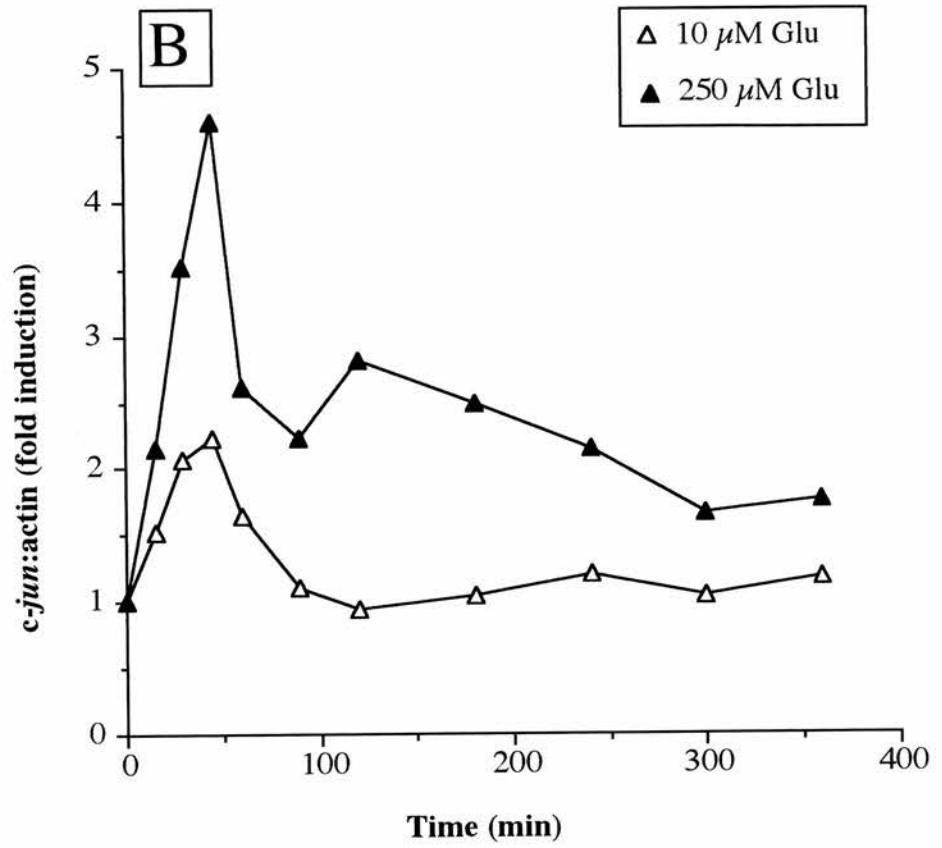
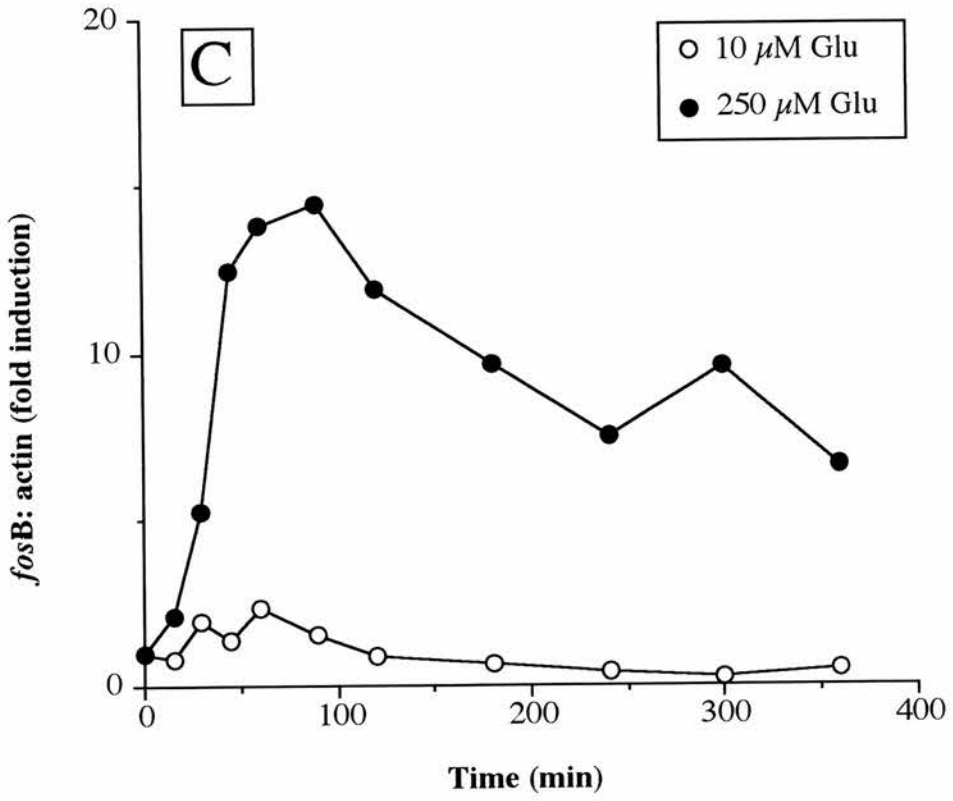


Figure 4.4 Time-course of Glu-induced, steady-state mRNA levels of c-*fos*, c-*jun* and *fos*B in 2 DIV cultured cerebellar granule cells.
Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old post-natal mouse cerebella. Granule cells were maintained in culture for 2 days before exposure to 10 μM or 250 μM Glu for up to 6h. Total RNA was prepared from the cultures, and the amount of c-*fos*, c-*jun*, *fos*B and β-actin mRNA was quantified. The results refer to clEG mRNA expression relative to that of β-actin. The β-actin gene has a constant expression during the time interval used and thus acts as a control for the loading of RNA.


B
10 μM Glu
250 μM Glu
c-jun:actin (fold induction)
0
1
2
3
4
5
0
100
200
300
400
Time (min)


C
10 μM Glu
250 μM Glu
fosB: actin (fold induction)
0
10
20
0
100
200
300
400
Time (min)

over basal 45 min following initial exposure. The level of mRNA induction had reverted to a basal level by 2h. Exposure of the cells to 250 μM Glu, in contrast, induced a greater response (an increase in induction to ~11-fold over basal was achieved by 45 min); this higher level of mRNA being essentially sustained throughout the 6h observation period. Unlike the situation found in 7 DIV cells exposed to toxic, 250 μM Glu (see Figure 3.6), there was no delay in mRNA production following exposure of 2 DIV cells to 250 μM Glu (Figure 4.4 A-C).

Induction of c-*jun* mRNA in response to both 10 μM and 250 μM Glu exhibited a temporal profile similar to that of c-*fos* induced by 10 μM Glu, namely a transient increase peaking at 45 min following exposure and returning to a basal level after 2h (Figure 4.4 B). The maximum fold induction attained with 10 μM Glu was ~2-fold over basal. However, stimulation of cells with 250 μM Glu did elicit a greater induction of c-*jun* mRNA than that observed with 10 μM Glu throughout the observation period, the maximum induction in this case being ~5-fold over basal.

Similarly, *fos*B mRNA induction was transient after stimulation with 10 μM Glu, peaking at ~2-fold over basal 1h following initial exposure and returning to basal levels by 2h (Figure 4.4C). However, following exposure to 250 μM Glu, there was a marked elevation (~15-fold) in the peak *fos*B mRNA induction when compared to that measured when cells were exposed to 10 μM Glu. The induction was somewhat sustained, only approaching basal levels very slowly during the 6h exposure period.

4.2.4 NMDA receptor channel antagonists, but not L-type Ca^{2+} channel blockers, are able to change the temporal profile of Glu-mediated c-*fos and fos*B *mRNA induction*

The pharmacology of c-*fos* and *fos*B mRNA induction by high levels of Glu was studied by stimulating cells with 250 μM Glu alone and co-administered with the selective NMDA receptor antagonists, APV (500 μM) and TCP (1 μM), and with the L-type VGCC blocker, nifedipine (5 μM). For c-*fos* and *fos*B, it was found that both APV and TCP were able to prevent the elevated, sustained induction of mRNA observed in the presence of 250 μM Glu alone (Figure

4.5). However, unlike the situation observed in 7 DIV cells (see Figure 3.7), coadministration of 250 μM Glu with 5 μM nifedipine did not result in a transient early gene response but rather the elevated profile observed with 250 μM Glu alone.

4.2.5 Investigation of the composition of the AP-1 transcription factor complex shows that culture conditions and Glu concentration matter

Cerebellar granule cells at 2 DIV were exposed to 'high' and 'low' concentrations of Glu and nuclear extracts probed for the presence of proteins capable of forming AP-1 transcription factor complexes. In all cases, a band signifying the presence of AP-1 transcription factor binding to the TRE probe was observed.

4.2.5.1 Cells maintained in serum-free medium

Cerebellar granule cells at 2 DIV were stimulated by addition of Glu at a final concentration of 10 μM to the growth medium. The growth medium had been replaced the day before with serum-free defined medium (see Section 2.2.5). Subsequent EMSA studies using nuclear extracts showed that binding of the radioactively-labelled DNA fragment by AP-1 complexes occurred in all cases (Figure 4.6). Addition of antibodies specific to each of the Fos and Jun family members showed that, with the exception of JunD, no Fos or Jun family members were part of the AP-1 transcription factor complex. In contrast, when cells were exposed to 250 μM Glu, c-Fos, Fra-2 and c-Jun in addition to JunD appeared to be involved in AP-1 complex formation (Figure 4.7).

4.2.5.2 Cells maintained in serum-containing medium

In 2 DIV cells, exposed to 10 μM Glu, in serum-containing medium, Fra-1, Fra-2, c-Jun and JunD were found to be components of AP-1 dimers which bound to the TRE *in vitro*, as shown by the super-shift obtained in the presence of antibodies specific to each of these proteins (Figure 4.8). When the cells were exposed to 250 μM Glu in serum-containing medium, Fra-1, Fra-2, c-Jun and JunD were again shown to be components of AP-1 transcription factor

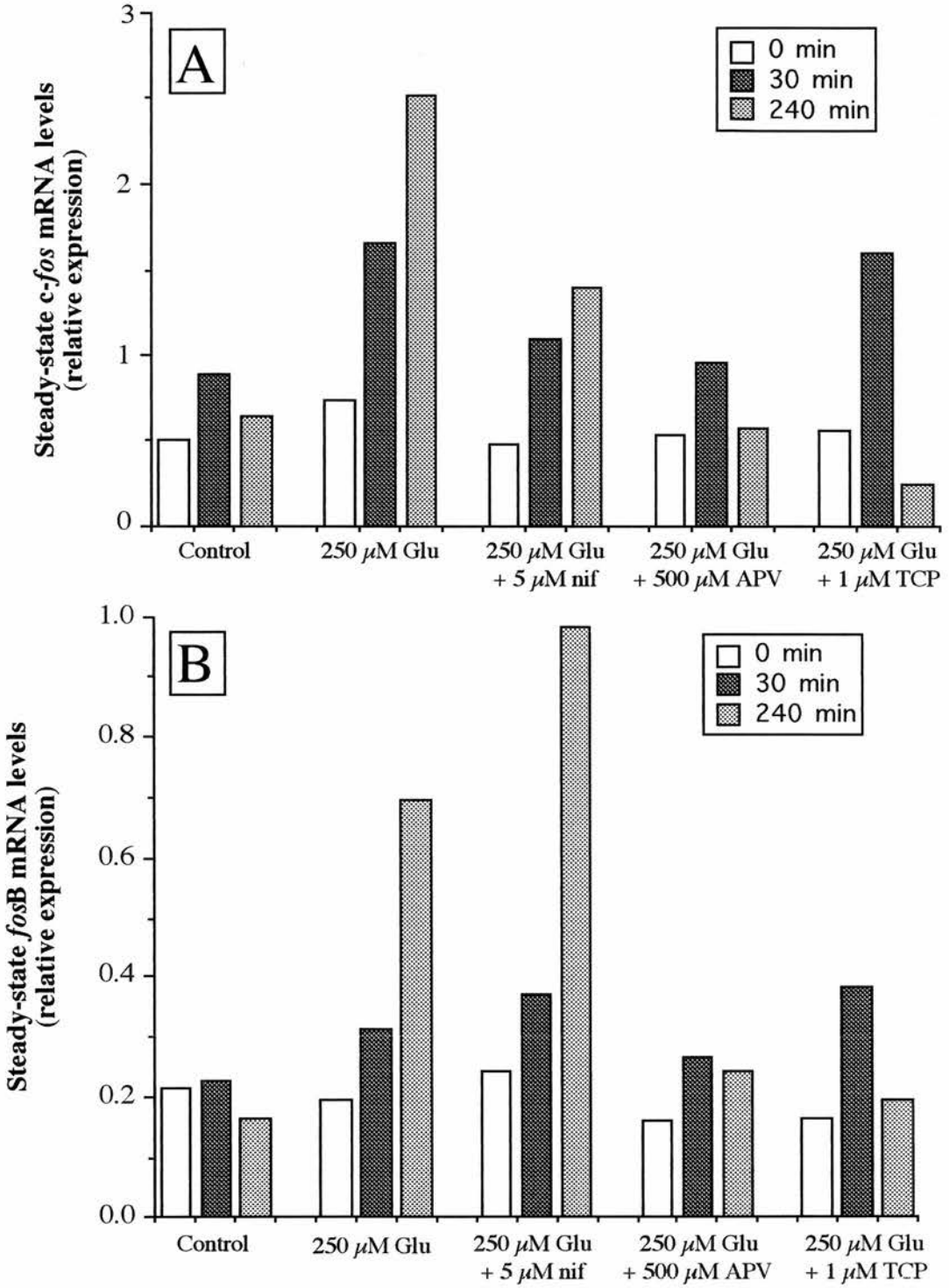


Figure 4.5 Pharmacology and temporal profiles of Glu-stimulated, steady-state c-*fos* (A) and *fos*B (B) mRNA levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old post-natal mouse cerebella. Granule cells were maintained in culture for 2 days before exposure for different times (0, 30 or 240 min) to 10 μM and 250 μM Glu alone or coadministered with various pharmacological antagonists, as indicated. After exposure, total RNA was prepared from the cultures, and the amount of c-*fos*, *fos*B and β-actin mRNA was quantified. The results represent the average of at least duplicate experiments and refer to c-*fos* or *fos*B mRNA expression relative to that of β-actin. The β-actin gene has a constant expression during the time interval used in this study; thus it acts as a control for the loading of RNA.

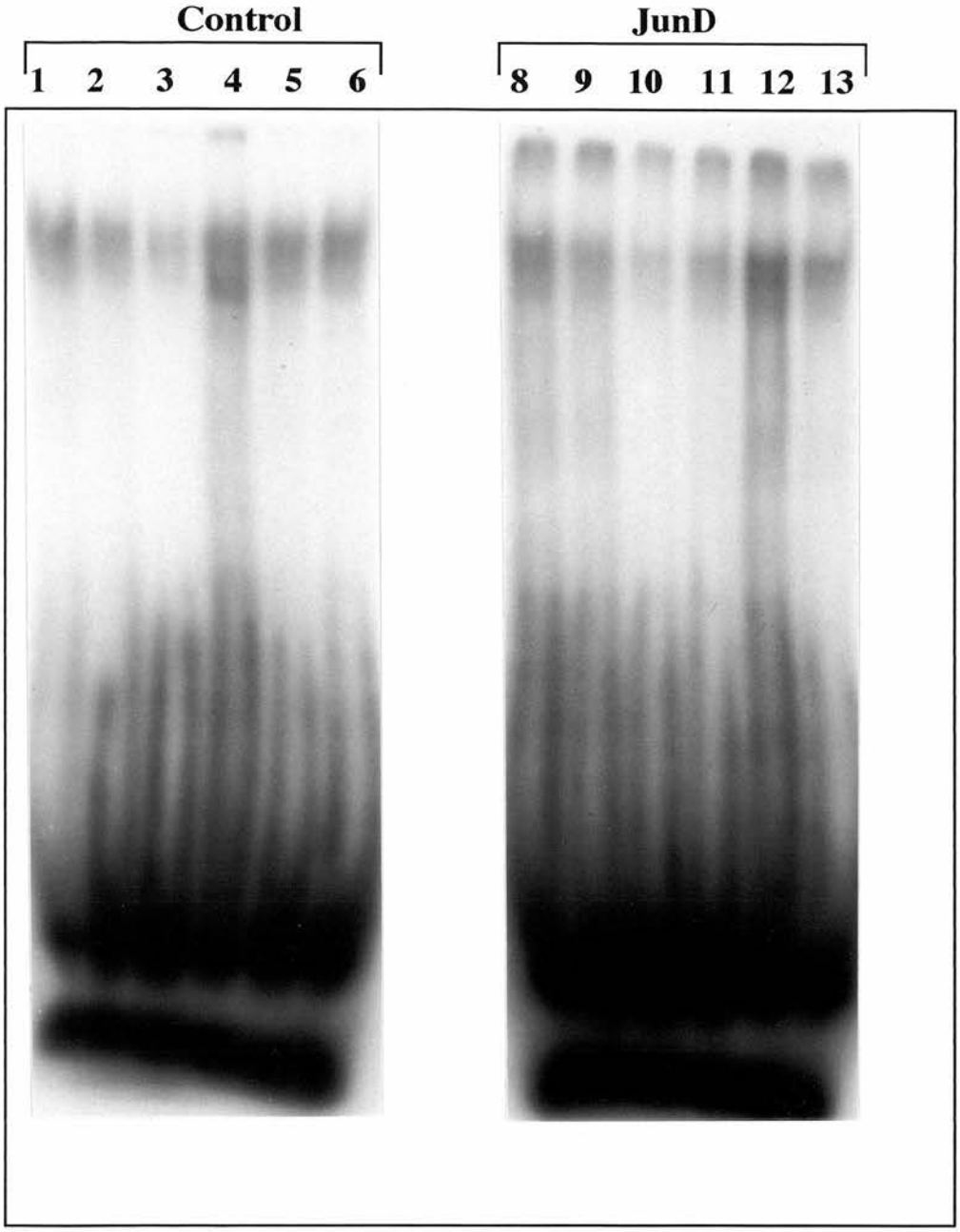


Figure 4.6 Electrophoretic mobility shift assay to evaluate the composition of AP-1 transcription factor complex following stimulation by a low concentration of Glu in the absence of serum.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 2 days. On day 1, the culture medium was exchanged for a serum-free, chemically-defined medium (containing 25 mM KCl; see Materials and Methods for details). On day 2, the day of the experiment, the medium was again changed, this time to a serum-free, chemically-defined medium (5 mM KCl), before exposure to 10 μM Glu for 0 min (lanes 1, 8); 15 min (lanes 2, 9); 30 min (lanes 3, 10); 45 min (lanes 4, 11); 60 min (lanes 5, 12) or 90 min (lanes 6, 13). Nuclear extracts were prepared and aliquots incubated with a 32P radioactively-labelled DNA fragment corresponding to the TRE, without (control; lanes 1-6) or with the addition of antibodies specific for JunD (lanes 8-13). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film.

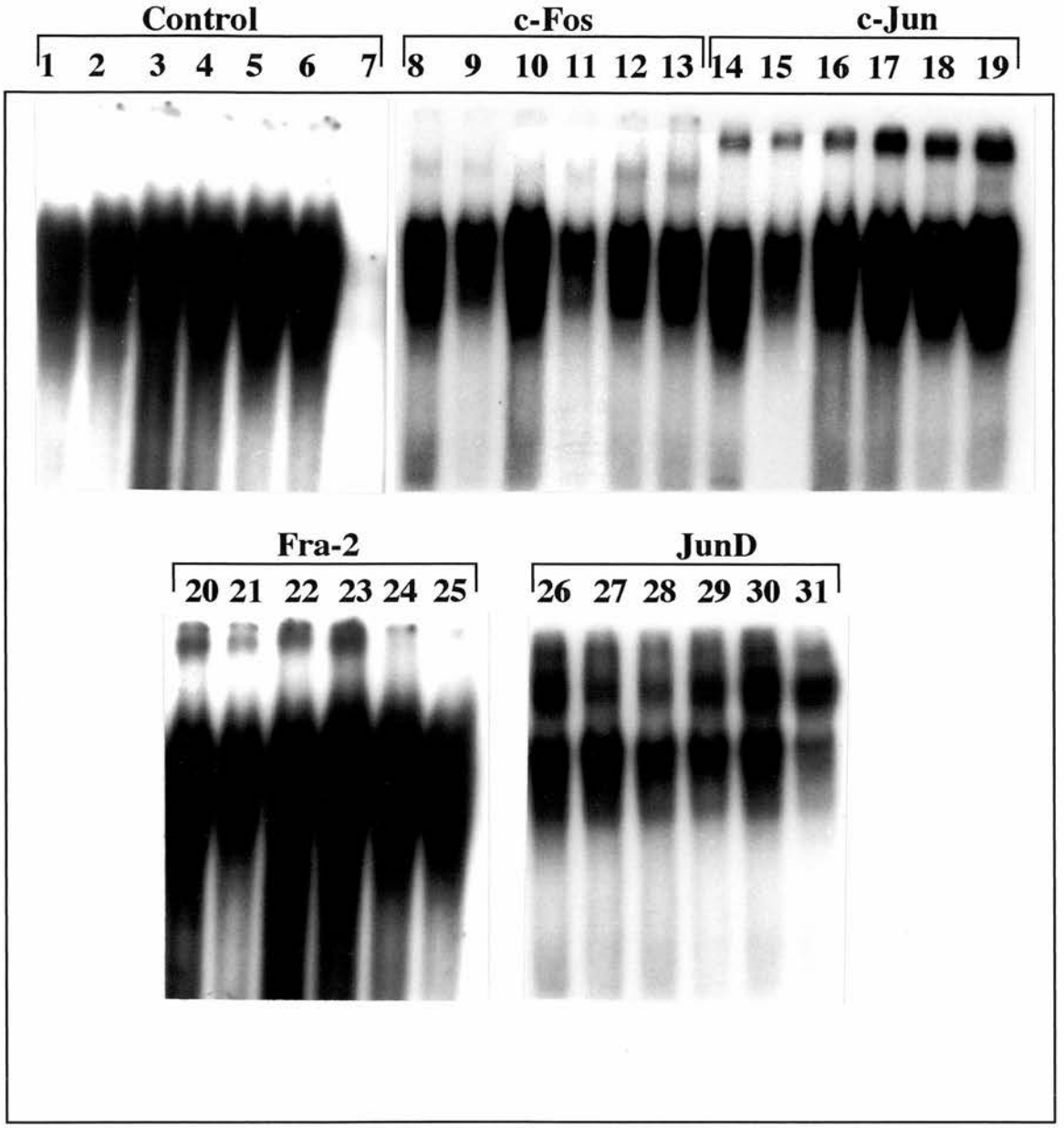


Figure 4.7 Electrophoretic mobility shift assay to evaluate the composition of AP-1 transcription factor complex following stimulation by a high concentration of Glu in the absence of serum.
Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 2 days. On day 1, the culture medium was exchanged for a serum-free, chemically-defined medium (containing 25 mM KCl; see Materials and Methods for details). On day 2, the day of the experiment, the medium was again changed, this time to a serum-free, chemically-defined medium (5 mM KCl), before exposure to 250 μM Glu for 0 min (lanes 1, 8, 14, 20, 26); 15 min (lanes 2, 9, 15, 21, 27); 30 min (lanes 3, 10, 16, 22, 28); 45 min (lanes 4, 11, 17, 23, 29); 60 min (lanes 5, 12, 18, 24, 30) or 90 min (lanes 6, 13, 19, 25, 31). Nuclear extracts were prepared and aliquots incubated with a 32P radioactively-labelled DNA fragment corresponding to the TRE, without (control; lanes 1-6) or with the addition of antibodies specific for c-Fos (lanes 8-13), c-Jun (lanes 14-19), Fra-2 (lanes 20-25) or JunD (lanes 26-31). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film. Lane 7: 0 min exposure, excess cold TRE added.

