ON THE NEUROPROTECTIVE ACTIONS OF FK506

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DECLARATION

I hereby declare that the thesis has been composed by myself, and, except where indicated in the text, describes work which is my own.

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ABBREVIATIONS USED

Ac-DEVD-amc	acetyl- Asp-Glu-Val-Asp-amino methyl coumarin
Ac-DEVD-cho	acetyl- Asp-Glu-Val-Asp-aldehyde
AMPA	α-amino-3 hydroxyl-5 methyl-4 isoxazole proprionic acid
ASK	Australian streptokinase trial
ATM	ataxia telangiectasia- mutated
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAD	caspase activated DNAse
cGMP	3',5'-cyclic guanosine monophosphate
CHAPS	3-[cyclohexylamino]-1-propanesulphonic acid
CT	computed tomography
DEVD	Asp-Glu-Val-Asp
DMEM	Dulbecco's
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothrietol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (ß -aminoethyl ether) N,N,N',N' tetraacetic acid
FCS	foetal calf serum
FKBP	FK binding protein
GABA	gamma amino butyric acid
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid
HRP	horse radish peroxidase
IL-2	interleukin 2
IP_3	inositol triphosphate
LDH	lactate dehydrogenase
L-NAME	L-nitro arginine methyl ester
MAP2	microtubule associated protein 2

MAPK	mitogen activated protein kinase
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MPP+	1-methyl-4 phenylpyridinium
mRNA	messenger ribonucleic acid
MTOR	mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyphenoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
NADPH	nicotine adenine dinucleotide phosphate
NFAT	nuclear factor of activated T-cells
NGF	nerve growth factor
NINDS	National Institute for Neurological Diseases and Stroke
NMDA	N-methyl D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
PARP	poly ADP-ribose polymerase
PBS	phosphate buffered saline
PI3K	phosphatidyl inositol 3' kinase
PKC	protein kinase C
RA	all trans- retinoic acid
RAFT	Rapamycin and FK506 target
rt-PA	recombinant tissue plasminogen activator
SNP	sodium nitroprusside
TACS	total anterior circulation syndrome
TBS	tris buffered saline
TUNEL	terminal UTP-transferase nick end labeling
zVAD	z-Val-Ala-Asp

ABSTRACT

The macrolide immunosuppressant FK506 has potent neuroprotective properties in focal and transient global cerebral ischaemia and improves motor performance following middle cerebral artery occlusion. In this thesis I seek to identify those actions of FK506 responsible for its neuroprotective properties.

These neuroprotective properties have been attributed to a calcineurin- mediated inhibition of nitric oxide synthase (NOS) activity. In cerebellar prisms from neonatal rats N- methyl D-aspartate (NMDA) induces a rapid rise in NOS activity which in turn stimulates the enzyme guanylate cyclase and leads to the accumulation of cGMP. I demonstrate that cGMP accumulation begins within a minute of NMDA stimulation and is inhibited by the NMDA receptor antagonist MK-801 and the broad spectrum NOS inhibitor L-NAME. The nitric oxide (NO) donor SNP also stimulates cGMP production, and this is not inhibited by either MK-801 or L-NAME. These results are consistent with NMDA inducing accumulation of cGMP through stimulation of NOS. Following NMDA stimulation FK506 augments protein phosphorylation in cerebellar prisms consistent with calcineurin inhibition. However, FK506 was entirely without effect on NMDA stimulated cGMP production, suggesting that its neuroprotective actions may not be mediated through NOS inhibition. Recent evidence supports the view that the neuroprotective effect of FK506 is not dependent on inhibition of neuronal NOS (nNOS) activity. In neuronal cultures lacking nNOS FK506 retains neuroprotective efficacy, and nNOS activity is not reduced by neuroprotective treatment with FK506 following middle cerebral artery occlusion in rats.

If FK506 is exerting its neuroprotective effects through other mechanisms, what might these be? FK506 is known to inhibit activation- induced apoptosis in T lymphocytes, and apoptosis is now recognised as an important mode of neuronal death in cerebral ischaemia. FK506 might therefore be reducing infarct size in focal cerebral ischaemia by inhibiting the apoptotic contribution to neuronal death.

To examine the role of FK506 in neuronal apoptosis I have examined apoptotic cell death in the SHSY-5Y neuroblastoma cell line. Initial experiments used differentiated SHSY-5Y cells at high passage. Neither their morphology nor their response to various toxins was constant. I therefore identified a source of low passage SHSY-5Y cells and compared their behaviour with high passage cells under differentiating conditions. Treatment of low passage cells with retinoic acid and serum reduction for 7 days resulted in the development of neuron- like morphology, with stabilisation of cell number and the development of immunopositivity for the neuronal marker MAP2 and the cell cycle G_0 marker $p21^{waf}$.

In terminally differentiated SHSY-5Y cells the calcium ionophore ionomycin caused rapid and simultaneous loss of mitochondrial respiratory activity and membrane integrity (determined using the MTS and LDH assays respectively) and had no effect on endogenous (PARP) or synthetic (Ac-DEVD-amc) Caspase 3 substrate cleavage. FK506 had no effect on ionomycin induced death. The broad spectrum protein kinase C inhibitor staurosporine also caused death in SHSY-5Y cells in a concentration dependent fashion, but the onset of the death was slower than with ionomycin. This was associated with increased cleavage of the synthetic caspase 3 substrate Ac-DEVD-amc. The decline in viability determined using the MTS assay, an index of mitochondrial function, was more pronounced than that seen using the LDH assay, a measure of membrane integrity. FK506 had no effect on staurosporine- induced death. Serum withdrawal caused the apoptosis of a proportion of SHSY-5Y cells, as evidenced by characteristic nuclear changes, caspase activation and cleavage of caspase substrates. FK506 partially reduced the decline in viability seen following serum withdrawal, but was without discernable effect on caspase activation.

In conclusion, I have demonstrated that FK506 can be without effect on NOS activity in brain tissue. Secondly, I describe the terminal differentiation of SHSY-5Y cells. In these cells both serum withdrawal and staurosporine cause a decline in viability that has some of the features of apoptosis; the effect of serum withdrawal, but not staurosporine, is partially reversed by FK506. These findings, interpreted along with

the published work of others, suggests that the neuroprotective effect of FK506 is not mediated through an effect on nNOS activity. While the neuroprotective actions of FK506 may involve inhibition of apoptosis, other mechanisms such as its reported neurotrophic