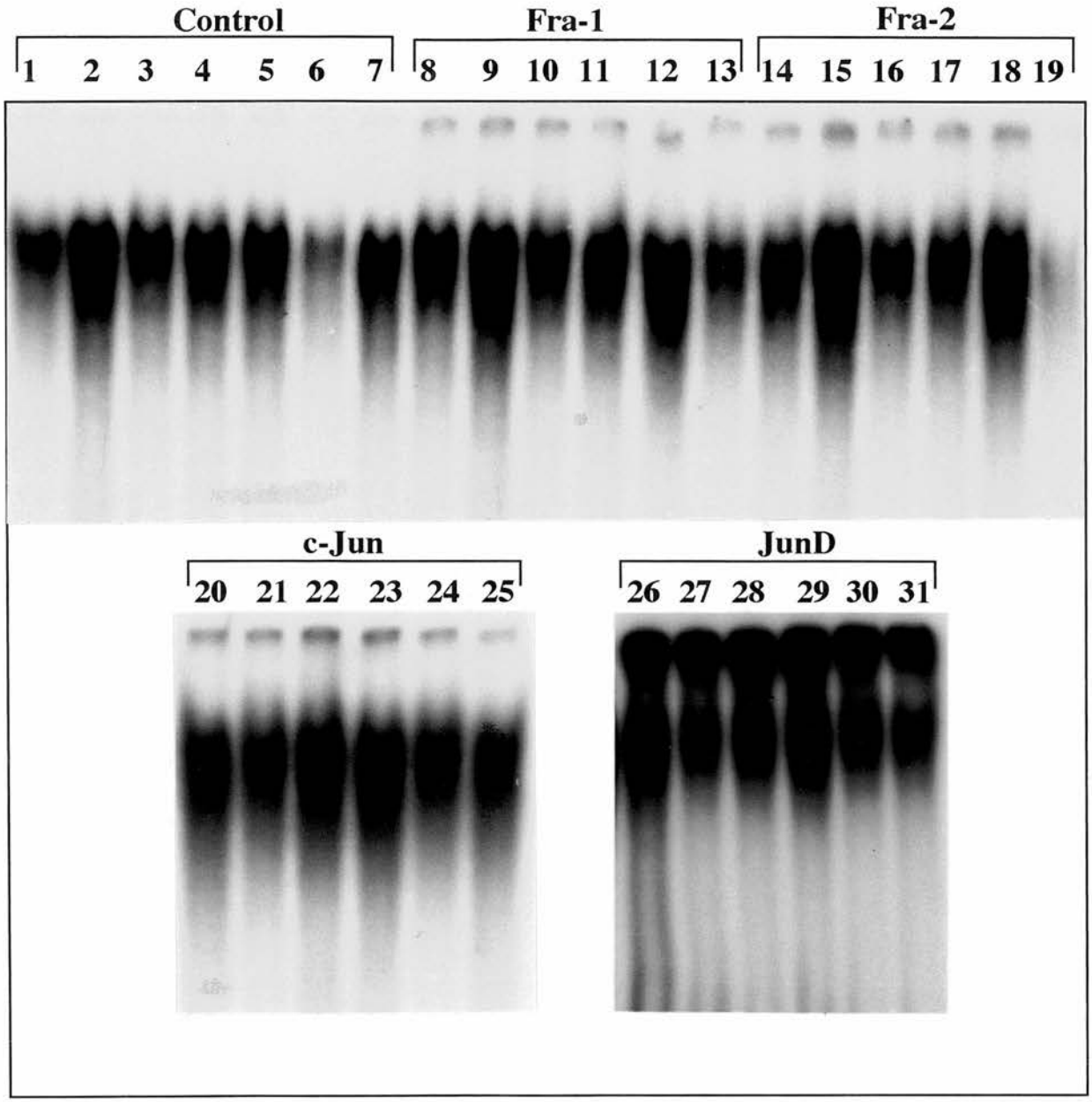


Figure 4.8 Electrophoretic mobility shift assay to evaluate the composition of the AP-1 transcription factor complex following stimulation by a low concentration of Glu in the presence of serum.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 2 days before exposure to 10 μM Glu for 0 min (lanes 1, 8, 14, 20, 26); 15 min (lanes 2, 9, 15, 21, 27); 30 min (lanes 3, 10, 16, 22, 28); 60 min (lanes 4, 11, 17, 23, 29); 90 min (lanes 5, 12, 18, 24, 30) or 240 min (lanes 6, 13, 19, 25, 31). Nuclear extracts were prepared and aliquots incubated with a 32P radioactively-labelled DNA fragment corresponding to the TRE, without (Control; lanes 1-6) or with the addition of antibodies specific for Fra-1 (lanes 8-13), Fra-2 (lanes 14-19), c-Jun (lanes 20-25) or JunD (lanes 26-31). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film. Lane 7: 15 min exposure, excess mutant TRE added.

complexes (Figure 4.9).

4.2.6 Translation of cIEGs to protein depends on concentration of agonist

Cerebellar granule cells at 2 DIV were exposed for 4h to either 10μM or 250 μM Glu. The cellular extracts were then separated by SDS-PAGE, blotted onto nitrocellulose and probed using specific antibodies for the presence of c-Fos, c-Jun and FosB protein (Figure 4.10A-C, respectively).

The expression of c-Fos protein after stimulation of cells with 10 μM Glu was fast and transient, peaking by 1h and returning to basal levels by 4h (Figure 4.10A). In contrast, expression of c-Fos following stimulation of cells with 250 μM Glu appeared more intense and peaked at 2h before decreasing towards basal levels by 4h.

Expression of c-Jun protein in cellular extracts after exposure to 10 μM Glu was again fast, peaking by 1h, and decreasing with time over the 4h observation period. In response to 250 μM Glu, however, c-Jun expression was more delayed, peaking by 2h, but appeared less intense than that stimulated by 10 μM Glu (Figure 4.10B).

The amount of FosB protein expressed following exposure to 10 μM Glu peaked by 1h, decreased by 2h and then peaked for a second time by 4h (Figure 4.10C). In contrast, expression of FosB following exposure of cells to 250 μM Glu appeared more intense, and increased throughout the 4h observation period.

4.2.7 Transient transfection studies

Cerebellar granule cells at 2 DIV were transfected with the -361luc plasmid in an attempt to analyse the functional aspects of the c-Fos protein detected using EMSA and Western blotting. The cells were exposed for periods up to 6h to 10 μM Glu, 250 μM Glu or 5 μM forskolin (which served as a positive control). As shown in Figure 4.11, cells stimulated with 10 μM Glu before being assayed for luciferase protein activity displayed an increased amount of functional

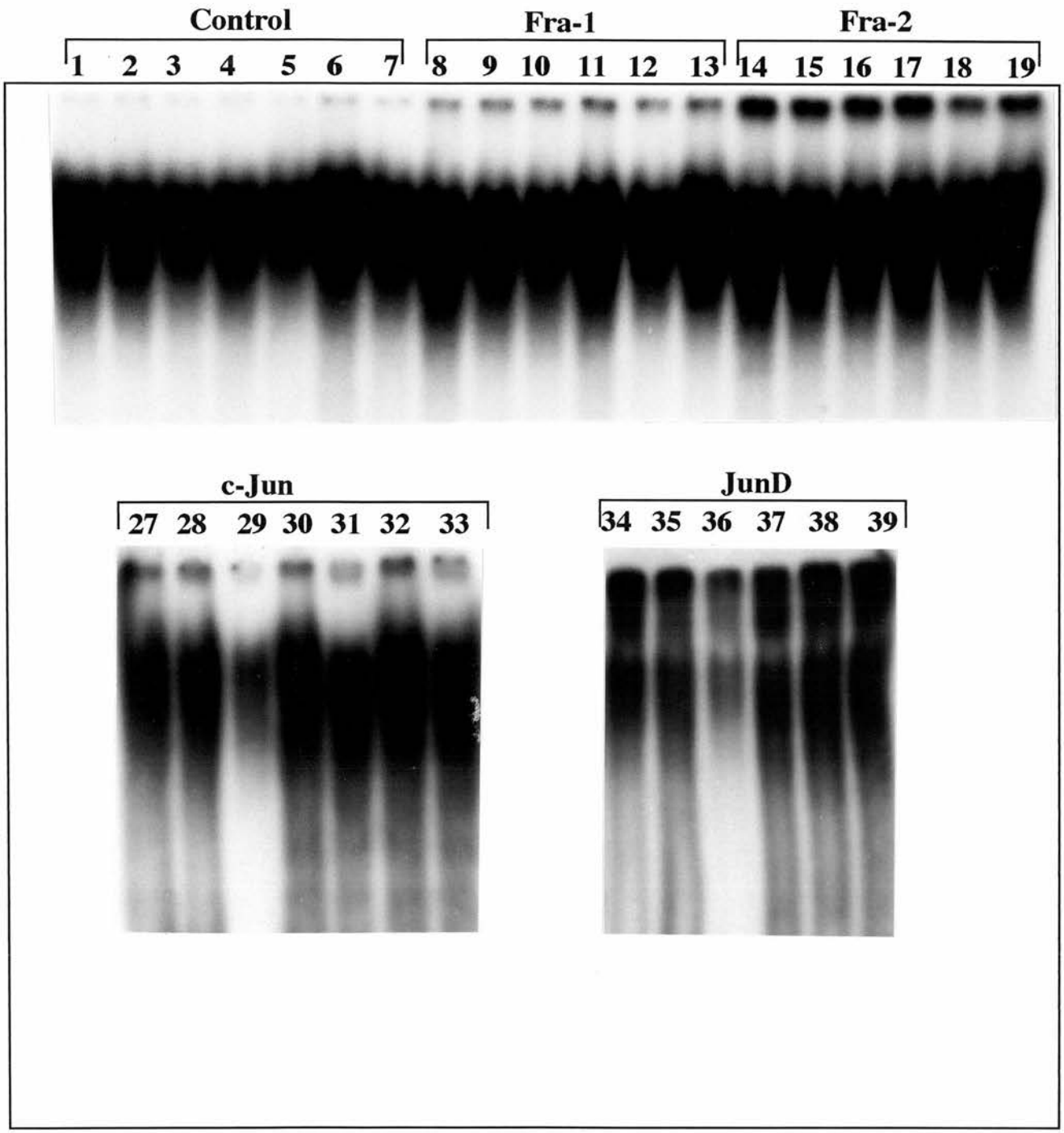


Figure 4.9 Electrophoretic mobility shift assay to evaluate the composition of the AP-1 transcription factor complex following stimulation by a high concentration of Glu in the presence of serum.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 2 days before exposure to 250 μM Glu for 0 min (lanes 1, 8, 14, 20, 27); 15 min (lanes 2, 9, 15, 21, 28); 30 min (lanes 3, 10, 16, 22, 29); 45 min (lane 23); 60 min (lanes 4, 11, 17, 24, 30); 90 min (lanes 5, 12, 18, 25, 31) or 240 min (lanes 6, 13, 19, 26, 32). Nuclear extracts were prepared and aliquots incubated with a 32P radioactively-labelled DNA fragment corresponding to the TRE, without (Control; lanes 1-6) or with the addition of antibodies specific for Fra-1 (lanes 8-13), Fra-2 (lanes 14-19), c-Jun (lanes 20-26) or JunD (lanes 27-32). After electrophoresis on a 10% native gel, the bands were visualised by exposure of the gel to X-ray film. Lane 7: 60 min exposure, excess mutant TRE added.

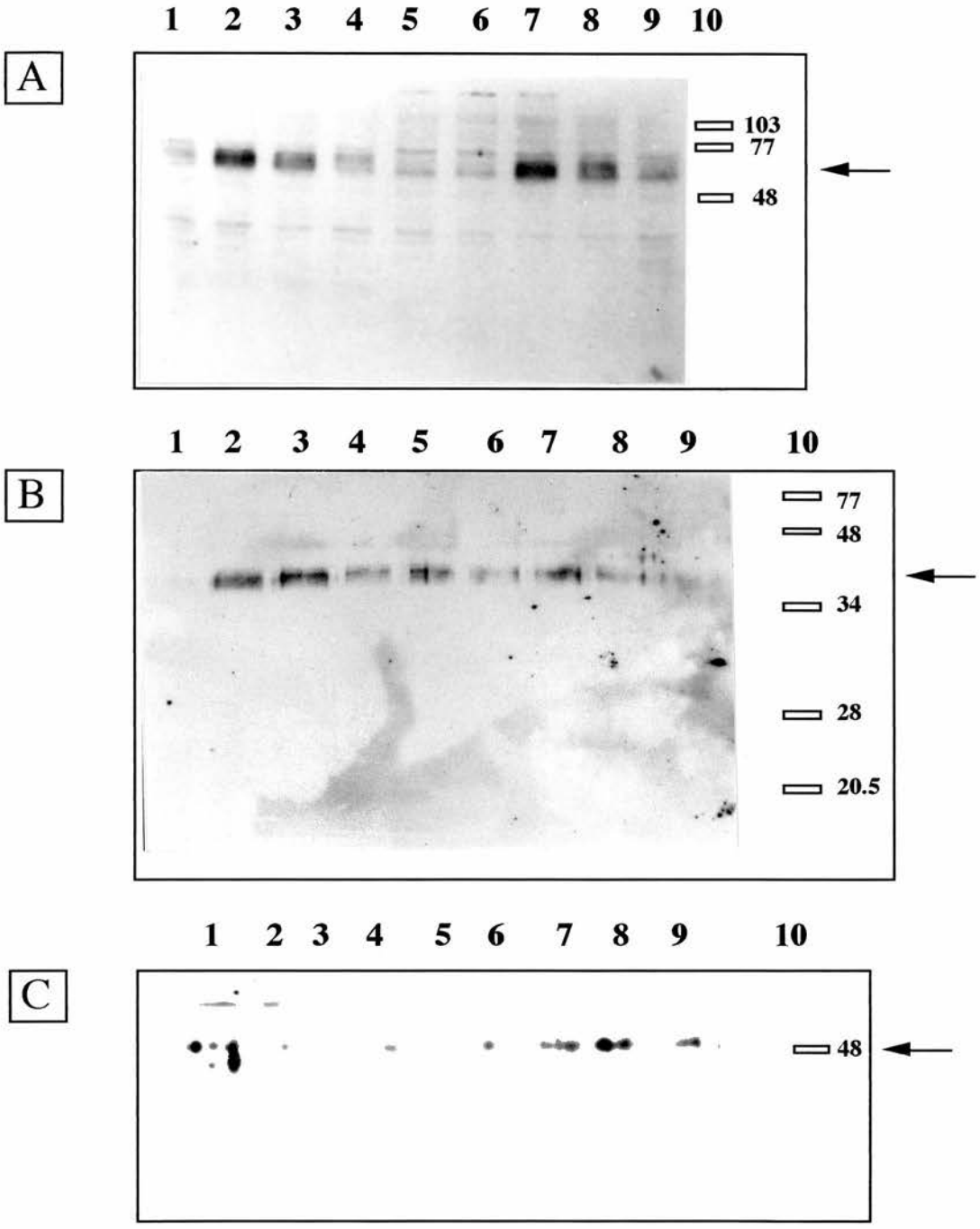


Figure 4.10 Expression of c-Fos, c-Jun and FosB protein in cerebellar granule cells in response to Glu exposure.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella. Granule cells were maintained in culture for 2 days before exposure to 10 μM or 250 μM Glu for up to 4h. 5 μM forskolin was used as a positive control. Cellular extracts were prepared and separated by SDS-PAGE on a 10% gel. The proteins were then blotted onto nitrocellulose and probed using specific antibodies for c-Fos (A), c-Jun (B) and FosB (C) protein. The presence of protein was visualised by enhanced chemiluminescence. Lanes 1 and 5: Control, no exposure. Lane 2: 10 μM Glu, 1h exposure. Lane 3: 10 μM Glu, 2h exposure. Lane 4: 10 μM Glu, 4h exposure. Lane 6: 250 μM Glu, 1h exposure. Lane 7: 250 μM Glu, 2h exposure. Lane 8: 250 μM Glu, 4h exposure. Lane 9: 5 μM forskolin, 4h exposure. Lane 10: molecular weight markers. Arrow indicates position of c-Fos (A), c-Jun (B) or FosB (C) protein band.

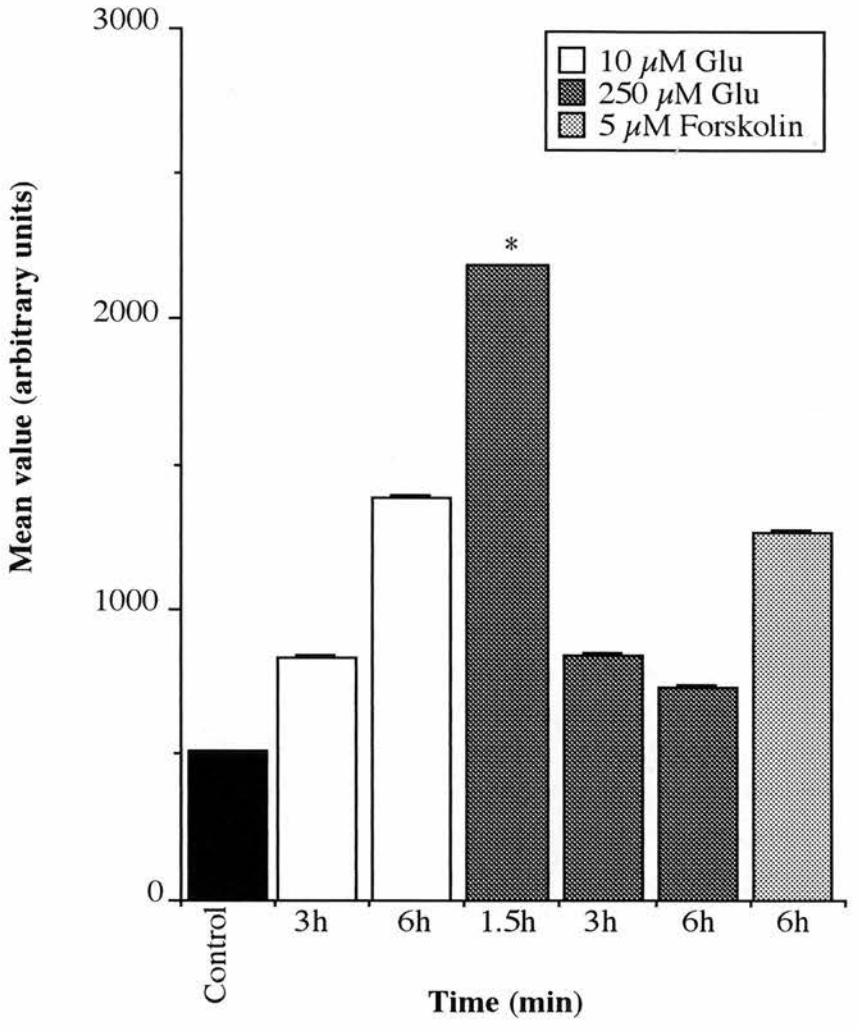


Figure 4.11 Analysis of c-Fos protein expression using the luciferase reporter gene assay.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 2 days before use. Cells were transfected with -361fosluc plasmid in serum-free medium and allowed to recover overnight. Granule cells were then exposed to agonist for up to 6h in serum-free medium before being assayed for luciferase protein expression using a luminometer. Data represent mean ± SEM values (n = 3). Asterisk indicates significant difference from control value.

protein expression over controls after a 3h exposure period, that had increased to ~3-fold of basal after 6h. When cells were exposed to 250 μM Glu, the response 1.5h after initial exposure was elevated to > 4-fold that of control, decreasing over the ensuing 4.5h to ~1.5-fold basal, although not significantly different from the control level. Forskolin (5 μM) elicited a response which was ~2.5-fold that of control, a lower increase than expected, since forskolin activates the CRE by elevating cAMP levels (Barthel *et al.*, 1996).

Section B: Investigation of the effect of K^+ ions on cerebellar granule cells

4.2.8 Investigation of KCl-mediated effects on cell viability and Ca^{2+} ion influx, immediate-early gene induction and protein expression in 2 DIV cerebellar granule neurons

Cerebellar granule cells at 2 DIV were exposed to increasing concentrations (25-55 mM) of KCl for 24h and assayed for cytotoxicity using MTT staining. Since the culture medium used contains 25 mM KCl, the absorbance obtained with medium alone was used as a basal or control value. As shown in Figure 4.12, increasing the concentration of KCl in the medium was unable to effect any cytotoxic response during the exposure period.

However, when cells were exposed to increasing concentrations of KCl but assayed for intracellular-free Ca^{2+} ion concentration, it was found that large amounts of Ca^{2+} ions were fluxed into the cell in response to depolarisation by K^+ ions (Figure 4.13); a 3-fold increase over basal (i.e. the level attained at a physiological $[K^+]_e$ of 5 mM) was observed at 55 mM KCl. The concentration range in this case was 5-55 mM, and the cells were exposed in HBS buffer. When the pharmacology of this Ca^{2+} ion influx was probed by coadministration of 55 mM KCl with NMDA receptor antagonists APV and TCP, and the L-type channel blocker nifedipine, only the presence of nifedipine was able to prevent the increase in $[Ca^{2+}]_i$ (Figure 4.14). Neither of the NMDA receptor antagonists were able to cause any decrease in the level of intracellular Ca^{2+}.

Cerebellar granule cells at 2 DIV were exposed for up to 6h to either 5 mM or 55 mM KCl. Exposure of cells to 5 mM KCl did not induce any appreciable increase in either c-*fos* or *fos*B mRNA during the 6h period (Figure 4.15). However, after stimulation with 55 mM KCl, the level of c-*fos* mRNA detected rose dramatically, beginning at around 1h after the start of the stimulation and continuing throughout the time of the experiment (6h). In contrast, there was no marked rise in the level of *fos*B mRNA (Figure 4.15).

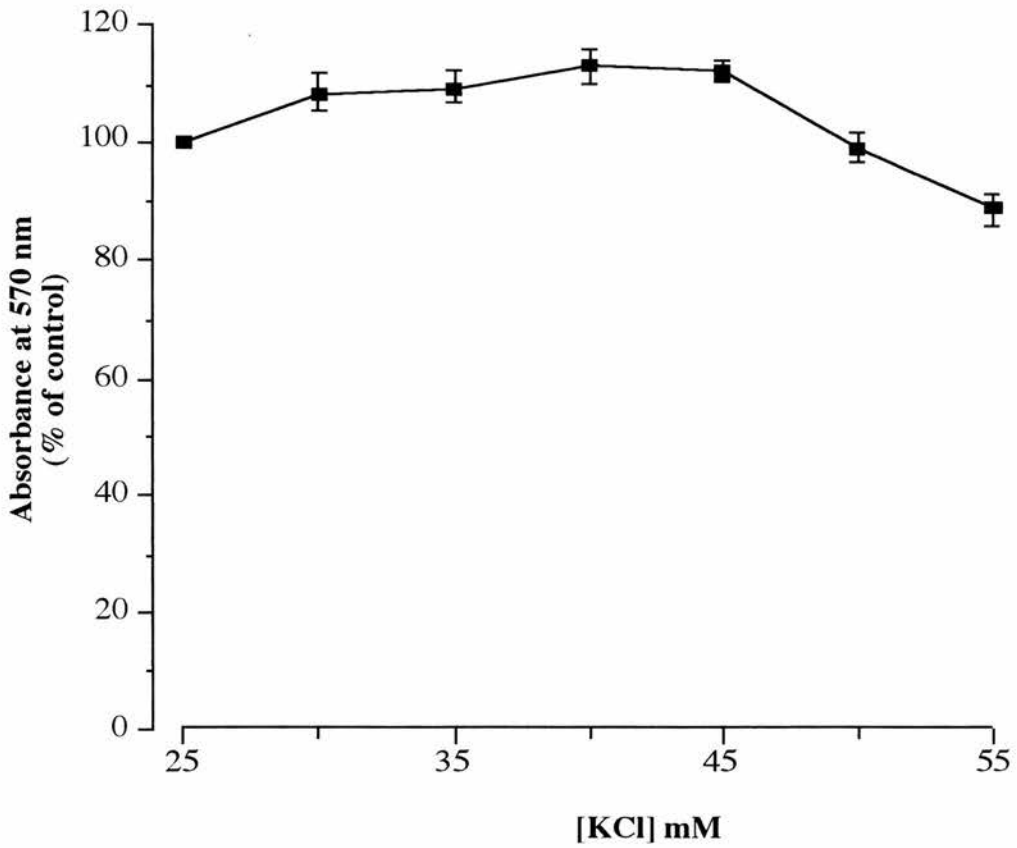


Figure 4.12 Survival of cerebellar granule cells in primary culture after exposure to increasing concentrations of KCl.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella, and were maintained in culture for 2 days before use. Cells were exposed (24h) in culture-conditioned medium to increasing concentrations of KCl before being assayed for cell viability using MTT staining. The absorbance at 570 nm was expressed as a percentage of control (untreated) cells, in this instance the 25 mM KCl value. Data represent mean ± SEM values (n = 8-16). The absorbance values for control cells were 0.167 ± 0.002.

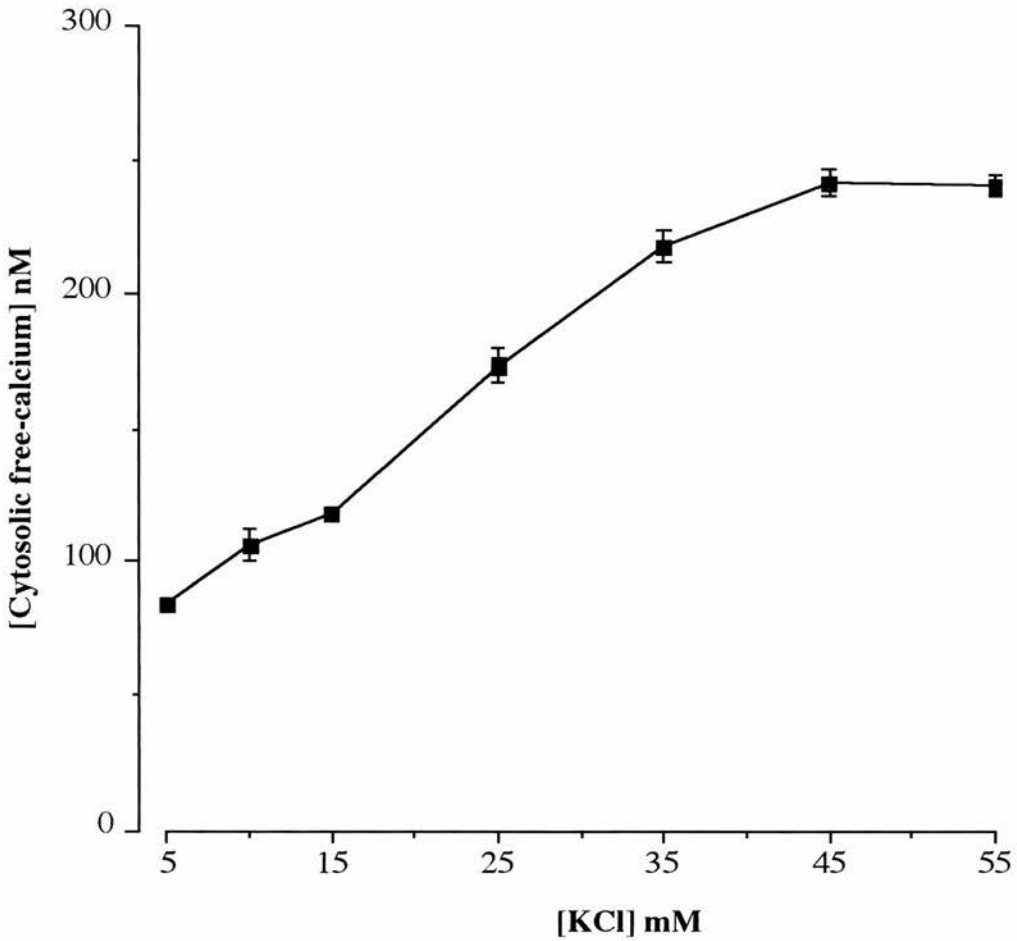


Figure 4.13 Concentration-dependent effect of KCl on stimulated increases in intracellular-free calcium levels in cultured cerebellar granule cells.
Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella, and were maintained in culture for 2 days before use. Granule cells were washed with balanced salt solution, loaded with Fluo-3/AM and exposed to increasing [KCl], as indicated. Data are the mean ± SEM values (n = 7-8).

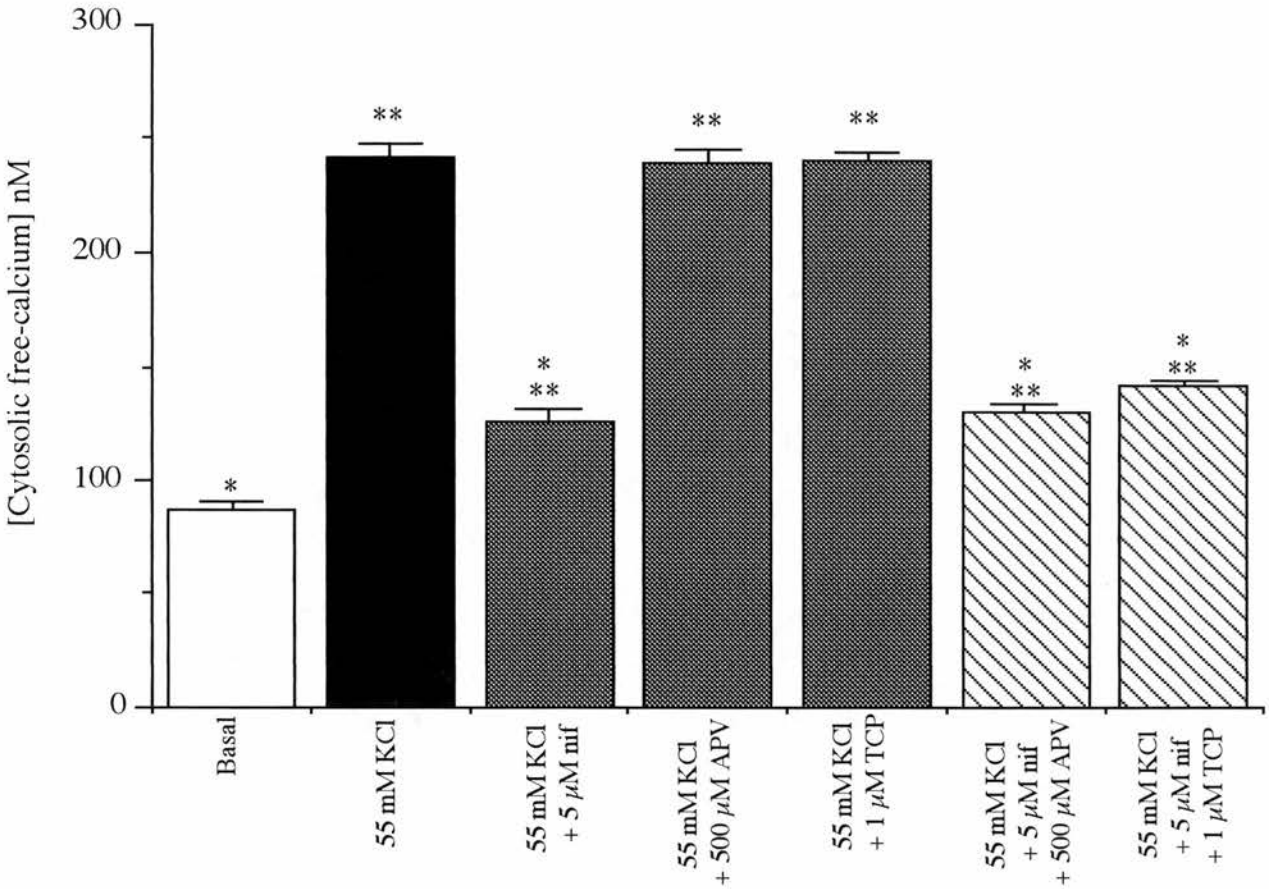


Figure 4.14 Pharmacology of KCl-stimulated increases in intracellular-free calcium levels in cultured cerebellar granule cells.

Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old postnatal mouse cerebella, and were maintained in culture for 2 days before use. Granule cells were washed with balanced salt solution, loaded with Fluo-3/AM and exposed to 55 mM KCl alone or coadministered with pharmacological agents, as indicated. Data are the mean ± SEM values (n = 8-16). *: indicates significant difference from 55 mM KCl alone; **: indicates significant difference from basal.

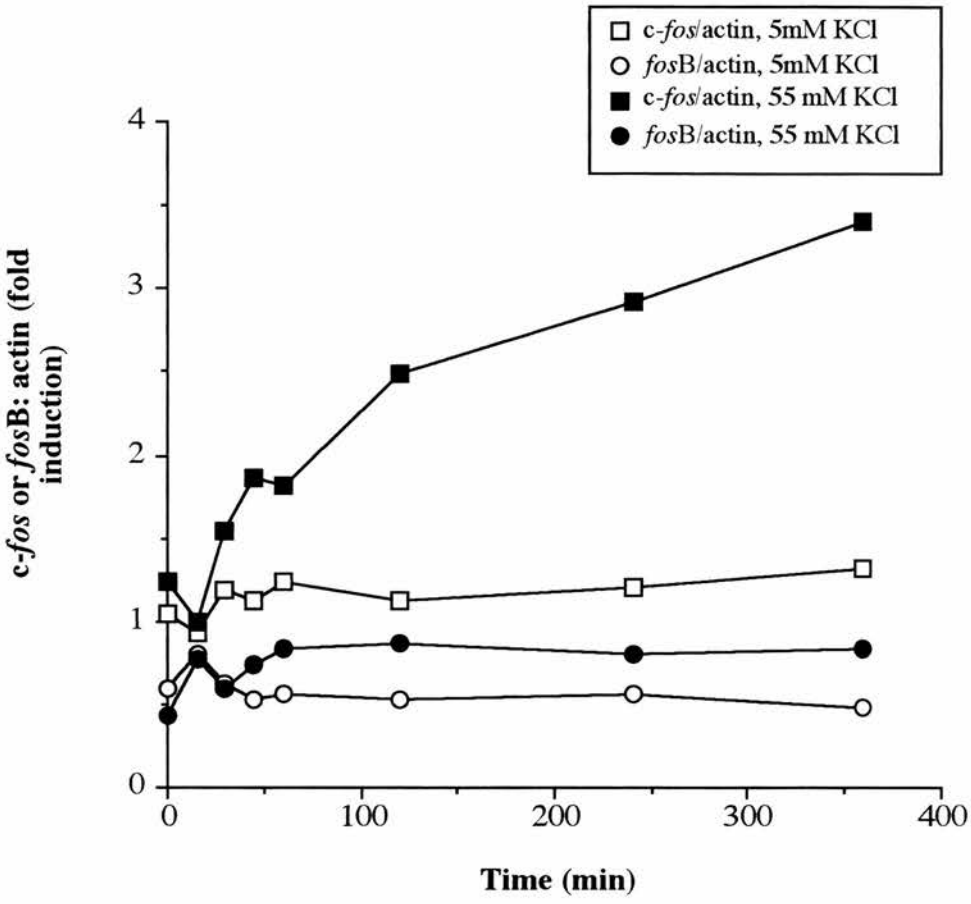


Figure 4.15 Timecourse of KCl-stimulated, steady-state c-*fos* and *fos*B mRNA levels in cultured cerebellar granule cells.
Primary cultures of cerebellar granule cells were prepared from suspensions of dissociated cells from 7 day-old post-natal mouse cerebella. Granule cells were maintained in culture for 2 days before exposure to 5 mM or 55 mM KCl for up to 6h. Total RNA was prepared from the cultures, and the amount of c-*fos*, *fos*B and β-actin mRNA was quantified. The results refer to c-*fos* or *fos*B mRNA expression relative to that of β-actin.

4.3 Discussion

4.3.1 Non-toxic Ca^{2+} influx and immediate-early gene induction mediated by Glu and K^+ ions

The distribution of receptors and voltage-gated Ca^{2+} channels in the developing brain is highly regulated, and is cell type-specific. In cultured cerebellar granule neurons, dihydropyridine-sensitive, L-type voltage-gated Ca^{2+} channels are the dominant channel-type at an early stage of development and thus control the influx of Ca^{2+} ions in immature cultured neurons (Evans and Pocock, 1999). In the present study, an increase in intracellular Ca^{2+} concentration, mediated by either Glu or K^+ ions, was nifedipine-sensitive; thus L-type voltage-gated Ca^{2+} channels are developed and active in 2 DIV cultured cerebellar granule cells. In both cases, the increase in $[Ca^{2+}]_i$ was only partially blocked by nifedipine, although this could be a concentration effect. Coadministration of KCl with both nifedipine and an NMDA receptor antagonist also failed to completely prevent the increase in $[Ca^{2+}]_i$; in fact, the presence of an NMDA receptor antagonist had no significant additive effect on the prevention of $[Ca^{2+}]_i$ increase by nifedipine. Thus it appears plausible that other mechanisms contribute to the overall increase in $[Ca^{2+}]_i$ (Simpson *et al.*, 1995). For example, activation of metabotropic Glu receptors is known to cause release of Ca^{2+} from intracellular stores (Sugiyama *et al.*, 1987); however, a role for K^+ ions in activation of these receptors has not been suggested.

Regulation of the composition of the NMDA receptor in developing cerebellum *in vivo* has been demonstrated (Sasner and Buonanno, 1996). The receptor subunit NMDAR1 (NR1) is thought to be essential for functional receptor formation, and heteromeric combinations of NR1 with one or more of the NR2 subunits (NR2A-D) are differentially expressed depending on location within the brain and stage of development. In the cerebellum, expression of the NR2B subunit is widespread for the first two weeks after birth, when it becomes suppressed and NR2C becomes the common partner for NR1. This regulation of subunit expression corresponds to the localisation of cerebellar granule cells in the external granule layer during the first two postnatal weeks, after which they migrate to the internal granule layer, and NR2C expression is up-regulated (Sasner and Buonanno, 1996). Additionally, alternative RNA splicing of NR1 can give

rise to up to eight splice variants, thereby allowing even more variation (Zukin and Bennett, 1995). In the *in vitro* model used in this study, differential expression of NMDA receptor subunits is also likely to cause regulation of the function of Glu-mediated NMDA receptor activation. Thus the finding here that exposure of 2 DIV cerebellar granule cells to up to 1 mM Glu has no effect on cell viability may be explained in the light of regulation of NMDA receptor composition as well as the finding elsewhere that Glu and NMDA are able to exert a trophic effect on developing cerebellar neurons (Balázs *et al.*, 1988a, c; 1989).

Induction of the immediate-early gene, c-*fos*, has been demonstrated in PC12 cells following depolarisation-induced Ca^{2+} influx via voltage-gated Ca^{2+} channels (Sheng *et al.*, 1990), and in particular via L-type voltage-gated Ca^{2+} channels (Murphy *et al.*, 1991; Rosen *et al.*, 1994). The cascade studied in the experiments reported by Sheng and colleagues involves the phosphorylation of CREB by CaM kinase, and the subsequent binding and activation of the CRE on the c-*fos* gene by CREB (Sheng *et al.*, 1990). This result corresponds to that reported herein in the context of 2 DIV cerebellar granule neurons, where c-*fos* mRNA induction was observed following depolarisation of the cell membrane with 'high' (55 mM) levels of K^+ ions, but no expression of c-Fos as part of the AP-1 transcription factor complex bound to the TRE was detected by EMSA studies. It is possible to speculate that this (K^+-stimulated) induction of c-*fos* mRNA was mediated by CREB, whereas its induction following exposure to 'low' (10 μM) and 'high' (250 μM) levels of Glu could arguably be induced via binding of either the CRE or the SRE. In the case of both Glu and K^+-mediated c-*fos* mRNA induction, any AP-1 transcription factor formed may have been signalled to bind the CRE of a target gene, rather than the TRE, since the consensus sequence of the former differs from the latter by only one base pair.

Induction of *fos*B mRNA was very low in response to K^+-induced depolarisation. Rapid, transient induction of *fos*B mRNA has been observed in NIH 3T3 cells in the presence of growth factors (Zerial *et al.*, 1989); it has also been shown to be activated in this cell line by serum, PDGF and PMA, this induction being regulated via the SRE on the *fos*B gene (Lazo *et al.*, 1992). The differential induction of *fos*B (and indeed c-*fos*) mRNA in 2 DIV cerebellar granule cells stimulated with 'low' and 'high' levels of Glu shown in this study is interesting, since neither

concentration of Glu was able to impose any toxicity on these cells. The intracellular Ca^{2+} concentration measured in 2 DIV cerebellar neurons following exposure to 250 μM Glu was, however, 2-fold higher than that observed following exposure to 10 μM Glu, suggesting that the level of mRNA induction may be a function of $[Ca^{2+}]_i$. Unlike the results observed in 7 DIV cells, the elevated, sustained profile of c-*fos* and *fos*B mRNA induction observed in 2 DIV cells was not affected by the presence of nifedipine, suggesting the action of different mechanisms of Ca^{2+}-dependent cIEG induction in immature and mature cultured cerebellar granule cells.

4.3.2 AP-1 transcription factor is formed and activated in immature cultured cerebellar granule cells

The regulation of signal transduction culminating in the induction of immediate-early genes and expression of their products is complex, and differs according to location and developmental stage. Binding of the TRE by the AP-1 transcription factor complex is the differential step between short-term, immediate regulation and long-term alteration of the cellular make-up via transcriptional regulation of late, target genes. Even taking only the Fos and Jun family members as possible components of the AP-1 transcription factor dimer, there are still many stimuli and conditions which induce different combinations of these proteins, allowing a host of possible final outcomes.

The results shown in this chapter from the EMSA studies give some interesting insights into the mechanism of glutamate stimulation of immature cultured cerebellar granule cells. The formation of, and binding of the TRE by, the AP-1 transcription factor in nuclear extracts following stimulation of cells with a low concentration (10 μM) of Glu in the absence of serum was demonstrated clearly by gel shift analysis (Figure 4.6). Under these conditions, no members of the Fos or Jun families of proteins, except for JunD, were detected using specific antibodies to each member protein. The presence of JunD may be explained by a large body of evidence supporting its constitutive expression (Hope *et al.*, 1994; Hou *et al.*, 1997). Other proteins, for example, the activating transcription factor family (ATFs), contain leucine zippers and form dimers with Fos and Jun family members which are subsequently able to bind to the TRE (Hai

and Curran, 1991). JunD is able to form heterodimers with members of the ATF family of transcription factors (Shimizu *et al.*, 1998), however, the JunD supershift detected allows the suggestion that the AP-1 transcription factor bound to the TRE is composed, at least in part, of JunD/JunD homodimers under these experimental conditions and at this developmental stage.

However, it may be that Fos and Jun family member proteins are present in the cell under physiological conditions until they are signalled to dimerise in the event of a non-physiological stimulus. When cellular extracts were analysed using SDS-PAGE and Western blotting, it was found to be the case that c-Fos, FosB and c-Jun were present after stimulation with 10 μM Glu (Figure 4.10). Thus it appears that these proteins are present in the cytoplasm in a monomeric form under non-toxic conditions. This finding corresponds with the theory that no *de novo* protein synthesis is required for the induction of cIEGs (Angel and Karin, 1991; Ginty *et al.*, 1992; Karin, 1995), since AP-1 dimers are known to auto-regulate induction of cIEGs (Angel *et al.*, 1988a; Sassone-Corsi *et al.*, 1988a,b; Chiu *et al.*, 1989; Schütte *et al.*, 1989; Boyle *et al.*, 1991; Ham *et al.*, 1995).

Expression of Fra-1 protein as part of the AP-1 transcription factor complex was only observed following stimulation of cells with 'low' and 'high' levels of Glu in the presence of serum (Figures 4.8 and 4.9), and not following this stimulation in serum-free medium. c-Fos has been reported as being serum-inducible in different systems (Treisman, 1985; Stumpo and Blackshear, 1986; Sassone-Corsi *et al.*, 1988a), and Fra-1 has been shown to be induced by stimulation with serum in fibroblasts (Cohen *et al.*, 1989). Fra-1 is known to dimerise with ATF-4 (Hai and Curran, 1991), and the binding of this dimer to the TRE may form part of the DNA-binding band which supershifted in the presence of anti-Fra-1 antiserum, shown in Figures 4.8 and 4.9.

As discussed above, Fra-1 protein was only expressed as part of the AP-1 complex in the presence of serum; in contrast, a supershift indicating the presence of c-Fos protein as a component of the AP-1 transcription factor complex was only observed in cells which were exposed to 'high' (250 μM) levels of Glu in the absence of serum (Figure 4.7). Expression of c-

Fos has been observed in *in vivo* development studies (for review see Dragunow and Preston, 1995).

In the results presented in this chapter, 'high' levels of Glu (250 μM) caused expression of Fra-2, c-Jun and JunD as constituents of the AP-1 dimer. c-Jun is known to form heterodimers with Fra-2, as well as acting as a homodimer. Thus it is possible that the cascade(s) activated in cultured 2 DIV cerebellar granule cells after exposure to 250 μM Glu is in some way, or in some situations, mediated by an AP-1 dimer containing c-Jun. It has been suggested that perhaps heterodimers of c-Fos/c-Jun may confer a programmed cell death signal, whereas c-Jun homodimers could cause a growth/survival signal to be activated (Walton *et al.*, 1998). However, in the experiments reported in this chapter involving 2 DIV cerebellar granule neurons, c-Jun was not detected as part of the AP-1 transcription factor complex in any situation which ultimately ended in significant, detectable cell death.

4.3.3 Transfection studies on 2 DIV cultured cerebellar granule cells

Cells at 2 DIV were transfected with the -361fosluc plasmid and subsequently exposed to 10 μM and 250 μM Glu prior to evaluation of functional protein expression using the luciferase reporter assay. Initially when this experiment was carried out, only the 3h and 6h timepoints were used for each concentration of Glu. However, the values obtained for protein expression following exposure to 250 μM Glu were lower than might be expected, and it was thought that use of these timepoints was perhaps inappropriate for the timescale of protein expression. In an attempt to address this, protein levels at 1.5h after initial exposure to 250 μM Glu were measured. The fact that this exposure caused an increase of activity ~4.5-fold over basal, when compared to ~1.5-fold increase over basal following exposure for 3h and 6h to 250 μM Glu (Figure 4.11), suggests that the protein expression occurring is fast and transient, in contrast to the protein expression detected using Western blotting (Figure 4.10A). This part of the work undertaken is incomplete; additional studies using a variety of plasmids would yield more information. However, significant amounts of time were spent troubleshooting the technique, such that it was decided to focus on more fruitful areas.

Chapter 5

General discussion, conclusions and suggestions for further work

The neurotransmitter Glu has been widely studied over a number of years due to its ability to have a role in neurotrophic effects during development as well as a role in toxicity in mature cells. The clinical implications of dysregulation of Glu metabolism in the brain have been mentioned in Chapter 1. This project was undertaken in a laboratory which seeks to further explore the mechanism by which Glu and other excitatory compounds elicit these cellular responses to toxicity. In this chapter, the results of each section are outlined in the context of both ages of cells employed to draw conclusions about the developmental consequences of the present study, and to suggest further work which could be carried out to more fully explain the mechanisms behind Glu-induced toxicity in mouse cerebellar granule cells.

5.1 Effects of Glu- and K^+-induced Ca^{2+} influx in 2 DIV cultured cerebellar granule cells in terms of cell viability and immediate-early gene induction

A comparative study of the effects of the excitatory amino acid Glu and the monovalent cation K^+ on 2 DIV cultured cerebellar granule cells revealed that these two stimuli elicit similar responses in terms of cell viability but differ in their ability to increase the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. No decrease in cell viability was observed in these cells following exposure to either 55 mM K^+ ions or up to 1000 μM Glu. However, in response to 100 μM Glu, a 6-fold increase in $[Ca^{2+}]_i$ over basal was observed, compared to a 3-fold increase following exposure of cells to 55 mM KCl. Glu-induced Ca^{2+} ion influx was partly antagonised by the L-type voltage-gated calcium channel blocker, nifedipine. This compound was clearly also effective in preventing the K^+-induced rise in intracellular Ca^{2+} levels; however, coadministration of the NMDA antagonists, APV and TCP, had no effect on the $[Ca^{2+}]_i$ increase observed following exposure to high levels of K^+ ions. K^+ ions are known to cause influx of Ca^{2+} ions by depolarising the cell membrane, thereby activating voltage-dependent Ca^{2+} channels. This can lead to induction of immediate-early genes, allowing transcriptional regulation of target genes (Bartel *et al.*, 1989). K^+ ions have no direct effect on glutamatergic ionotropic receptors, although depolarisation of the cell membrane has been suggested as a cause of relief of the Mg^{2+} block on NMDA receptor channels (Bading *et al.*, 1995). Glu itself is a ligand for several glutamatergic

receptors, some of which contain a Ca^{2+}-permeable ion channel as an integral part of their structure; in particular, the NMDA and certain AMPA receptors (see Section 1.1 for discussion). Thus, antagonism of these ionotropic receptors alone might be expected to prevent the increase in Ca^{2+} ion concentration within the cell. However, the action of nifedipine on the rise of Ca^{2+} following exposure to Glu suggests a more complex mechanism. The activation of ionotropic receptors and resultant Ca^{2+} influx could cause a change in the membrane potential, and thus open voltage-gated Ca^{2+} channels, hence explaining the nifedipine-sensitivity of the Glu-mediated rise in $[Ca^{2+}]_i$. This dual-influx situation could stimulate activation of more than one signal transduction pathway, leading to clEG induction via multiple binding sites. In this case, several target genes could either be activated or suppressed by the resultant AP-1 transcription factors.

Blockade of L-type Ca^{2+} channels had no effect, however, on the steady-state mRNA levels of c-*fos* and *fos*B induced in 2 DIV cerebellar granule cells exposed to 250 μM Glu. Whilst antagonism of NMDA receptor channels by APV or TCP altered the mRNA induction profile from sustained to transient, nifedipine had no such effect. This result suggests a strong link between NMDA receptor activation and immediate-early gene induction, as has been observed elsewhere (Didier *et al.*, 1989). However, pre-incubation with 50 μM NMDA has been shown to protect 9 DIV cerebellar granule neurons from toxicity due to $\leq$ 2 mM Glu, but to have no effect on c-*fos* or c-*jun* mRNA induction (Weller *et al.*, 1994). Thus both nifedipine and NMDA receptor antagonists have changing roles during the development of these cells, possibly involving distinct pathways at different stages of development. In contrast to the similar induction profiles observed for c-*fos* and *fos*B mRNA following exposure to 250 μM Glu, cells exposed to 55 mM KCl produced significant levels of c-*fos* mRNA, but not *fos*B mRNA. The profile of *fos*B mRNA induction was similar to that observed following stimulation of cells with 5 mM KCl. This result suggests that c-*fos* and *fos*B are regulated via distinct pathways in 2 DIV cerebellar granule cells; in the case of c-*fos*, one or more pathways which are inducible by both Glu and KCl (e.g. CaM kinase IV or MAP kinase activation, leading to CREB or SRF phosphorylation and binding of target genes) may be inducible, and in the case of *fos*B, a pathway which is inducible by Glu but not KCl (e.g. PKA activation, which can occur as a result

of NMDA-mediated increase in $[Ca^{2+}]_i$ or by metabotropic receptor activation, leading to adenylate cyclase activation and phosphorylation of CREB by PKA) may be the only possible option. Induction of c-*fos* mRNA has been demonstrated following activation of voltage-gated Ca^{2+} channels by K^+ ions and the subsequent increase of $[Ca^{2+}]_i$ in PC12 cells (Morgan and Curran, 1986), and, in whole rat brain preparations, the induction of c-*fos* mRNA has been shown to be stimulated by KA and NMDA via distinct mechanisms (Sonnenberg *et al.*, 1989b).

5.2 Effects of Glu- and K^+-induced Ca^{2+} influx in 7 DIV cultured cerebellar granule cells in terms of cell viability and immediate-early gene induction

Not only the amount of Ca^{2+} influx (Garthwaite and Garthwaite, 1986b; Barthel *et al.*, 1996) but also the route of that Ca^{2+} influx is important in regulation of cellular mechanisms, and in particular in transcription of immediate-early genes (Gallin and Greenberg, 1995; Sattler *et al.*, 1998). The results presented here involving exposure of 7 DIV cultured cerebellar granule cells to high and low levels of both Glu and K^+ ions clearly show this to be the case. Whilst exposure of these cells to high levels (up to 1000 μM) of Glu caused a 90% decrease in absorbance, stimulation with high levels (up to 55 mM) of K^+ ions failed to exert any decrease in cell viability. However, both compounds at these concentrations caused a 7-8-fold increase over basal in $[Ca^{2+}]_i$. Thus an equal amount of Ca^{2+} influx caused very different results depending on the route of entry. Similar results regarding Glu-mediated Ca^{2+} influx in cultured cortical neurons have been reported (Sattler *et al.*, 1998). As discussed in Section 3.3.1, both Glu- and K^+-mediated Ca^{2+} influx could be significantly decreased by the coadministration of the stimulus with nifedipine, clearly implicating L-type voltage-gated Ca^{2+} channels in Ca^{2+} influx mediated by both of these stimuli. However, it would appear that the endpoints of these two induction pathways are different; *i.e.* that the role of c-*fos* and its encoded protein in each case would involve binding of a distinct Jun family protein and targeting of a distinct late gene.

Comparing the effects of Glu and K^+ ions on induction of immediate-early gene mRNA revealed that although 10 μM Glu, 5 mM K^+ and 55 mM K^+ were all non-toxic stimuli, they each exhibited a characteristic mRNA induction profile. Whilst the c-*fos*, *fos*B and c-*jun* response to

10 μM Glu was fast and transient, peaking at 30-60 min following initial exposure and reverting to basal levels within 6h, the level of both c-*fos* and *fos*B mRNA observed following stimulation of 7 DIV cultured cerebellar granule cells with 55 mM KCl continued to increase throughout the 6h observation period; conversely, no induction of either c-*fos* or *fos*B mRNA was observed following exposure to 5 mM KCl. These induction profiles correlate to a certain extent with the amount of Ca^{2+} influx observed in the presence of each of these stimuli: 5 mM KCl was taken as the basal level of $[Ca^{2+}]_i$ (Figure 3.16), 10 μM Glu exerted a 4.5-fold increase over basal, whilst exposure to 55 mM KCl caused an increase of ~6.5-fold over basal. Thus, the greater the increase in $[Ca^{2+}]_i$, the longer the delay in mRNA levels returning to basal levels following non-toxic stimulation. Toxic concentrations of Glu (250 μM) stimulated a similar increase in $[Ca^{2+}]_i$ levels to 55 mM KCl, with the mRNA levels peaking at 2-3h before returning to a basal level by 6h.

In addition, *fos*B mRNA induction following exposure to 10 μM Glu appeared to peak for a second time at 5h. The reason for this second peak is unknown (time constraints meant that it was not possible to repeat this experiment to ascertain the validity of this result). NIH-3T3 cells subjected to serum stimulation expressed *fos*B mRNA rapidly and transiently, peaking at 15-30 min, whilst expression of *fos*B/SF was delayed, peaking at 1h following stimulation (Mumberg *et al.*, 1991). However, Hollen *et al.*, (1997) reported that FosB protein is expressed in quinolinate-lesioned rat striatum between 4h and 8h, whereas the expression of FosB/SF begins at 6h and is still apparent at 30h after initial stimulus. A similar phenomenon has been reported with c-*jun* mRNA expression following transient forebrain ischaemia, where the first peak is observed at 1-2h following ischaemia, but a second peak is observed at 24-48h (Wessel *et al.*, 1991). The probe used in the present study corresponds to the entire *fos*B sequence, and thus would be expected to recognise both the long and short forms of *fos*B, so it is conceivable that the first and second peak observed in Figure 3.6C correspond to induction of *fos*B and *fos*B/SF mRNA, respectively.

Measurement of the steady-state levels of c-*fos* and *fos*B mRNA induced by low and high concentrations of Glu and KCl allowed investigation into the pharmacology of mRNA induction by these stimuli. Since the 250 μM Glu-mediated, 'delayed' c-*fos* mRNA profile was prevented by coadministration with TCP, while cells exposed to 10 μM Glu exhibited a normal, 'transient'

profile, it is reasonable to conclude that this 'delayed' profile results from the toxic stimulus. However, the L-type Ca^{2+} channel blocker, nifedipine, also had an effect on the mRNA levels expressed by cells exposed to 250 μM Glu, altering the profile from 'delayed, sustained' to 'transient'. Since nifedipine has no effect on Glu-mediated toxicity in these cells (Griffiths *et al.*, 1998), the 'delayed, sustained' profile is not solely associated with toxicity, but rather with both toxicity and also Ca^{2+} influx which may or may not be related to the toxic response. This suggestion is supported by the fact that exposure of cells to high (55 mM) KCl stimulated the 'delayed, sustained' profile of c-*fos* mRNA, which was nifedipine-sensitive but also unconnected with toxicity. Hou and colleagues have demonstrated that KCl-induced expression of c-Fos and JunD proteins as components of the AP-1 transcription factor complex in rat cerebellar granule cell cultures can be prevented by blockade of the L-type voltage-gated Ca^{2+} channels by nifedipine (Hou *et al.*, 1997). Reduction in basal and kainate-induced expression of immediate-early genes has also been demonstrated in cortical neurons by antagonists of L-type voltage-gated Ca^{2+} channels (Murphy *et al.*, 1991), indicating the complexity of the signalling pathways involving c-*fos* mRNA induction.

5.3 Developmental aspects of Glu-mediated effects on cerebellar granule cells in culture

5.3.1 The role of the neurotransmitter Glu changes with development of the brain and central nervous system

Investigation of the effect of increasing concentrations of Glu on cultured cerebellar granule cells at two different ages (2 DIV and 7 DIV) using the MTT assay showed that this endogenous neurotransmitter is highly toxic to 7 DIV cells, but has no cytotoxic effect on 2 DIV cells. The distribution of Glu transporters (Bar-Peled *et al.*, 1997; Furuta *et al.*, 1997) and voltage-gated Ca^{2+} channels (Rossi *et al.*, 1994; Verderio *et al.*, 1995; Parri and Lansman, 1996; Harrold *et al.*, 1997) changes during the development of a variety of cell types, in culture as well as *in vivo*. Differential expression of Glu transporters (both glial and neuronal) during development allows for regulation of the extracellular Glu concentration. EAAT2 has been

identified as the Glu transporter important in migration of neurons towards the cerebellum (Komuro and Rakic, 1993). By regulating the extracellular Glu concentration, EAAT2 is able to control NMDA receptor activation and subsequent Ca^{2+} influx in order to allow correct migration, since overactivation of this receptor type can cause dysregulation of the migration process (Marret *et al.*, 1996).

In addition, the composition of NMDA (Sheng *et al.*, 1994; Dunah *et al.*, 1996; Portera-Cailliau *et al.*, 1996) and AMPA (Burnashev *et al.*, 1992) receptors has been found to change with development. NMDA receptor subunits are divided into two subtypes: NMDAR1 (NR1), which is required for receptor channel function, and the four NR2 subunits (NR2A-D), which define the properties of the receptor channel. It is thought that the NR1 subunit is essential for receptor formation, and can be combined with one or more of the NR2 subunits. Within each subunit, alternative splicing creates several different variants, allowing greater variation of receptor composition, and by application, of receptor function. Several studies using subunit-specific antibodies have shown that individual subunit expression and thus NMDA receptor composition changes according to the region and developmental stage of the brain (Dunah *et al.*, 1996; Portera-Cailliau *et al.*, 1996), but as yet little is known about the precise combinations responsible for particular functions, e.g. the trophic or toxic effects of Glu. The process of RNA editing of AMPA subunits (GluR2) giving rise to Ca^{2+}-permeability of AMPA receptors is thought to be regulated by mechanisms involving Glu receptor activation and subsequent Ca^{2+} influx (Burnashev *et al.*, 1992).

Use of a fluorimetric assay to measure $[Ca^{2+}]_i$ in these cells after exposure to increasing concentrations of Glu revealed that there was no significant difference between the final (nM) concentration of Ca^{2+} ions after stimulation of either cell age group with 100 μM Glu (at both ages, this concentration exerted the maximum increase in $[Ca^{2+}]_i$). In addition, the $[Ca^{2+}]_i$ following exposure to 250 μM Glu when compared to that following 10 μM Glu was doubled. However, the maximum fold increase over basal values (i.e. final/basal) were ~8-fold for 7 DIV cells, compared to ~5-fold for 2 DIV cells. Thus, the same concentration of Glu caused a greater influx of Ca^{2+} ions in mature cells than in young, less developed cells, thereby echoing the

findings cited above, namely, that there is a degree of receptor development which occurs between these two ages. Wahl *et al.* (1993) reported that receptor development occurs *in vitro* on a similar timescale to that which happens *in vivo*.

When the pharmacology of Ca^{2+} ion influx was investigated, nifedipine was found to reduce the $[Ca^{2+}]_i$ stimulated by 250 μM Glu by approximately one-third at both ages of cells. The activation effect of glutamatergic receptor stimulation on voltage-gated Ca^{2+} channels has been shown to stimulate induction of, among other genes, c-*fos* (Morgan and Curran, 1986).

5.3.2 Immediate-early gene mRNA induction: developmental effects

Induction of c-*fos* mRNA in mature, 7 DIV cultured cerebellar granule cells exposed to low levels of Glu (10-50 μM) has been demonstrated previously (Szekely *et al.*, 1989; Grayson *et al.*, 1990). These workers carried out their experiments on 7-9 DIV cells in which Glu-mediated c-*fos* mRNA induction was shown to be rapid and transient, and reversible by inhibitors of the NMDA receptor (Grayson *et al.*, 1990). In addition, exposure of BALB/c 3T3 cells to serum allows similar c-*fos*, c-*jun* and *jun*B mRNA induction profiles to be observed (Ryder and Nathans, 1988). Exposure of 8 or 9 DIV cerebellar granule cells to Glu for as little as 5 min has been shown to elicit the delayed, elevated response shown in Figure 3.6A of this study (Szekely *et al.*, 1989). When cells were exposed to Glu (50 μM) in conjunction with NMDA (100 μM), it was found that this non-toxic concentration of NMDA was able to protect cerebellar granule cells from the toxic potential of 50 μM Glu (Marini and Paul, 1992). These workers have suggested a neuroprotective role for low concentrations of agonists of the NMDA receptor (e.g. NMDA, Glu) in 7 DIV cultured cerebellar granule cells, which may involve the induction of c-*fos* mRNA as part of the mechanism (Marini and Paul, 1992). NMDA receptor activation and subsequent c-*fos* mRNA expression has been implicated in NMDA-dependent survival of cerebellar granule neurons in culture (Didier *et al.*, 1989). The results reported herein are in line with these findings, since 10 μM Glu caused a rapid, transient increase in c-*fos* mRNA induction, whilst being unable to cause any toxicity.

In cells at both ages, the induction of c-*fos* and *fos*B mRNA was sustained at a higher level for longer than that of c-*jun*. At 7 DIV, the toxic dose of Glu caused a delayed induction profile, whereas at 2 DIV the profile was elevated, but not delayed. In the case of c-*fos* and, to a lesser extent, *fos*B, mRNA levels were sustained, but not delayed in 2 DIV cerebellar granule cells. At 7 DIV, in the case of both c-*fos* and *fos*B, the L-type Ca^{2+} channel blocker nifedipine and the NMDA receptor antagonist TCP were able to revert the delayed profile to the transient profile after stimulation with 250 μM Glu. However, at 2 DIV, only the NMDA receptor antagonists TCP and APV were able to cause this change, and nifedipine had no significant effect. From this evidence it appears that at 7 DIV, Ca^{2+} influx via NMDA receptor binding of extracellular Glu causes Glu-mediated toxicity, but L-type channels are also a major route of influx of Ca^{2+} ions. However, at 2 DIV, there is no role apparent for L-type Ca^{2+} channels from the mRNA induction profiles, despite the earlier observation that nifedipine is able to reduce the amount of Ca^{2+} ion influx to the cell in response to Glu (Figure 4.4). L-type Ca^{2+} channels are thought to be the main route of Ca^{2+} influx required for survival of cerebellar granule cells in culture (Marchetti and Usai, 1996), and are also abundant in the early developmental stages of these cells (Harrold *et al.*, 1997). Thus the role of these channels changes, with development, from being necessary for Ca^{2+} influx to promote survival at 2 DIV, to being involved in transcriptional activation of immediate-early genes at 7 DIV, which is connected with excitotoxicity.

5.3.3 Implications of age on immediate-early gene protein expression and AP-1 transcription factor composition

The use of transient transfection of both cell lines and primary cell cultures is a useful technique for probing the activation of expression of specific proteins (Gorman *et al.*, 1982; Loeffler *et al.*, 1990; Ye *et al.*, 1990; Barthel *et al.*, 1993). The results obtained from the transfection experiments carried out in this study have been discussed in each of the relevant chapters. This technique was not satisfactorily mastered, and the results are included in this thesis as an indication of part of the work which was carried out as part of this project. However, no

real conclusions can be made from the results presented in sections 3.2.6 and 4.2.7. This is an area where much further work could be carried out, since trouble-shooting of the technique was not completed during the time available.

More fruitful results were, however, obtained from studies using EMSA. Experiments using this technique formed a large part of the total work, and a summary of the results obtained is shown in Figure 5.1[3]. There were three parameters studied:

i) age of cells *in vitro* (2 or 7 DIV)

ii) concentration of Glu used as stimulus (10 μM or 250 μM)

iii) medium (N-DMEM or chemically-defined, serum-free) used during exposure of cells to agonist.

Since serum is not a chemically-defined substance, and can differ quite markedly in its constituent parts, many serum-free, chemically-defined media have been developed in an attempt to combat the 'unpredictability' of serum-containing media and to sustain neural cells in a more reproducible manner. In some of the experiments reported herein, cells were plated out in serum-containing N-DMEM, and then transferred after 1 or 5 days to a serum-free, chemically-defined medium (described in Materials and Methods). The main difference between this chemically-defined medium and N-DMEM is that the former does not contain growth factors which are present in foetal calf serum, but contains alternative substances which allow cell growth and development. However, in addition, the concentration of KCl in the serum-free culture medium was reduced to 5 mM, one-fifth of the concentration required for survival in culture in serum-containing medium. This requirement for slightly depolarising levels of KCl (25 mM) is manifested at 3 DIV in rat cerebellar granule cells (Gallo *et al.*, 1987), and the resulting increase in $[Ca^{2+}]_i$ is mediated by L-type voltage-gated Ca^{2+} channels (Marchetti and Usai, 1996). Investigation using the MTT assay showed that during the time-scale of the experiment (up to 7h maximum period in serum-free, low

---

[3]**Figure 5.1 Summary of results obtained in EMSA studies.**

Dimers marked are those thought to be possible between members of the Fos and Jun families. Dimers involving interaction between these proteins and other bZIP family members (e.g. ATFs, CREB etc.) have not been included since no data were collected with regard to formation of dimers containing these proteins.

7 DIV

[Glu]	Outcome	Condition	Proteins	Dimers
10 μM	non-toxic	+ serum	c-Fos Fra-2 JunD	c-Fos/JunD Fra-2/JunD JunD/JunD
10 μM	non-toxic	- serum	Fra-2 JunD	Fra-2/JunD JunD/JunD
250 μM	toxic	+ serum	c-Fos Fra-2 FosB JunD	c-Fos/JunD Fra-2/JunD FosB/JunD JunD/JunD
250 μM	toxic	- serum	FosB JunB c-Jun JunD	FosB/JunB FosB/c-Jun FosB/JunD JunB/c-Jun JunB/JunD JunB/JunB c-Jun/JunD c-Jun/c-Jun JunD/JunD

2 DIV

[Glu]	Outcome	Condition	Proteins	Dimers
10 μM	non-toxic	+ serum	Fra-1 Fra-2 c-Jun JunD	Fra-1/c-Jun Fra-1/JunD Fra-2/c-Jun Fra-2/JunD c-Jun/JunD c-Jun/c-Jun JunD/JunD
10 μM	non-toxic	- serum	JunD	JunD/JunD
250 μM	non-toxic	+ serum	Fra-1 Fra-2 c-Jun JunD	Fra-1/c-Jun Fra-1/JunD Fra-2/c-Jun Fra-2/JunD c-Jun/c-Jun c-Jun/JunD JunD/JunD
250 μM	non-toxic	- serum	c-Fos Fra-2 c-Jun JunD	c-Fos/c-Jun c-Fos/JunD Fra-2/c-Jun Fra-2/JunD c-Jun/c-Jun c-Jun/JunD JunD/JunD

KCl, chemically-defined medium), no significant cell death occurred (Figure 3.26). A comparative study of experiments undertaken in serum-free versus serum-containing medium allowed several interesting observations to be made.

The first observation is that JunD is present in all conditions, regardless of age, Glu concentration or medium, making a strong case for its expression as a constitutive protein, a finding in agreement with those demonstrated in different preparations (Hope *et al.*, 1994; Hou *et al.*, 1997). There seems to be no negative effect of any of the parameters on the expression and dimerisation of this protein to form a TRE binding protein. However, in some instances (e.g. 7 DIV, 10 μM Glu), almost all the AP-1 binding band was supershifted to the JunD band, which may indicate that a higher proportion of the AP-1 dimers present under those circumstances contained JunD.

Secondly, the findings from EMSA studies undertaken in the presence of serum suggest that the 'toxic' AP-1 transcription factor complex is likely to comprise a combination of FosB, c-Fos, Fra-2 and JunD proteins. By comparing the dimeric possibilities (see Figure 5.1) it is possible to eliminate certain dimers from having no role in the excitotoxic event. In 7 DIV cells exposed for up to 4h to 250 μM Glu in serum-containing medium, where marked necrosis is evident (Figure 3.1B), the possible dimers comprising the 'toxic' AP-1 transcription factor complex are c-Fos/JunD, Fra-2/JunD, JunD/JunD and FosB/JunD. Of these it is suggested that only the FosB/JunD dimer is implicated in the excitotoxic event since the other three dimers are components of the AP-1 complex in 7 DIV cells under non-toxic conditions, namely when cells are exposed for up to 4h to 10 μM Glu; and/or in 2 DIV cells, were no toxicity is observed at either concentration of Glu. In addition, participation of FosB in the AP-1 transcription factor complex was not detected following exposure of 7 DIV cerebellar granule cells to 55 mM KCl, a non-toxic event. In support of this theory, expression of FosB and JunD as components of the AP-1 transcription factor complex in cultured rat cerebellar granule cells has been demonstrated (Hou *et al.*, 1997).

Further support of a role for FosB/JunD in excitotoxicity comes from key pharmacological experiments. Thus, in 7 DIV cells, coadministration of 250 μM Glu with 1 μM TCP (a selective

NMDA receptor antagonist) in serum-containing medium prevented FosB and c-Fos protein involvement in AP-1 complex formation, thus only Fra-2/JunD and JunD/JunD are permissible dimers under these circumstances. Since TCP also prevents excitotoxicity induced by 250 μM Glu, these findings provide further evidence to indicate the lack of functional involvement of either Fra-2/JunD or JunD/JunD dimers as AP-1 complexes involved in Glu-mediated excitotoxicity but further enhances a possible role for the FosB/JunD heterodimer. This result also provides strong evidence to support a NMDA receptor-mediated signalling pathway for induction of c-*fos* and *fos*B mRNAs and consequent expression of both c-Fos and FosB proteins.

In contrast, APV has been shown to completely prevent all TRE binding, rather than supershifting of specific proteins, after exposure of 6-7 DIV cerebellar granule cells to 100 μM NMDA (Sakurai *et al.*, 1992). Hiroi *et al.* (1998) have found that, in mice subjected to chronic electroconvulsive seizures, the frontal cortex brain homogenate contained AP-1 transcription factor complexes displaying high levels of both FosB and JunD proteins. Thus they report that an AP-1 heterodimer comprising FosB and JunD is produced following potentially lethal stimulation of the frontal cortex. A consequence of AP-1 transcription factor complex formation and binding of the TRE is transcriptional regulation of target genes, thus allowing speculation that the FosB/JunD heterodimer is involved in either the regulation of a downstream toxic event, or a failed neuroprotective function. These workers have also reported the presence of a TRE sequence in the coding sequence for the NMDA receptor subunit, NMDAR1. They further report that the FosB/JunD heterodimer is able to bind to the TRE on this gene, allowing the possibility of a feedback loop involving NMDA receptor formation and activation under 'toxic' circumstances (Hiroi *et al.*, 1998). In papilloma-producing 308 mouse keratinocytes, okadaic acid was observed to stimulate post-translational processing of existing FosB/JunD dimers by ERK-1/2 (Rosenberger *et al.*, 1999). In contrast to those results observed using EMSA, FosB protein was detected using Western blotting in 2 DIV cerebellar granule cells exposed to high levels of both Glu and KCl. It may be that FosB protein is present in the cytosol under non-toxic/physiological conditions, and is only triggered to combine with a Jun family protein to form an AP-1 transcription factor complex following exposure to a toxic stimulus. The C-terminal activation

domain present in all Fos family member proteins is important for transcriptional activation (Skinner *et al.*, 1997), but there may also be a phosphorylation site which causes stimulation of dimerisation. c-Jun homodimers have been suggested as existing in an inactive, phosphorylated form in resting cells, requiring to be dephosphorylated before DNA binding can occur (Boyle *et al.*, 1991).

Expression and integration of c-Fos protein into the AP-1 transcription factor complex was observed in both cases in 7 DIV cells (but not 2 DIV cells) in the presence of serum, and was only observed under one condition in the absence of serum (2 DIV, 250 μM Glu). The serum-inducibility of c-Fos, along with other bZIP proteins, is well documented (Müller *et al.*, 1984; Treisman, 1985; Treisman, 1986; Mohun *et al.*, 1987; Gilman, 1988; Chen *et al.*, 1996). However, the apparent involvement of c-Fos in toxicity, particularly that induced by Glu, has also been reported (see Gass and Herdegen (1995) for review), whereas in this study it appears that the involvement of c-Fos in TRE binding is not exclusively linked to Glu-induced toxicity, since c-Fos is present as part of the AP-1 transcription factor after exposure of cells to both 10 μM and 250 μM Glu. The involvement of NMDA receptor activation in c-Fos expression in the context of cell survival has been observed (Didier *et al.*, 1989). Thus, it is conceivable that the Jun family member which forms the AP-1 dimer by forming a leucine zipper with a c-Fos protein is responsible for the distinct roles observed in these experiments. The c-Fos/c-Jun heterodimer could only be expressed as the AP-1 transcription factor complex, using the results obtained in this study, in 2 DIV cells exposed to 250 μM Glu in the presence of serum. Thus it is possible to speculate that high levels of Glu in 2 DIV cells trigger a specific response which involves the formation of an AP-1 complex composed of c-Fos and c-Jun. This is not a toxic event, and thus the mRNA profiles of c-*fos* and c-*jun* under these conditions are different to those produced by 7 DIV cells exposed to 250 μM Glu, suggesting levels of regulation which are beyond the scope of the present study. In addition, nifedipine is not involved in the regulation of the induction of this elevated profile, whereas this antagonist of L-type voltage-gated Ca^{2+} channels was able to prevent the toxic profile of mRNA being produced in 7 DIV cells. The target genes for these transcription factor complexes have still not been elucidated, and it remains to be seen what effect

a c-Fos/c-Jun heterodimer would have on 2 DIV cells in terms of specific target gene binding and activation. c-Fos is also able to bind to ATF-4 (Hai and Curran, 1991; Herdegen, 1996), allowing other possibilities of AP-1 transcription factor binding which were not explored in this study. c-Jun has been suggested as being involved in neuronal death in rats following hypoxic ischaemia or status epilepticus (Dragunow *et al.*, 1993), although its function as a neuroprotective or survival factor has also been observed following axotomy (Herdegen *et al.*, 1993). Herdegen and colleagues later established that c-Jun was expressed following axotomy but that some cells were subsequently triggered to survive, whilst others underwent programmed cell death. These workers postulate that c-Jun must be expressed before other transcriptional components can be expressed and thus either allow the cell to survive or induce apoptosis (Herdegen *et al.*, 1997).

Coadministration of 7 DIV cells with 250 μM Glu and the NMDA receptor antagonist, TCP, in the presence of serum prevented the participation of both FosB and c-Fos in AP-1 transcription factor complexes, suggesting an NMDA receptor-linked signalling pathway for expression of these proteins. This result may be evidence for the NMDA receptor-dependent nature of Glu-mediated cIEG induction. Further work using non-NMDA receptor antagonists and other receptor channel blockers would allow greater insight into the regulation and composition of AP-1 transcription factors under different conditions.

All of the antibodies used for the EMSA studies were obtained from Santa Cruz Biotechnology, Inc., and are reported to be non-cross-reactive with any other Fos or Jun family member (see Appendix 1). However, FosB/SF, a naturally-occurring, alternatively spliced variant of FosB, lacks the C-terminal 101 amino acids. Since the epitope of the antibody for FosB used was in the central region of the protein, FosB/SF will be detected by this antibody as well as FosB. FosB/SF is able to form heterodimers and bind TRE; however, heterodimers containing FosB/SF were found to be unable to activate transcription of genes containing a TRE in F9 teratocarcinoma stem cells (Nakabeppu and Nathans, 1991; Yen *et al.*, 1991). Since Fos proteins are unable to form homodimers (Nakabeppu and Nathans, 1989), the inability of FosB/SF to activate transcription reported by these workers means that FosB/SF binding to Jun proteins has the potential to inhibit the Jun family's transcriptional activational ability (Nakabeppu and

Nathans, 1991). In addition, it has been shown that the presence of FosB/SF has a negative effect on the transcriptional and transformational activity of c-Fos and FosB, by competing for c-Jun proteins and AP-1 binding sites (Yen *et al.*, 1991; Nakabeppu and Nathans, 1991). The inability of FosB/SF to be activated is due to its shortened C-terminal domain. FosB is phosphorylated in this area by as yet unidentified kinases, which causes activation of the protein and subsequent transcriptional activation (Skinner *et al.*, 1997). However, the presence of FosB/SF in the nuclear extracts would have no effect on the assay, since only binding of TRE and not transcriptional activation is measured. It is conceivable, however, that the FosB band would be less intense in the presence of an antibody which detected only FosB, and not FosB/SF.

Investigation of Fos and Jun family protein expression in response to Glu using Western blotting gave information on the levels of each protein over a 4h period. At 7 DIV, the presence of c-Fos protein in response to both 10 μM and 250 μM Glu mirrors the results found using EMSA as the detection system, although since Western blotting is a more quantitative technique, it is possible to say that a greater amount of c-Fos protein was produced after cellular exposure to 250 μM Glu than was produced following exposure to 10 μM Glu. The expression of FosB in 7 DIV cells was also consistent with the EMSA results, being only induced in response to 250 μM Glu.

At 2 DIV, expression of c-Fos protein was transient following exposure to 10 μM Glu, peaking at 1h. This corresponds with the mRNA profile, allowing time for translation to occur. In response to 250 μM Glu, however, the expression of c-Fos protein was delayed, rising above basal at 2h, and remaining at a high level up to 4h. Thus the sustained nature of the response to high Glu is concurrent with the mRNA profile, although the delay in expression was not predicted.

c-Jun protein expression in response to 10 μM Glu in 2 DIV cells was strong for the first two hours and then had decreased by 4h. In contrast, the expression of c-Jun protein after stimulation with 250 μM Glu was delayed, reaching maximal levels at 1h and then decreasing rapidly. There is no apparent correspondence of these results with the c-*jun* mRNA results obtained. However, the regulation of c-Jun is complex, involving phosphorylation and negative auto-regulation (Angel *et al.*, 1988; Boyle *et al.*, 1991), and thus this investigation could be

expanded in further work to fully explain the Glu-induced expression of this immediate-early gene and its associated protein. In contrast with c-Jun, the protein expression and mRNA induction profiles of FosB correlated very well, with very low levels of mRNA and protein expressed after 10 μM Glu, but large levels of both expressed after a 250 μM Glu insult. As with c-Fos, the protein expression under these circumstances lagged behind the mRNA induction, which can possibly be accounted for by the time required for protein synthesis, although it would be expected that this would take less time than the discrepancy observed here.

The end-point of a stimulus-transcription coupling cascade is the regulation of the target gene, since the induction and expression of immediate-early genes is not an end in itself. Whilst the physiological target of, for example, the FosB/JunD heterodimer, activated following a toxic stimulation of 7 DIV cerebellar granule cells, is unknown, there are several genes whose expression is thought to be activated or repressed by various AP-1 transcription factor complexes (Sharp and Sagar, 1994). In addition, autoregulation of Fos and Jun family members by AP-1 containing the specific gene-encoded protein has been suggested (Angel *et al.*, 1988).

5.4 Final comments

Stimulus-transcription coupling within the mammalian system comprises a highly regulated and complex set of mechanisms. Receptor and ion channel distribution and composition varies at the cellular and sub-cellular level, and according to development. In addition, the route as well as the amount of Ca^{2+} ion influx determines the final outcome of the signalling pathway. Induction of the immediate-early gene, c-*fos*, has been implicated in a myriad of different organisms and cell types, with very different consequences depending on the circumstances of activation of expression. Thus the genes targeted by any ensuing AP-1 transcription factors differ depending on the particular cell type, the stimulus and the signalling mechanism activated by that stimulus.

The main conclusions from this study support the theory that FosB and c-Fos expression are induced by different stimuli, and that expression of FosB is possibly correlated with a high level of toxicity. Specifically, in this study it was found that the only possible dimer composed of

Fos and Jun proteins (i.e. excluding ATF/CREB interactions) was FosB/JunD, a heterodimer recently implicated in chronic electroconvulsive seizures in the rat frontal cortex (Hiroi *et al.*, 1998).

References

Abate, C., Luk, D., Gagne, E., Roeder, R.G. and Curran, T. (1990a) Fos and Jun cooperate in transcriptional regulation via heterologous activation domains. *Mol. Cell. Biol.* **10**: 5532-5535.

Abate, C., Luk, D., Gentz, R., Rauscher, F.J.III and Curran, T. (1990b) Expression and purification of the leucine zipper and DNA-binding domains of Fos and Jun: both Fos and Jun contact DNA directly. *Proc. Natl. Acad. Sci. USA* **87**: 1032-1036.

Abe, T., Sugihara, H., Nawa, H., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1992) Molecular characterisation of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca^{2+} signal transduction. *J. Biol. Chem.* **267**: 13361-13368.

Agre, P., Johnson, P.F. and McKnight, S.L. (1989) Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. *Science* **246**: 922-926.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987) Phorbol ester-inducible genes contain a common *cis* element recognised by a TPA-modulated *trans* -acting factor. *Cell* **49**: 729-739.

Angel, P., Hattori, K., Smeal, T and Karin, M. (1988) The *jun* proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* **55**: 875-885.

Angel, P. and Karin, M. (1991) The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochim. Biophys. Acta* **1072**: 129-157.

Ankarcrona, M., Dypbukt, J.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S.A. and Nicotera, P. (1995) Glutamate-induced neuronal death: A succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15**: 961-973.

Aronheim, A., Zandi, E., Hennemann, H., Elledge, S.J. and Karin, M. (1997) Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. *Mol. Cell. Biol.* **17**: 3094-3102.

Bading, H., Ginty, D.D. and Greenberg, M.E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signalling pathways. *Science* **260**: 181-186.

Bading, H., Segal., M.M., Sucher, N.J., Dudek, H., Lipton, S.A. and Greenberg, M.E. (1995) *N*-Methyl-D-aspartate receptors are critical for mediating the effects of glutamate on intracellular calcium concentration and immediate-early gene expression in cultured hippocampal neurons. *Neuroscience* **64**: 653-664.

Balázs, R., Jørgensen, O.S. and Hack, N. (1988a) *N* -Methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. *Neuroscience* **27**: 437-451.

Balázs, R., Gallo, V. and Kingsbury, A. (1988b) Effect of depolarisation on the maturation of cerebellar granule cells in culture. *Dev. Brain Res.* **40**: 269-276.

Balázs, R., Hack, N. and Jørgensen, S.O. (1988c) Stimulation of the *N* -methyl-D-aspartate receptor has a trophic effect on differentiating cerebellar granule cells. *Neurosci. Lett.* **87**: 80-86.

Balázs, R., Hack, N., Jørgensen, O.S. and Cotman, C.W. (1989) *N*-Methyl-D-aspartate promotes the survival of cerebellar granule cells: pharmacological characterization. *Neurosci. Lett.* **101**: 2441-246.

Ball, A.R. Jr., Bos, T.J., Löliger, C., Nagata, L.P., Nishimura, T., Su, H., Tsuchie, T. and Vogt, P.K. (1988) jun: oncogene and transcriptional regulator. *Cold Spring Harbor Symposia on Quantitative Biology* **53**: 687-693.

Bar-Peled, O., Ben-Hur, H., Beigon, A., Groner, Y., Dewhurst, S., Furuta, A. and Rothstein, J.D. (1997) Distribution of glutamate transporter subtypes during human brain development. *J. Neurochem.* **69**: 2571-2580.

Bartel, D.P., Sheng, M., Lau, L.F. and Greenberg, M.E. (1989) Growth factors and membrane depolarisation activate distinct programs of early response gene expression: dissociation of *fos* and *jun* induction. *Genes and Dev.* **3**: 304-313.

Barthel, F., Remy, J-S., Loeffler, J-Ph. and Behr, J-P. (1993) Gene transfer optimization with lipospermine-coated DNA. *DNA Cell. Biol.* **12**: 553-560.

Barthel, F. and Loeffler, J-Ph. (1995) β_2-adrenoreceptors stimulate c-*fos* transcription through multiple cyclic AMP- and Ca^{2+}-responsive elements in cerebellar granule neurons. *J. Neurochem* . **64**: 41-51.

Barthel, F., Boutillier, A-L., Trouslard, J. and Loeffler, J-Ph. (1996) Fine tuning of calcium entry into neurons regulates adenosine 3',5'-monophosphate-dependent transcription by several distinct mechanisms. *Neuroscience* **70**: 1053-1065.

Bazán, N.G. (1970) Effects of ischaemia and electroconvulsive shock on free fatty acid pool in the brain. *Biochim. Biophys. Acta* **218**: 1-10.

Bazán, N.G. (1989) Arachidonic acid in the modulation of excitable membrane function and at the onset of brain damage. *Ann. N.Y. Acad. Sci.* **559**: 1-16.

Belcher, S.M. and Howe, J.R. (1997) Characterisation of RNA editing of the glutamate-receptor subunits GluR5 and GluR6 in granule cells during cerebellar development. *Mol. Brain Res.* **52**: 130-138.

Bishop, J.M. (1985) Viral oncogenes. *Cell* **42**: 23-38.

Bito, H., Deisseroth, K. and Tsien, R.W. (1997) Ca^{2+}-dependent regulation in neuronal gene expression. *Curr. Opin. Neurobiol.* **7**: 419-429.

Blitzblau, R., Gupta, S., Djali, S., Robinson, M.B. and Rosenberg, P.A. (1996) The glutamate transport inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate indirectly evokes NMDA receptor mediated neurotoxicity in rat cortical cultures. *Eur. J. Neurosci.*. **8**: 1840-1852.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P. and Lipton S.A. (1995) Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. USA* **92**: 7162-7166.

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B. and Behr, J-P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo* : Polyethylenimine. *Proc. Natl. Acad. Sci. USA* **92**: 7297-7301.

Boyle, W.J., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M. and Hunter, T. (1991) Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell* **64**: 573-584.

Burgoyne, R.D. and Cambray-Deakin, M.A. (1988) The cellular neurobiology of neuronal development: the cerebellar granule cell. *Brain Res. Rev.* **13**: 77-101.

Burnashev, N., Monyer, H., Seeburg, P.H. and Sakmann, B. (1992) Divalent cation permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* **8**: 189-198.

Carine, K. and Hudig, D. (1984) Assessment of a role for phospholipase A_2 and arachidonic acid metabolism in human lymphocyte natural cytotoxicity. *Cell. Immunol.* **87**: 270-283.

Carter, D.A. (1992) Neurotransmitter-stimulated immediate-early gene responses are organised through differential postsynaptic receptor mechanisms. *Mol. Brain Res.* **16**: 111-118.

Castellazzi, M., Spyrou, G., La Vista, N., Dangy, J-P., Piu, F., Yaniv, M. and Brun, G. (1991) Overexpression of c-*jun*, *jun*B or *jun*D affects cell growth differently. *Proc. Natl. Acad. Sci. USA* **88**: 8890-8894.

Chang, J., Skowronek, M.D., Cherney, M.L. and Lewis, A.J. (1984) Differential effects of putative lipoxygenase inhibitors on arachidonic acid metabolism in cell-free and intact cell preparations. *Inflammation* **8**: 143-155.

Chang, J.Y. and Wang, J-Z. (1997) Morphological and biochemical changes during programmed cell death of rat cerebellar granule cells. *Neurochem. Res.* **22**: 43-48.

Chaudhry, F.A., Lehre, K.P., van Lookeren Campagne, M., Ottersen, O.P., Danbolt, N.C. and Storm-Mathisen, J. (1995) Glutamate transporters in glial plasma membranes: highly differentiated localisations revealed by quantitative ultrastructural immunocytochemistry. *Neuron* **15**: 711-720.

Chen, Q., Harris, C., Brown, C.S., Howe, A., Surmeier, D.J. and Reiner, A. (1995a) Glutamate-mediated excitotoxic death of cultured striatal neurons is mediated by non-NMDA receptors. *Exp. Neurol.* **136**: 212-224.

Chen, S-C., Curran, T. and Morgan, J.I. (1995b) Apoptosis in the nervous system: New revelations. *J. Clin. Path.* **48**: 7-12.

Chen, R-H., Juo, P.C-H., Curran, T. and Blenis, J. (1996) Phosphorylation of c-Fos at the C-terminus enhances its transforming activity. *Oncogene* **12**: 1493-1502.

Cheung, N.S., Carroll, F.Y., Larm, J.A., Beart, P.M. and Giardina, S.F. (1998) Kainate-induced apoptosis correlates with c-*Jun* activation in cultured cerebellar granule cells. *J. Neurosci. Res.* **52**: 69-82.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T. and Karin M. (1988) The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* **54**: 541-552.

Chiu, R., Angel, P. and Karin, M. (1989) JunB differs in its biological properties from, and is a negative regulator of, c-Jun. *Cell* **59**: 979-986.

Choi, D.W., Maulucci-Gedde, M. and Kriegstein, A.R. (1987) Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* **7**: 357-368.

Choi, D.W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**: 623-634.

Choi, D.W. (1992) Excitotoxic cell death. *J. Neurobiol.* **23**: 1261-1276.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**: 156-159.

Cohen, D.R. and Curran, T. (1988) *fra-1* : a serum-inducible, cellular immediate-early gene that encodes a Fos-related antigen. *Mol. Cell. Biol.* **8**: 2063-2069.

Cohen, D.R., Ferreira, P.C.P., Gentz, R., Franza, B.R.Jr. and Curran, T. (1989) The product of a *fos*-related gene, *fra*-1, binds cooperatively to the AP-1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes. *Genes and Dev.* **3**: 173-184.

Colotta, F., Polentarutti, N., Sirone, M. and Mantovani, A. (1992) Expression and involvement of c-*fos* and c-*jun* proto-oncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines. *J. Biol. Chem.* **267**: 18278-18283.

Copani, A., Bruno, V.M.G., Barresi, V., Battaglia, G., Condorelli, D.F. and Nicoletti, F. (1995) Activation of metabotropic glutamate receptors prevents neuronal apoptosis in culture. *J. Neurochem.* **64**: 101-108.

Courtney, M.J., Lambert, J.J. and Nicholls, D.G. (1990) The interactions between plasma membrane depolarisation and glutamate receptors action in the regulation of cytoplasmic free calcium in cultured cerebellar granule cells. *J. Neurosci.* **10**: 3873-3879.

Coyle, J.T. and Puttfarcken, P. (1993) Oxidative stress, glutamate and neurodegenerative disorders. *Science* **262**: 689-695.

Curran, T. and Teich, N.M. (1982) Candidate product of the FBJ murine osteosarcoma virus oncogene: characterisation of a 55,000-Dalton phosphoprotein. *J. Virol.* **42**: 114-122.

Deng, T. and Karin, M. (1994) c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. *Nature* **371**: 171-175.

Dessi, F., Charriaut-Marlangue, C., Khrestchatisky, M. and Ben-Ari, Y. (1993) Glutamate-induced neuronal death is not a programmed cell death in cerebellar culture. *J. Neurochem.* **60**: 1953-1955.

Dessi, F., Charriaut-Marlangue, C. and Ben-Ari, Y. (1994) Glutamate-induced neuronal death in cerebellar culture is mediated by two distinct components: A sodium-chloride component and a calcium component. *Brain Res.* **650**: 49-55.

Didier, M., Roux, P., Piechaczyk, M., Verrier, B., Bockaert, J. and Pin, J-Ph. (1989) Cerebellar granule cell survival and maturation induced by K^+ and NMDA correlate with c-*fos* proto-oncogene expression. *Neurosci. Lett.* **107**: 55-62.

Didier, M., Roux, P., Piechaczyk, M., Mangeat, P., Devilliers, G., Bockaert, J. and Pin, J-Ph. (1992) Long-term expression of the c-*fos* protein during the *in vitro* differentiation of cerebellar granule cells induced by potassium or NMDA. *Mol. Brain Res.* **12**: 249-258.

Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nuc. Acids Res.* **11**: 1475-1489.

D'Mello, S.R., Galli, C., Ciotti, T. and Calissano, P. (1993) Induction of apoptosis in cerebellar granule neurons by low potassium: Inhibition of death by insulin-like growth factor I and cAMP. *Proc. Natl. Acad. Sci. USA* **90**: 10989-10993.

Dobrzanski, P., Noguchi, T., Kovary, K., Rizzo, C.A., Lazo, P.S. and Bravo, R. (1991) Both products of the fosB gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. *Mol. Cell. Biol.* **11**: 5470-5478.

Dony, C. and Gruss, P. (1987) Proto-oncogene c-*fos* expression in growth regions of fetal bone and mesodermal web tissue. *Nature* **328**: 711-714.

Dragunow, M., Young, D., Hughes, P., MacGibbon, G., Lawlor, P., Singleton, K., Sirimanne, E., Beilharz, E. and Gluckman, P. (1993) Is c-Jun involved in nerve cell death following status epilepticus and hypoxic-ischaemic brain injury? *Mol. Brain Res.* **18**: 347-352.

Dragunow, M. and Preston, K. (1995) The role of inducible transcription factors in apoptotic nerve cell death. *Brain Res. Rev.* **21**: 1-28.

Drejer, J., Honore, T., Meier, E. and Schousboe, A. (1986) Pharmacologically distinct glutamate receptors on cerebellar granule cells. *Life Sci.* **38**: 2077-2085.

Dunah, A.W., Yasuda, R.P., Wang, Y., Luo, J., Dávila-García, M.I., Gbadegesin, M., Vicini, S. and Wolfe, B.B. (1996) Regional and ontogenic expression of the NMDA receptor

subunit NR2D protein in rat brain using a subunit-specific antibody. *J. Neurochem.* **67**: 2335-2345.

Edwards, S.A., Rundell, A.Y.K. and Adamson, A.D. (1988) Expression of c-*fos* antisense RNA inhibits differentiation of F9 cells to parietal endoderm. *Dev. Biol.* **129**: 91-102.

Edwards, D.R. (1994) Cell signalling and the control of gene transcription. *Trends Pharmacol. Sci.* **15**: 239-244.

Evans, G.J.O. and Pocock, J.M. (1999) Modulation of neurotransmitter release by dihydropyridine-sensitive calcium channels involves tyrosine phosphorylation. *Eur. J. Neurosci.* **11**: 279-292.

Ferhat, L., Khrestchatisky, M., Roisin, M-P. and Barbin, G. (1993) Basic fibroblast growth factor-induced increase in *zif*/268 and c-*fos* mRNA levels is Ca^{2+} dependent in primary cultures of hippocampal neurons. *J. Neurochem.* **61**: 1105-1112.

Ferreira, S.H. and Vane, J.R. (1974) New aspects of the mode of action of nonsteroid anti-inflammatory drugs. *Ann. Rev. Pharmacol.* **14**: 57-73.

Finkbeiner, S. and Greenberg, M.E. (1998) Ca^{2+} channel-regulated neuronal gene expression. *J. Neurobiol.* **37**: 171-189.

Finkel, M.P., Biskis, B.O. and Jinkins, P.B. (1966) Virus induction of osteosarcomas in mice. *Science* **151**: 698-700.

Frandsen, A., Drejer, J. and Schousboe, A. (1989) Direct evidence that excitotoxicity in cultured neurons is mediated via *N* -methyl-D-aspartate (NMDA) as well as non-NMDA receptors. *J. Neurochem.* **53**: 297-299.

Frandsen, A. and Schousboe, A. (1990) Development of excitatory amino acid induced cytotoxicity in cultured neurons. *Int. J. Dev. Neurosci.* **8**: 209-216.

Frandsen, A. and Schousboe, A. (1993) Excitatory amino acid-mediated cytotoxicity and calcium homeostasis in cultured neurons. *J. Neurochem.* **60**: 1202-1211.

Fried, M. and Crothers, D.M. (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nuc. Acids Res.* **11**: 1475-1489.

Furuta, A., Rothstein, J.D. and Martin, L.J. (1997) Glutamate transporter protein subtypes are expressed differentially during rat CNS development. *J. Neurosci.* **17**: 8363-8375.

Gallin, W.J. and Greenberg, M.E. (1995) Calcium regulation of gene expression in neurons: the mode of entry matters. *Curr. Opin. Neurobiol.* **5**: 367-374.

Gallo, V., Ciotti, M.T., Coletti, A., Aliosi, F. and Levi, G. (1982) Selective release of glutamate from cerebellar granule cells differentiating in culture. *Proc. Natl. Acad. Sci. USA* **79**: 7919-7923.

Gallo, V., Kingsbury, A., Balázs, R. and Jørgenson, O.S. (1987) The role of depolarisation in the survival and differentiation of cerebellar granule cells in culture. *J. Neurosci.* **7**: 2203-2213.

Garner, M.M. and Revzin, A. (1981) A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Eschericia coli* lactose operon regulatory system. *Nuc. Acids Res.* **9**: 3047-3060.

Garthwaite, G. and Garthwaite, J. (1986a) *In vitro* neurotoxicity of excitatory acid analogues during cerebellar development. *Neuroscience* **17**: 755-767.

Garthwaite, G. and Garthwaite, J. (1986b) Neurotoxicity of excitatory amino acid receptor agonists in rat cerebellar slices: dependence on calcium concentration. *Neurosci. Lett.* **66**: 193-198.

Garthwaite, G. and Garthwaite, J. (1989) Neurotoxicity of excitatory amino acid receptor agonists in young rat hippocampal slices. *J. Neurosci. Met.* **29**: 33-42.

Gass, P. and Herdegen, T. (1995) Neuronal expression of AP-1 proteins in excitotoxic neurodegenerative disorders following nerve fibre lesions. *Prog. Neurobiol.* **47**: 257-290.

Gentz, R., Rauscher, F.J. III, Abate, C. and Curran, T. (1989) Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. *Science* **243**: 1695-1699.

Ghosh, A., Ginty, D.D., Bading, H. and Greenberg, M.E. (1994) Calcium regulation of gene expression in neuronal cells. *J. Neurobiol.* **25**: 294-303.

Ghosh, A. and Greenberg, M.E. (1995) Calcium signalling in neurons: molecular mechanisms and cellular consequences. *Science* **268**: 239-247.

Gillardon, F., Eschenfelder, C., Uhlmann, E., Hartschuh, W. and Zimmermann, M. (1994) Differential regulation of c-*fos*, *fos*B, c-*jun*, *jun*B, *bcl* -2 and *bax* expression in rat skin following single or chronic ultraviolet irradiation and *in vivo* modulation by antisense oligodeoxynucleotide superfusion. *Oncogene* **9**: 3219-3225.

Gillardon, F., Bäurle, J, Grüsser-Cornehls, U. and Zimmermann, M. (1995) DNA fragmentation and activation of c-Jun in the cerebellum of mutant mice (weaver, Purkinje cell degeneration). *NeuroReport* **6**: 1766-1768.

Gille, H., Sharrock, A.D. and Shaw, P.E. (1992) Phosphorylation of transcription factor $p62^{TCF}$ by MAP kinase stimulates ternary complex formation at c-*fos* promoter. *Nature* **358**: 414-417.

Gilman, M.Z. (1988) The c-*fos* serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. *Genes and Dev.* **2**: 394-402.

Ginty, D.D. (1997) Calcium regulation of gene expression: Isn't that spatial? *Neuron* **18**: 183-186.

Ginty, D.D., Bading, H. and Greenberg, M.E. (1992) Trans-synaptic regulation of gene expression. *Curr. Opin. Neurobiol.* **2**: 312-316.

Gonzales, G.A. and Montminy, M.R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**: 675-680.

Gorman, A.M., Scott, M.P., Rumsby, P.C., Mercdith, C. and Griffiths, R. (1995) Excitatory amino acid-induced cytotoxicity in primary cultures of mouse cerebellar granule cells

correlates with elevated, sustained c-*fos* proto-oncogene expression. *Neurosci. Lett.* **191**: 116-120.

Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**: 1044-1051.

Grayson, D.R., Szekely, A.M. and Costa, E. (1990) Glutamate-induced gene expression in primary cerebellar neurons. In: *Neurotoxicity of Excitatory Amino Acids*, Ed: A. Guidotti, Raven Press, Ltd., New York, pp 185-202.

Greenamyre, J.T. and Porter, R.H.P. (1994) Anatomy and physiology of glutamate in the CNS. *Neurology* **44** (Suppl. 8) S7-S11.

Greenberg, M.E. and Ziff, E.B. (1984) Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. *Nature* **311**: 433-438.

Greenberg, M.E., Ziff, E.B. and Greene, L.A. (1986) Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. *Science* **234**: 80-83.

Griffiths, R., Grieve, A., Frandsen, A. and Schousboe, A. (1994) Mixed actions of TMB-8 as a Ca^{2+} antagonist in cultured mouse cortical neurones. *NeuroReport* **5**: 605-608.

Griffiths, R., Malcolm, C., Ritchie, L., Frandsen, A., Schousboe, A., Scott, M., Rumsby, P. and Meredith, C. (1997) Association of c-*fos* mRNA expression and excitotoxicity in primary cultures of mouse neocortical and cerebellar neurons. *J. Neurosci. Res.* **48**: 533-542.

Griffiths, R., Ritchie, L., Lidwell, K., Grieve, A., Malcolm, C.S., Scott, M. and Meredith, C. (1998) Calcium influx via L-type voltage-gated channels mediates the delayed, elevated increases in steady-state c-*fos* mRNA levels in cerebellar granule cells exposed to excitotoxic levels of glutamate. *J. Neurosci. Res.* **52**: 641-652.

Hai, T. and Curran, T. (1991) Cross-family dimerisation of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. USA* **88**: 3720-3724.

Halazonetis, T.D., Georgopoulos, K., Greenberg, M.E. and Leder, P. (1988) c-Jun dimerises with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* **55**: 917-924.

Haliday, E.M., Ramesha, C.S. and Ringold, G. (1991) TNF induces c-*fos* via a novel pathway requiring conversion of arachidonic acid to a lipoxygenase metabolite. *EMBO J.* **10**: 109-115.

Ham, J., Babij, C., Whitfield, J., Pfarr, C.M., Lallemand, D., Yaniv, M. and Rubin, L.L. (1995) A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* **14**: 927-939.

Harrold, J., Ritchie, J., Nicholls, D., Smith, W., Bowman, D. and Pocock, J. (1997) The development of Ca^{2+} channel responses and their coupling to exocytosis in cultured cerebellar granule cells. *Neuroscience* **77**: 683-694.

Hassanain, H.H., Dai, W. and Gupta, S.L. (1993) Enhanced gel mobility shift assay for DNA-binding factors. *Analytical Biochemistry* **213**: 162-167.

Henley, J.M., Bruton, R. and Richmond, S.A. (1998) Metabotropic glutamate receptors. In *Amino Acid Neurotransmission*, F.A. Stephenson and A.J. Turner (Eds) Portland Press, London, pp 113-128.

Herdegen, T., Fiallos-Estrada, C.E., Schmid, W., Bravo, R. and Zimmermann, M. (1992) The transcription factors c-JUN, JUND and CREB, but not FOS and KROX-24, are differentially regulated in axotomised neurons following transection of rat sciatic nerve. *Mol. Brain Res.* **14**: 155-165.

Herdegen, T., Brecht, S., Kummer, W., Mayer, B., Leah, J., Bravo, R. and Zimmermann, M. (1993) Persisting expression of JUN and KROX transcription factors and increase of nitric oxide synthase immunoreactivity in rat central neurons following axotomy. *J. Neurosci.* **13**: 4130-4146.

Herdegen, T. (1996) Jun, Fos and CREB/ATF transcription factors in the brain: Control of gene expression under normal and pathophysiological conditions. *Neuroscientist* **2**: 153-161.

Herdegen, T., Skene, P. and Bähr, M. (1997) The c-*jun* transcription factor - bipotential mediator of neuronal death, survival and regeneration. Trends *Neurosci.* **20**: 227-231.

Hertz, L., Yu, A.C.H. and Schousboe, A. (1992) Uptake and metabolism of malate in neurons and astrocytes in primary cultures. *J. Neurosci. Res.* **33**: 289-296.

Hill, C.S. and Treisman, R. (1995) Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* **80**: 199-211.

Hirai, S-I., Ryseck, R-P., Mechta, F., Bravo, R. and Yaniv, M. (1989) Characterisation of *jun*D: a new member of the *jun* proto-oncogene family. *EMBO J.* **8**: 1433-1439.

Hiroi, N., Marek, G.J., Brown, J.R., Ye, H., Saudou, F., Vaidya, V.A., Duman, R.S., Greenberg, M.E. and Nestler, E.J. (1998) Essential role of the *fos*B gene in molecular, cellular and behavioural actions of chronic electroconvulsive seizures. *J. Neurosci.* **18**: 6952-6962.

Hollen, K.M., Nakabeppu, Y. and Davies, S.W. (1997) Changes in expression of ΔFosB and the Fos family proteins following NMDA receptor activation in the rat striatum. *Mol. Brain Res.* **47**: 31-43.

Hollmann, M., Hartley, M. and Heinemann, S. (1991) Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* **252**: 851-853.

Hollmann, M. and Heinemann, S.F. (1993) Cloned glutamate receptors. *Ann. Rev. Neurosci.* **17**: 37-108.

Holt, J.T., Venkat Gopal, T., Moulton, A.D. and Nienhuis, A.W. (1986) Inducible production of c-*fos* antisense RNA inhibits 3T3 cell proliferation. *Proc. Natl. Acad. Sci. USA* **83**: 4794-4798.

Hong, S-C.L. and Levine, L. (1976) Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory corticosteroids. *Proc. Natl. Acad. Sci. USA* **73**: 1730-1734.

Hope, B.T., Kelz, M.B., Duman, R.S. and Nestler, E.J. (1994) Chronic electroconvulsive seizure (ECS) treatment results in expression of a long-lasting AP-1 complex in brain with altered composition and characteristics. *J. Neurosci.* **14**: 4318-4328.

Hou, Y-N., Cebers, G., Terenius, L. and Liljequist, S. (1997) Characterization of NMDA- and AMPA-induced enhancement of AP-1 DNA binding activity in rat cerebellar granule cells. *Brain Res.* **754**: 79-87.

Hughes, P. and Dragunow, M. (1995) Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. *Pharmacol. Rev.* **47**: 133-178.

Hume, R.I., Dingledine, R. and Heinemann, S. (1991) Identification of a site in glutamate receptor subunits that controls calcium permeability. *Science* **253**: 1028-1031.

Humphries, S.E., Whitall, R., Minty, A., Bucking, M. and Williamson, R. (1981) There are approximately 20 actin genes in the human genome. *Nuc. Acids Res.* **9**: 4895-4908.

Imler, J.L., Schatz, C., Wasylyk, C., Chatton, B. and Wasylyk, B. (1988) A Harvey-ras responsive transcription element is also responsive to a tumour-promoter and to serum. *Nature* **332**: 275-278.

Jensen, J. B., Schousboe, A. and Pickering, D.S. (1998a) AMPA receptor mediated excitotoxicity in neocortical neurons is developmentally regulated and dependent upon receptor desensitisation. *Neurochem. Int.* **32**: 505-513.

Jensen, J. B., Schousboe, A. and Pickering, D.S. (1998b) Development of calcium-permeable a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in cultured neocortical neurons visualised by cobalt staining. *J. Neurosci. Res.* **54**: 273-281.

Jensen, K.F., Ohmstede, C-A., Fisher, R.S. and Sahyoun, N. (1991) Nuclear and axonal localisation of Ca^{2+}/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex. *Proc. Natl. Acad. Sci. USA* **88**: 2850-2853.

Jensen, J.B., Schousboe, A. and Pickering, D.S. (1999) Role of desensitisation and subunit expression for kainate receptor-mediated neurotoxicity in murine neocortical cultures. *J. Neurosci. Res.* **55**: 208-217.

Juurlink, B.H.J. and Hertz, L. (1993) Ischaemia-induced death of astrocytes and neurons in primary culture - pitfalls in quantifying neuronal cell death. *Dev. Brain Res.* **71**: 239-246.

Kacich, R.L., Williams, L.T. and Coughlin, S.R. (1988) Arachidonic acid and cyclic adenosine monophosphate stimulation of c-*fos* expression by a pathway independent of phorbol ester-sensitive protein kinase C. *Mol. Endocrinol.* **2**: 73-77.

Karin, M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* **270**: 16483-16486.

Karin, M. and Hunter, T. (1995) Transcriptional control by protein phosphorylation: Signal transmission from the cell surface to the nucleus. *Curr. Biol.* **5**: 747-757.

Kardos, J., Elster, L., Damgaard, I., Krogsgaard-Larsen, P. and Schousboe, A. (1995) Role of $GABA_B$ receptors in intracellular Ca^{2+} homeostasis and possible interaction between $GABA_A$ and $GABA_B$ receptors in regulation of transmitter release in cerebellar granule cells. *J. Neurosci. Res.* **39**:646-655.

Kasof, G.M., Mahanty, N.K., Miller, L.D.P., Curran, T., Connor, J.A. and Morgan, J.I. (1995) Spontaneous and evoked glutamate signalling influences Fos-LacZ expression and

pyramidal cell death in hippocampal slice cultures from transgenic rats. *Mol. Brain Res.* **34**: 197-208.

Katsuki, H., Akino, N., Okuda, S. and Saito, H. (1995) Antioxidants, but not cAMP or high K^+, prevent arachidonic acid toxicity on neuronal cultures. *NeuroReport* **6**: 1101-1104.

Kerppola, T.K. and Curran, T. (1991) Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: Implications for transcription factor cooperativity. *Cell* **66**: 317-326.

Kerppola, T.K. and Curran, T. (1994) Maf and Nrl can bind to AP-1 sites and form heterodimers with Fos and Jun. *Oncogene* **9**: 675-684.

Koh, J-Y., Goldberg, M.P., Hartley, D.M. and Choi, D.W. (1990) Non-NMDA receptor-mediated neurotoxicity in cortical culture. *J. Neurosci.* **10**: 693-705.

Komuro, H. and Rakic, P. (1993) Modulation of neuronal migration by NMDA receptors. *Science* **260**: 95-97.

Komuro, H. and Rakic, P. (1996) Intracellular Ca^{2+} fluctuations modulate the rate of neuronal migration. *Neuron* **17**: 275-285.

König, H., Ponta, H., Rahmsdorf, U., Büscher, M., Schöntal, A., Rahmsdorf, H.J. and Herrlich, P. (1989) Autoregulation of *fos*: the dyad symmetry element as the major target of repression. *EMBO J.* **8**: 2559-2566.

Kouzarides, T. and Ziff, E. (1988) The role of the leucine zipper in the fos-jun interaction. *Nature* **336**: 646-651.

Kovács, K.J. (1998) c-Fos as a transcription factor: a stressful (re)view from a functional map. *Neurochem. Int.* **33**: 287-297.

Kovary, K. and Bravo, R. (1991) Expression of different Jun and Fos proteins during the G_0-to-G_1 transition in mouse fibroblasts: In vitro and in vivo associations. *Mol. Cell. Biol.* **11**: 2451-2459.

Kure, S., Tominaga, T., Yoshimoto, T., Tada, K. and Narisawa, K. (1991) Glutamate triggers internucleosomal DNA cleavage to neuronal cells. *Biochem. Biophys. Res. Comm.* **179**: 39-45.

Lafon-Cazal, M., Fagni, L., Guiraud, M-J., Mary, S., Lerner-Natoli, M., Pin, J-P., Shigemoto, R. and Bockaert, J. (1999) mGluR7-like metabotropic glutamate receptors inhibit NMDA-mediated excitotoxicity in cultured mouse cerebellar granule neurons. *Eur. J. Neurosci.* **11**: 663-672.

Landschulz, W.H., Johnson, P.F. and McKnight, S. L. (1988) The Leucine Zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science* **240**: 1759-1764.

Larm, J.A., Cheung, N.S. and Beart, P.M. (1997) Apoptosis induced via AMPA-selective glutamate receptors in cultured murine cortical neurons. *J. Neurochem.* **69**: 617-622.

Lazo, P.S., Dorfman, K., Noguchi, T., Mattéi, M-G. and Bravo, R. (1991) Structure and mapping of the fosB gene. FosB downregulates the activity of the fosB promoter. *Nuc. Acids Res.* **20**: 343-350.

Lee, W., Mitchell, P. and Tjian, R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49**: 741-752.

Lehmann, A. (1987) Pharmacological protection against the toxicity of *N* -methyl-D-aspartate in immature rat cerebellar slices. *Neuropharmacology* **26**: 1751-1761.

Lehre, K.P., Levy, L.M., Ottersen, O.P., Storm-Mathisen, J. and Danbolt, N.C. (1995) Differential expression of two glutamate transporters in rat brain: quantitative and immunocytochemical observations. *J. Neurosci.* **15**: 1835-1853.

Lerea, L.S. and McNamara, J.O. (1993) Ionotropic glutamate receptor subtypes activate c-*fos* transcription by distinct calcium-requiring intracellular signalling pathways. *Neuron* **10**: 31-41.

Lin, W.W., Wang, C.W. and Chuang, D-M. (1997) Effects of depolarisation and NMDA antagonists on the survival of cerebellar granule cells: A pivotal role for protein kinase C isoforms. *J. Neurochem.* **68**: 2577-2586.

Lipton, S.A. (1992) Models of neuronal injury in AIDS: another role for the NMDA receptor? *Trends Neurosci.* **15**: 75-79.

Lipton, S.A. and Rosenberg, P.A. (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *In: Mechanisms of Disease (Ed. F.H. Epstein)* in *The New England Journal of Medicine* **330**: 613-622.

Liu, H-N. and Almazan, G. (1995) Glutamate induces c-*fos* proto-oncogene expression and inhibits proliferation in oligodendrocyte progenitors: receptor characterization. *Eur. J. Neurosci.* **7**: 2355-2363.

Loeffler, J-Ph., Barthel, F., Feltz, P., Behr, J-P., Sassone-Corsi, P. and Feltz, A. (1990) Lipopolyamine-mediated transfection allows gene expression studies in primary neuronal cells. *J. Neurochem.* **54**: 1812-1815.

Lu, Y.M., Yin, H.Z., Chiang, J. and Weiss, J.H. (1996) Ca^{2+}-permeable AMPA/kainate and NMDA channels: High rate of Ca^{2+} influx underlies potent induction of injury. *J. Neurosci.* **16**: 5457-5465.

Maki, Y., Bos, T.J., Davis, C., Starbuck, M. and Vogt, P.K. (1987) Avian sarcoma virus 17 carries the *jun* oncogene. *Proc. Natl. Acad. Sci. USA* **84**: 2848-2852.

Malcolm, C.S., Ritchie, L. Grieve, A. and Griffiths, R. (1996a) Glutamate toxicity in primary cerebellar cultures from mouse brain is unaffected by changes in cGMP levels. *NeuroReport* **7**: 1650-1654.

Malcolm, C.S., Ritchie, L. Grieve, A. and Griffiths, R. (1996b) A prototypic intracellular calcium antagonist, TMB-8, protects cultured cerebellar granule cells against the delayed, calcium-dependent component of glutamate neurotoxicity. *J. Neurochem.* **66**: 2350-2360.

Malcolm, C., Griffiths, R., Rumsby, P. and Meredith, C. (1997) Differential profiles of c-*fos* expression within primary cultures of mouse cerebellar granule cells stimulated with the excitatory amino acid L-glutamate: Correlation with excitotoxic outcome and derivation of a predictive index. *Toxicology in Vitro* **11**: 505-507.

Marchetti, C. and Usai, C. (1996) High affinity block by nimodipine of the internal calcium elevation in chronically depolarised rat cerebellar granule neurons. *Neurosci. Lett.* **207**: 77-80.

Marini, A.M. and Paul, S.M. (1992) *N*-methyl-D-aspartate receptor-mediated neuroprotection in cerebellar granule cells requires new RNA and protein synthesis. *Proc. Natl. Acad. Sci. USA* **89**: 6555-6559.

Marret, S., Gressens, P. and Evrard, P. (1996) Arrest of neuronal migration by excitatory amino acids in hamster developing brain. *Proc. Natl. Acad. Sci. USA* **93**: 15463-15468.

Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R. and Nakanishi, S. (1991) Sequence and expression of a metabotropic glutamate receptor. *Nature* **349**: 760-765.

Mayer, M.L. and Westbrook, G.L. (1987) The physiology of excitatory amino acids in the vertebrate central nervous system. *Prog. Neurobiol.* **28**: 197-276.

Mayer, M.L., MacDermott, A.B., Westbrook, G.L., Smith, S.J. and Barker, J.L. (1987) Agonist- and voltage-gated calcium entry in cultured mouse spinal cord neurons under voltage clamp measured using arsenazo III. *J. Neurosci.* **7**: 3230-3244.

Meredith, C., Scott, M., Rumsby, P., Frandsen, A., Schousboe, A., Gorman, A. and Griffiths, R. (1996) Sustained c-*fos* expression is associated with excitotoxicity during the development of neuronal cells *in vitro* . *Biochem. Soc. Trans.* **24**: 6S.

Messer, A. (1977) The maintenance and identification of mouse cerebellar granule cells in monolayer culture. *Brain Res.* **130**: 1-12.

Metz, R., Kouzarides, T. and Bravo, R. (1994) A C-terminal domain in FosB, absent in FosB/SF and Fra-1, which is able to interact with the TATA binding protein, is required for altered cell growth. *EMBO J.* **13**: 3832-3842.

Michaelis, E.K. (1998) Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and ageing. *Prog. Neurobiol.* **54**: 369-405.

Miller, T.M. and Johnson, E.M. Jr. (1996) Metabolic and genetic analyses of apoptosis in potassium/serum-deprived rat cerebellar granule cells. *J. Neurosci.* **16**: 7487-7495.

Miyazawa, K., Iimori, Y., Makino, M., Mikami, T. and Miyasaka, K. (1985) Effects of some non-steroidal anti-inflammatory drugs and other agents on cyclooxygenase and lipoxygenase activities in some enzyme preparations. *Jap. J. Pharmacol.* **38**: 199-205.

Mody, I. and MacDonald, J.F. (1995) NMDA receptor-dependent excitotoxicity: The role of intracellular calcium release. *Trends Pharmacol. Sci.* **16**: 356-359.

Mohun, T., Garrett, N. and Treisman, R. (1987) *Xenopus* cytoskeletal actin and human c-*fos* gene promoters share a conserved protein-binding site. *EMBO J.* **6**: 667-673.

Monstein, H-J. and Folkesson, R. (1991) Phorbol 12-myristate-13-acetate (PMA) stimulates a differential expression of cholecystokinin (CCK) and c-*fos* mRNA in a human neuroblastoma line. *FEBS Lett.* **293**: 145-148.

Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. and Goodman, R.H. (1986) Identification of a cyclic AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83**: 6682-6686.

Morgan, J.I. and Curran, T. (1986) Role of ion flux in the control of c-*fos* expression. *Nature* **322**: 552-555.

Morgan, J.I. and Curran, T. (1995) The immediate-early gene response and neuronal death and regeneration. *Neuroscientist* **1**: 68-75.

Mosmann, T. (1983) Rapid colourimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Met.* **65**: 55-63.

Müller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984) Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. *Nature* **312**: 716-720.

Mumberg, D., Lucibello, F.C., Schuermann, M. and Müller, R. (1991) Alternative splicing of *fos*B transcripts results in differentially expressed mRNAs encoding functionally antagonistic proteins. *Genes and Dev.* **5**: 1212-1223.

Murphy, S.N., Thayer, S.A. and Miller, R.J. (1987) The effects of excitatory amino acids on intracellular calcium in single mouse striatal neurons *in vitro*. *J. Neurosci.* **7**: 4145-4158.

Murphy, T.H., Worley, P.F. and Baraban, J.M. (1991) L-type voltage-sensitive calcium channels mediate synaptic activation of immediate-early genes. *Neuron* **7**: 625-635.

Nakabeppu, Y., Ryder, K. and Nathans, D. (1988) DNA binding activities of three murine Jun proteins: Stimulation by Fos. *Cell* **55**: 907-915.

Nakabeppu, Y. and Nathans, D. (1989) The basic region of Fos mediates specific DNA binding. *EMBO J.* **8**: 3833-3841.

Nakabeppu, Y. and Nathans, D. (1991) A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. *Cell* **64**: 751-759.

Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* **1**: 841-845.

Nicholls, D.G. and Sanchez-Prieto, J. (1998) Neurotransmitter release mechanisms. In *Amino Acid Neurotransmission*, F.A. Stephenson and A.J. Turner (Eds) Portland Press, London, pp 1-23.

Nicoletti, F., Wroblewski, J.T., Fadda, E. and Costa, E. (1988) Pertussis toxin inhibits signal transduction at a specific metabotropic glutamate receptor in primary cultures of cerebellar granule cells. *Neuropharmacology* **27**: 551-556.

Nicotera, P., Bellomo, G. and Orrenius, S. (1992) Calcium-mediated mechanisms in chemically induced cell death. *Ann. Rev. Pharmacol. Toxicol.* **32**: 449-470.

Nicotera, P., Ankarcrona, M., Bonfoco, E., Orrenius, S. and Lipton, S.A. (1996) Neuronal apoptosis versus necrosis induced by glutamate or free radicals. *Apoptosis* **1**: 5-10.

Nishina, H., Sato, H., Suzuki, T., Sato, M. and Iba, H. (1990) Isolation and characterisation of *fra-2* , an additional member of the *fos* gene family. *Proc. Natl. Acad. Sci. USA* **87**: 3619-3623.

Norman, C. and Treisman, R. (1988) Analysis of serum response element function *in vitro. Cold Spring Harbour Symposia on Quantitative Biology* **53**: 719-726.

Ohmstede, C-A., Jensen, K.F. and Sahyoun, N. (1989) Ca^{2+}/calmodulin-dependent protein kinase enriched in cerebellar granule cells. Identification of a novel neuronal calmodulin-dependent protein kinase. *J. Biol. Chem.* **264**: 5866-5875.

Okuda, S., Saito, H. and Katsuki, H. (1994) Arachidonic acid: Toxic and trophic effects on cultured hippocampal neurons. *Neuroscience* **63**: 691-699.

Olney, J.W., Ho, O.L. and Rhee, V. (1971) Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system. *Exp. Brain. Res.* **14**: 61-76.

Parri, H.R. and Lansman, J.B. (1996) Multiple components of Ca^{2+} channel facilitation in cerebellar granule cells: Expression of facilitation during development in culture. *J. Neurosci.* **16**: 4890-4902.

Pennypacker, K.R., Thai, L., Hong, J-S. and McMillian, M.K. (1994) Prolonged expression of AP-1 transcription factors in the rat hippocampus after systemic kainate treatment. *J. Neurosci.* **14**: 3998-4006.

Pennypacker, K.R., Hudson, P.M., Hong, J-S. and McMillian, M.K. (1995) DNA binding activity of CREB transcription factors during ontogeny of the central nervous system. *Dev. Brain Res.* **86**: 242-249.

Portera-Cailliau, C., Price, D.L. and Martin, L.J. (1996) *N*-Methyl-D-aspartate receptor proteins NR2A and NR2B are differentially distributed in the developing rat central nervous system as revealed by subunit-specific antibodies. *J. Neurochem.* **66**: 692-700.

Promega (1996) Protocols and Applications Guide (Third Edition) ©1996 Promega Corporation

Pulverer, B.J., Kyriakis, J.M., Auruch, J., Nikolakaki, E. and Woodgett, J.R. (1991) Phosphorylation of c-jun mediated by MAP kinases. *Nature* **353**: 670-674.

Rauscher, F.J. III, Volalas, P.J., Franza, B.R. Jr. and Curran, T. (1988a) Fos and Jun bind cooperatively to the AP-1 site: Reconstitution in vitro. *Genes and Dev.* **2**: 1687-1699.

Rauscher, F.J. III, Cohen, D.R., Curran, T., Bos, T., Vogt, P.K., Bohmann, D., Tjian, R. and Franza, B.R. Jr. (1988b) Fos-associated protein p39 is the product of the *jun* proto-oncogene. *Science* **240**: 1010-1016.

Resink, A., Boel, G.J. and Balazs, R. (1992) Treatment with excitatory amino acids or high K^+ and NMDA receptors in cerebellar granule cells. *NeuroReport* **3**: 757-760.

Risse, G., Jooss, K., Neuberg, M., Brüller, H-J. and Müller, R. (1989) Asymmetrical recognition of the palindromic AP-1 binding site (TRE) by Fos protein complexes. *EMBO J.* **8**: 3825-3832.

Rosen, L.B., Ginty, D.D., Weber, M.J. and Greenberg, M.E. (1994) Membrane depolarisation and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron* **12**: 1207-1221.

Rosenberger, S.F., Finch, J.S., Gupta, A. and Bowden, G.T. (1999) Extracellular signal-regulated kinase 1/2-mediated phosphorylation of JunD and FosB is required for okadaic acid-induced activator protein 1 activation. *J. Biol. Chem.* **274**: 1124-1130.

Rosenthal, M.D., Vishwanath, B.S. and Franson, R.C. (1989) Effects of aristolochic acid on phospholipase A_2 activity and arachidonate metabolism of human neutrophils. *Biochim. Biophys. Acta* **1001**: 1-8.

Rossi, P., D'Angelo, E., Magistretti, J., Toselli, M. and Taglietti, V. (1994) Age-dependent expression of high-voltage activated calcium currents during cerebellar granule cell development in situ. *Pflügers Archiv: Eur. J. Physiol.* **429**: 107-116.

Rothstein, J.D., Martin, L., Levy, A.I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N. and Kuncl, R.W. (1994) Localisation of neuronal and glial transporters. *Neuron* **13**: 713-725.

Rothstein, J.D., van Kammen, M., Levy, A.I., Martin, L.J., and Kuncl, R.W. (1995) Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Ann. Neurol.* **38**: 73-84.

Rozek, D. and Pfeifer, G.P. (1993) In vivo protein-DNA interactions at the c-*jun* promoter: preformed complexes mediate the UV response. *Mol. Cell. Biol.* **13**: 5490-5499.

Rüther, U., Garber, C., Komitowski, D., Müller, R. and Wagner, E.F. (1987) Deregulated c-*fos* expression interferes with normal bone development in transgenic mice. *Nature* **325**: 412-416.

Ryder, K. and Nathans, D. (1988) Induction of proto-oncogene c-*jun* by serum growth factors. *Proc. Natl. Acad. Sci. USA* **85**: 8464-8467.

Ryder, K., Lau, L.F. and Nathans, D. (1988) A gene activated by growth factors is related to the oncogene v-*jun*. *Proc. Natl. Acad. Sci. USA* **85**: 1487-1491.

Sakurai, H., Kurusu, R., Sano, K., Tomofusa and Tsuda, M. (1992) Stimulation of cultured cerebellar granule cells via glutamate receptors induces TRE- and CRE-binding activities mediated by common DNA-binding complexes. *J. Neurochem.* **59**: 2067-2075.

Sasner, M. and Buonanno, A. (1996) Distinct *N*-methyl-D-aspartate receptor 2B subunit gene sequences confer neural and developmental specific expression. *J. Biol. Chem.* **271**: 21316-21322.

Sassone-Corsi, P., Sisson, J.C. and Verma, I.M. (1988a) Transcriptional autoregulation of the proto-oncogene *fos*. *Nature* **334**: 314-319.

Sassone-Corsi, P., Lamph, W.W., Kamps, M. and Verma, I.M. (1988b) fos-associated cellular p39 is related to nuclear transcription factor AP-1. *Cell* **54**: 553-560.

Sassone-Corsi, P., Visvader, J., Ferland, L., Mellon, P. and Verma, I.M. (1988c) Induction of proto-oncogene fos transcription through the adenylate cyclase pathway: characterisation of a cAMP-responsive element. *Genes and Dev.* **2**: 1529-1538.

Sassone-Corsi, P., Ransone, L.J., Lamph, W.W. and Verma, I.M. (1988d) Direct interaction between fos and jun nuclear oncoproteins: role of the 'leucine zipper' domain. *Nature* **336**: 692-695.

Sattler, R., Charlton, M.P., Hafner, M. and Tymianski, M. (1998) Distinct influx pathways, not calcium load, determine neuronal vulnerability to calcium neurotoxicity. *J. Neurochem.* **71**: 2349-2364.

Schoepp, D.D. and Conn, P.J. (1993) Metabotropic glutamate receptors in brain function and pathology. *Trends Pharmacol. Sci.* **14**: 13-20.

Schönthal, A., Herrlich, P., Rahmsdorf, H.J. and Ponta, H. (1988) Requirement for *fos* gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. *Cell* **54**: 325-334.

Schousboe, A., Meier, E., Drejer, J. and Hertz, L. (1989) Preparation of primary cultures of mouse (rat) cerebellar granule cells. in: A. Shahar, J. de Vellis, A. Vernadalis, and B. Haber (Eds.) A Dissection and Tissue Culture Manual of the Nervous System. Alan R. Liss, New York, pp. 203-206.

Schousboe, A., Frandsen, A. and Krogsgaardlarsen, P. (1992) Pharmacological and functional characterisation of excitatory amino acid-mediated cytotoxicity in cerebral cortical neurons. *Cell Biol. Toxicol.* **8**: 93-100.

Schreiber, S.S., Tocco, G., Najm, I., Finch, C.E., Johnson, S.A. and Baudry, M. (1992) Absence of c-*fos* induction in neonatal rat brain after seizures. *Neurosci.Lett.* **136**: 31-35.

Schütte, J., Viallet, J., Nau, M., Segal, S., Fedorko, J. and Minna, J. (1989) *jun*-B inhibits and c-*fos* stimulates the transforming and *trans* -activating activities of c-*jun* . *Cell* **59**: 987-997.

Seeburg, P.H. (1993) The molecular biology of mammalian glutamate receptor channels. *Trends Neurosci.* **16**: 359-365.

Sharp, F.R. and Sagar, S.M. (1994) Alterations in gene expression as an index of neuronal injury: heat shock and the immediate-early gene response. *Neurotoxicology* **15**: 51-60.

Sheng, M., Dougan, S.T., McFadden, G. and Greenberg, M.E. (1988) Calcium and growth factor pathways of c-*fos* transcriptional activation require distinct upstream regulatory sequences. *Mol. Cell. Biol.* **8**: 2787-2796.

Sheng, M., McFadden, G. and Greenberg, M.E. (1990) Membrane depolarisation and calcium induce c-*fos* transcription via phosphorylation of transcription factor CREB. *Neuron* **4**: 571-582.

Sheng, M., Thompson, M.A. and Greenberg, M.E. (1991) CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252**: 1427-1430.

Sheng, M., Cummings, J., Roldan, L.A., Jan, Y.N. and Jan, L.Y. (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* **368**: 144-147.

Shimizu, M., Nomura, Y., Suzuki, K., Ichikawa, E., Takeuchi, A., Suzuki, M., Nakamura, T., Nakajima, T. and Oda, K. (1998) Activation of the rat cyclin a promoter by ATF2 and Jun family members and its suppression by ATF4. *Exp. Cell Res.* **239**: 93-103.

Shimokawa, N. and Miura, M. (1996) Detection of living cells that express AP-1 using a fluorolabelled DNA probe. *FEBS Lett.* **388**: 16-20.

Simpson, C.S. and Morris, B.J. (1994) Basic fibroblast growth factor induces c-*fos* expression in primary cultures of rat striatum. *Neurosci. Lett.* **170**: 281-285.

Simpson, P.B., Challiss, R.A.J. and Nahorski, S.R. (1995) Neuronal Ca^{2+} stores: activation and function. *Trends Neurosci.* **18**: 299-306.

Skaper, S.D., Floreani, M., Negro, A., Facci, L. and Giusti, P. (1998) Neurotrophins rescue cerebellar granule neurons from oxidative stress-mediated apoptotic death: Selective involvement of phosphatidylinositol 3-kinase and the mitogen-activated protein kinase pathway. *J. Neurochem.* **70**: 1859-1868.

Skinner, M., Qu, S., Moore, C. and Wisdom, R. (1997) Transcriptional activation and transformation by FosB protein require phosphorylation of the carboxyl-terminal activation domain. *Mol. Cell. Biol.* **17**: 2372-2380.

Smeyne, R.J., Vendrell, M., Hayward, M., Baker, S.J., Miad, G.G., Schilling, K., Robertson, L.M., Curran, T. and Morgan, J.I. (1993) Continuous c-*fos* expression precedes programmed cell death *in vivo*. *Nature* **363**: 166-169.

Sommer, B., Köhler, M., Sprengel, R and Seeburg, P.H. (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* **67**: 11-19.

Sonnenberg, J.L., Macgregor-Leon, P.F., Curran, T. and Morgan, J.I. (1989a) Dynamic alterations occur in the levels and composition of transcription factor AP-1 complexes after seizure. *Neuron* **3**: 359-365.

Sonnenberg, J.L., Mitchelmore, C., Macgregor-Leon, P.F., Hempstead, J., Morgan, J.I. and Curran, T. (1989b) Glu receptor agonists increase the expression of Fos, Fra and AP-1 binding activity in the mammalian brain. *J. Neurosci. Res.* **24**: 72-80.

Stumpo, D.J. and Blackshear, P.J. (1986) Insulin and growth factor effects on c-*fos* expression in normal and protein kinase C-deficient 3T3-L1 fibroblasts and adipocytes. *Proc. Natl. Acad. Sci. USA* **83**: 9453-9457.

Suffys, P., Beyaert, R., Van Roy, F. and Fiers, W. (1987) Reduced tumour necrosis factor-induced cytotoxicity by inhibitors of the arachidonic acid metabolism. *Biochem. Biophys. Res. Comm.* **149**: 735-743.

Sugioka, M., Fukuda, Y. and Yamashita, M. (1998) Development of glutamate-induced intracellular Ca^{2+} rise in the embryonic chick retina. *J. Neurobiol.* **34**: 113-125.

Sugiyama, H., Ito, I. and Hiromo, C. (1987) A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature* **325**: 531-533.

Sun, P., Enslen, H., Myung, P.S. and Maurer, R.A. (1994) Differential activation of CREB by calcium/calmodulin-dependent protein kinases types II and IV involves phosphorylation of a site that negatively regulates activity. *Genes and Dev.* **8**: 2527-2539.

Szekely, A.M., Barbaccia, M.L., Alho, H. and Costa, E. (1989) In primary cultures of cerebellar granule cells the activation of NMDA-sensitive glutamate receptors induces c-*fos* mRNA expression. *Mol. Pharmacol.* **35**: 401-408.

Tatter, S.B., Galpern, W.R. and Isacson, O. (1995) Neurotrophic factor protection against excitotoxic neuronal death. *Neuroscientist* **1**: 286-297.

Thomas, R.J. (1995) Excitatory amino acids in health and disease. *Journal of the American Geriatric Society* **43**: 1279-1289.

Treisman, R. (1985) Transient accumulation of c-*fos* RNA following serum stimulation requires a conserved 5' element and c-*fos* 3' sequences. *Cell* **42**: 889-902.

Treisman, R. (1986) Identification of a protein-binding site that mediates transcriptional response of the c-*fos* gene to serum factors. *Cell* **46**: 567-574.

Treisman, R. (1992) The serum response element. *Trends Biochem. Sci.* **17**: 423-426.

Treisman, R. (1994) Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Opin. Genet. Dev.* **4**: 96-101.

Tsuda, M. (1996) Cascade of gene expression induced by calcium signals in neurons. *Neurochem. Int.* **29**: 443-451.

Van Beveren, C., Van Straaten, F., Curran, T., Müller, R. and Verma, I.M. (1983) Analysis of FBJ-MSV provirus and c-*fos* (mouse) gene reveals that viral and cellular *fos* gene products have different carboxy termini. *Cell* **32**: 1241-1255.

Van Haasteren, G., Li, S., Muda, M., Susini, S. and Schlegel, W. (1999) Calcium signalling and gene expression. *Journal of Receptor and Signal Transduction Research* **19**: 481-492.

Van Wauwe, J. and Goossens, J. (1983) Effects of antioxidants on cyclooxygenase and lipoxygenase activities in intact human platelets: Comparison with indomethacin and ETYA. *Prostaglandins* **26**: 725-730.

Vane, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biology* **231**: 232-235.

Vendrell, M., Curran, T. and Morgan, J.I. (1993) Glutamate, immediate-early genes and cell death in the nervous system. *Ann. N.Y. Acad. Sci.* **679**: 132-141.

Verderio, C., Coco, S., Fumigalli, G. and Matteoli, M. (1995) Calcium-dependent glutamate release during neuronal development and synaptogenesis: Different involvement of *w*-agatoxin IVA- and *w*-conotoxin GVIA-sensitive channels. *Proc. Natl. Acad. Sci. USA* **92**: 6449-6453.

Verity, M.A. (1992) Ca^{2+}-dependent processes as mediators of neurotoxicity. *NeuroToxicology* **13**: 139-148.

Vinson, C.R., Sigler, P.B. and McKnight, S.L. (1989) Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* **246**: 911-916.

Vishwanath, B.S., Fawzy, A.A. and Franson, R.C. (1988) Oedema-inducing activity of phospholipase A_2 purified from human synovial fluid inhibition by aristolochic acid. *Inflammation* **12**: 549-561.

Visvader, J., Sassone-Corsi, P. and Verma, I.M. (1988) Two adjacent promoter elements mediate nerve growth factor activation of the c-*fos* gene and bind distinct nuclear complexes. *Proc. Natl. Acad. Sci. USA* **85**: 9474-9478.

Vogt, P.K. and Bos, T.J. (1989) The oncogene *jun* and nuclear signalling. *Trends Biochem. Sci.* **14**: 172-175.

Volterra, A., Bezzi, P., Rizzini, B.L., Trotti, D., Ullensvang, K., Danbolt, N. and Racagni, G. (1996) The competitive transport inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate triggers excitotoxicity in rat cortical neuron-astrocyte co-cultures via glutamate release rather than uptake inhibition. *Eur. J. Neurosci.* **8**: 2019-2028.

Wahl, P., Ragsdale, D., Schousboe, A. and Miledi, R. (1993) Expression of neurotransmitter receptors by mRNAs from neurons developing in vitro: A *Xenopus* oocyte expression study. *J. Neurochem.* **60**: 57-65.

Walton, M., MacGibbon, G., Young, D., Sirimanne, E., Williams, C., Gluckman, P. and Dragunow, M. (1998) Do c-Jun, c-Fos , and amyloid precursor protein play a role in neuronal death or survival? *J. Neurosci. Res.* **53**: 330-342.

Weller, M., Montpied, P. and Paul, S.M (1994) NMDA receptor-mediated excitoprotection of cultured cerebellar granule neurons fails to alter glutamate-inducted expression of c-*fos* and c-*jun* mRNAs. *Mol. Brain Res.* **22**: 227-235.

Wessel, T.C., Joh, T.H. and Volpe, B.T. (1991) In situ hybridisation analysis of c-*fos* and c-*jun* expression in the rat brain following transient forebrain ischaemia. *Brain Res.* **567**: 231-240.

Yamamoto, K.K., Gonzalez, G.A., Biggs, W.H.III and Montminy, M.R. (1988) Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* **334**: 494-498.

Ye, G-N., Daniell, H. and Sanford, J.C. (1990) Optimization of delivery of foreign DNA into higher-plant chloroplasts. *Plant Molecular Biology* **15**: 809-819.

Yen, J., Wisdom, R., Tratner, I and Verma, I.M. (1991) An alternative spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins. *Proc. Natl. Acad. Sci. USA* **88**: 5077-5081.

Zerial, M., Toschi, L., Ryseck, R-P., Schuermann, M., Müller, R. and Bravo, R. (1989) The product of a novel growth factor activated gene, fosB, interacts with JUN proteins enhancing their DNA binding activity. *EMBO J.* **8**: 805-813.

Zirrgiebel, U., Ohga, Y., Carter, B., Berninger, B., Inagaki, N., Thoenen, H. and Lindholm, D. (1995) Characterisation of TrkB receptor-mediated signalling pathways in rat cerebellar granule neurons: involvement of protein kinase C in neuronal survival. *J. Neurochem.* **65**: 2241-2250.

Zukin, R.S. and Bennett, M.V.L. (1995) Alternatively spliced isoforms of the NMDAR1 receptor subunit. *Trends Neurosci.* **18**: 306-313.

Appendices

Appendix 1: Fos and Jun family antibodies used

c-Fos: Santa Cruz cat # sc-52 X.

Affinity-purified goat polyclonal IgG. Raised against a peptide corresponding to amino acids 3-16 of the amino terminus of c-Fos p62 (human origin, identical to mouse sequence). Non cross-reactive with FosB, Fra-1 or Fra-2.

FosB: Santa Cruz cat # sc-48 X.

Affinity-purified rabbit polyclonal IgG. Raised against a peptide corresponding to amino acids 102-117 (central domain) of FosB (mouse origin). Non cross-reactive with c-Fos, Fra-1 or Fra-2.

Fra-1: Santa Cruz cat # sc-183 X.

Rabbit polyclonal IgG. Raised against a peptide corresponding to amino acids 3-22 mapping at the amino terminus of Fra-1 (mouse origin). Non cross-reactive with c-Fos, FosB or Fra-2.

Fra-2: Santa Cruz cat # sc-171 X.

Rabbit polyclonal IgG. Raised against a peptide corresponding to amino acids 288-302 within the carboxy terminus of Fra-2 (human origin). Non cross-reactive with c-Fos, FosB or Fra-1.

c-Jun: Santa Cruz cat # sc-45 X.

Affinity-purified goat polyclonal IgG. Raised against a peptide corresponding to amino acids 91-105 of the amino terminal domain of c-Jun p39 (mouse origin, identical to human and rat sequence). Non cross-reactive with JunB or JunD.

JunB: Santa Cruz cat # sc-46 X.

Goat polyclonal IgG. Raised against a peptide corresponding to amino acids 45-61 mapping within the amino terminal domain of JunB p39 (mouse origin, differs from human sequence by two amino acids). Non cross-reactive with c-Jun or JunD.

JunD: Santa Cruz cat # sc-74 X.

Goat polyclonal IgG. Raised against a peptide corresponding to amino acid residues 329-341 mapping at the carboxy terminus of JunD p39 (mouse origin; identical to human and rat sequences). Non cross-reactive with c-Jun or JunB.

Appendix 2: Optimisation of Western blotting protocol

The advent of specific recognition techniques for use with DNA, RNA and protein caused an increase in speed and in confidence of results. The technique which allows detection of a specific protein using antibodies is known as Western blotting (so called because the analogous DNA technique was named Southern blotting after the researcher who developed the assay).

This technique has been widely used over a number of years to quantitatively measure the expression of proteins. Proteins contained in cell extracts are separated according to molecular weight by SDS-polyacrylamide gel electrophoresis. Subsequently, the proteins are transferred onto nitro-cellulose membrane, which is then incubated in a solution of a specific antibody to the protein of interest. The membrane is then incubated in a solution containing a secondary antibody which recognises the first (primary) antibody but which is also linked to an enzyme, e.g. horseradish peroxidase, which, using enhanced chemiluminescence (ECL) reagents, can be visualised on X-ray film. Other detection systems can involve use of a enzyme which undergoes a visible colour reaction when exposed to substrate, e.g. alkaline phosphatase. The technique is widely recognised as a specific and quantitative method for protein recognition.

As with many techniques, Western blotting often requires 'tailoring' to the specific system being studied; in this case, the concentration of proteins and antibodies used must be finely tuned. All of the protein-specific (primary) antibodies used were supplied by Santa Cruz Biotechnology, whilst the secondary (horseradish peroxidase-linked) antibodies were obtained from Amersham and Dakopatts.

During the course of experimentation in this section, many small alterations to the original protocol (extensively used across the scientific community, including several different laboratories in the department where this study was carried out) were made in an attempt to optimise the quality of the results obtained (Table A2).

Table A2 Areas of change in Western blotting protocol
Concentration of primary antibody
Source of antibody
Concentration of secondary antibody
Composition of blocking/incubation buffer
Composition of washing buffer
Length of incubation time in each antibody
Temperature of incubation in antibody (related to length of time)
Method of blotting (semi-dry/wet)

Initial cell lysis method used

Originally, the method recommended in the Santa Cruz leaflet accompanying the antibody was used to produce cell lysates for subsequent electrophoresis. This involved harvesting the cells in RIPA buffer (1% Igepal CA-630; 0.5% sodium deoxycholate; 0.1% sodium dodecylsulphate; made up in PBS. Before use, 10 μl/ml of 10 mg/ml PMSF; 30 μl/ml aprotinin and 10 μl/ml of 100 mM sodium orthovanadate were added). Cerebellar granule cells were grown in 6-well plates in N-DMEM for 2 or 7 days before being stimulated with either 10 μM or 250 μM Glu for various periods of time, up to 4h. After the designated exposure, the growth medium was removed and the cells rinsed with PBS at room temperature. The 6-well plates were then put in an ice bath and the rest of the procedure was carried out on ice. 0.2 ml RIPA buffer was added to each well and the cells harvested with a scraper into an Eppendorf microfuge tube. The 6-well plate was once again washed with 0.1 ml RIPA buffer and the two lysates combined. The pooled lysate was then passed through a 21-gauge needle in order to shear the DNA. A further 3 μl of 10 mg/ml PMSF were added and the lysate left on ice for 30 minutes, after which it was spun in a microfuge at 4°C for 20 minutes. The supernatant was removed and assayed for protein concentration using the Bradford method, then frozen until required.

Determining the optimum antibody concentration to use

Before using the antibody purchased from Santa Cruz (Cat. No. sc-253), it was decided to perform a dot blot experiment in order to ascertain the optimum amount of antibody to use. A piece of nitro-cellulose membrane was cut to fit the dot plate apparatus and soaked in transfer buffer, then washed with PBS. It was then incubated in 10 μg/ml c-fos peptide (Cat. No. sc-253-P) for 3 hours at room temperature, after which it was incubated in blocking buffer (1x TBS; 5% milk powder; 0.001% Tween 20) overnight at 4 °C. The membrane was then washed thoroughly with PBS. Serial dilutions of the antibody were added to one of the dot plates as shown in Figure A2.1A. The plates were then placed together with the membrane in between, clamped and left for 30 minutes. The membrane was flash-washed with PBS three times, then washed thoroughly with PBS. Following the washes, the membrane was incubated for 30 minutes in the secondary antibody (Amersham anti-rabbit IgG, HRP-conjugated; 1:5000 dilution in blocking buffer). The membrane was again washed with PBS and then was exposed to X-ray film after treatment with ECL reagents. From the resulting pattern (Figure A2.1B), it was decided to use the c-Fos antibody at 0.1 μg/ml final concentration.

Initial Western blotting protocol used

In order to analyse the amount of c-Fos protein present in each cell extract, after isolation by the RIPA buffer method described above the proteins in the cell extracts were separated by electrophoresis. 30 μl of each sample was loaded onto a 15% SDS-polyacrylamide gel (12.5% (w/v) acrylamide; $1x10^{-3}$% (w/v) bisacrylamide; 0.4M Tris.HCl, pH 8.7; 0.1% (w/v) SDS; 0.1% (v/v) TEMED; 0.03% (w/v) ammonium persulphate), which was run at 40 mA for 1 hour. The proteins were then blotted onto nitro-cellulose membrane using a semi-dry blotter for 1 hour at 60 mA per gel-sized piece of membrane. The blotting sandwich contained 4 layers of Whatman paper which had been soaked in transfer buffer (48 mM Tris; 39 mM glycine; 20% methanol;

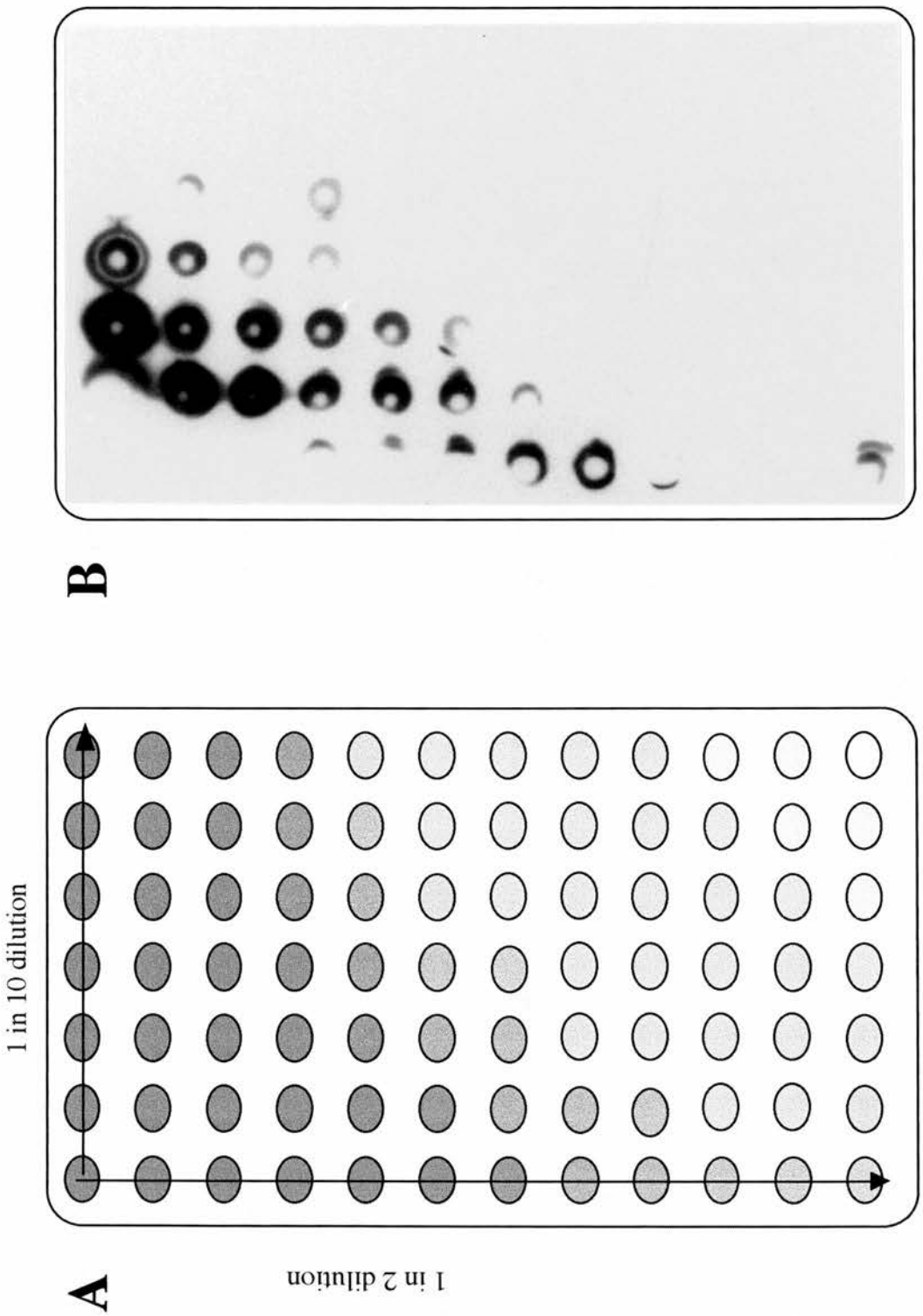


Figure A2.1 Dot blot schematic (A) and result (B)

0.0375% SDS) on each side of the gel/membrane. After transfer of the proteins from the gel, the membrane was then incubated in blocking buffer (1x TBS; 5% milk powder; 0.001% Tween 20) for 10 minutes with shaking before being incubated in primary antibody (0.1 μg/ml in blocking buffer) for 30 minutes with shaking. The membrane was then washed in blocking buffer for 3x 10 minutes, with the buffer being changed each time, before being incubated with the secondary antibody (1:5000 dilution in blocking buffer) for 30 minutes with shaking. After washing as before, the membrane was washed with TTBS (blocking buffer without milk powder) and then developed using a 1:1 solution of Amersham ECL reagents. The blot was then exposed to X-ray film.

The protocol described above was used several times; however, the signal obtained was very weak so it was decided to increase the primary (c-Fos) antibody concentration to 1 μg/ml. This change improved the results in terms of signal intensity; however, it was not particularly specific since many protein bands were visible. In addition, when the gels were stained after blotting, it was observed that there was still a significant amount of protein left in the gel.

Changes to initial Western blotting protocol

The next protocol which was used contained the following changes. Before blotting, the nitro-cellulose was soaked in a modified transfer buffer (25 mM Tris; 0.192 M glycine; 15% methanol; 0.0375% SDS) for 15 minutes. The gels were also soaked in transfer buffer for 5 minutes before blotting. After blotting, the nitro-cellulose was incubated in Ponceau S solution (0.2% Ponceau S in 3% TCA) for 2 minutes in order to visualise and mark the biotinylated molecular markers (Vectastain ABC Kit, Vector Laboratories, Peterborough, UK). The membrane was blocked using TBB (50 mM Tris; 150 mM NaCl; 0.5% Tween 20; 1% bovine serum albumin; 0.5% milk powder) for 1 hour at room temperature with shaking. After this, the membrane was incubated overnight at 4°C in primary antibody (0.5 μg/ml in TBB). The membrane was then washed for 10 minutes with TBS (50 mM Tris; 150 mM NaCl; pH

8.0), 10 minutes with TWB (50 mM Tris; 150 mM NaCl; 0.5% Tween 20) and then again with TBS for a further 10 minutes. The secondary antibody was anti-rabbit IgG, HRP-conjugated (Sigma Cat. no. A-9169) and was diluted 1: 10000 in TBB. The membrane was incubated in this mixture for 2 hours at room temperature with shaking. After washing as before, the membrane was incubated in a 1:1 mixture of Amersham ECL reagents for 30 seconds before being exposed to X-ray film.

Use of this second method seemed to have solved the problem of non-specific binding, but the molecular weight of the bands detected did not seem to correspond with c-Fos or the Fos-related antigens. In addition, the separation of the proteins was not optimal, so it was decided to separate the cell extracts using a 12.5% gel (12.5% (w/v) acrylamide; $1x10^{-3}$% (w/v) bisacrylamide; 0.37 M Tris.HCl, pH 8.7; $1x10^{-3}$% (w/v) sodium dodecyl sulphate; 25% (v/v) H_2O; $83x10^{-3}$% (v/v) TEMED; 332 μg/ml ammonium persulphate).

Third Western blotting protocol

This third method involved separating the proteins contained in the cellular extracts using a 10% SDS-polyacrylamide gel (13% (v/v) acrylamide/bisacrylamide mix (30:1.034); 490 mM Tris.HCl, pH 8.8; 0.13% (w/v) SDS; 0.08% (w/v) ammonium persulphate; 0.1% (v/v) TEMED). The cellular extracts themselves were obtained using a more simple method than mentioned above. This involved treating the cells as before with agonist, then aspirating the medium after the desired time period, washing the cells with PBS and then harvesting the cells in 1x sample buffer (150 μl) (62.5 mM Tris.HCl, pH 6.8; 2% (w/v) SDS; 10% (v/v) glycerol; 5% (v/v) 2-mercaptoethanol; 1% (w/v) bromophenol blue). For a detailed explanation of this method, see Section 2.2.9. The antibodies used from this point onwards were Santa Cruz Cat No. sc-52 X (c-Fos), sc-45 X (c-Jun) and sc-48 X (FosB).

Whilst trying to optimise the Western blotting method for the c-Fos antibody used, I went to the University of Strasbourg to learn transient transfection techniques. During my time there, I carried out some Western blots, again using murine primary

cerebellar granule cells and the method described in Section 2.2.9, but which were maintained in a defined medium (2% defined medium; 1% (w/v) insulin; 0.01% (w/v) T3; 0.5μl/ml gentamycin sulphate; 25 mM KCl). Thus the cells were treated with glutamate in a serum-free medium, and so further investigation into the effect of serum on the ability of Glu to cause c-Fos induction was possible.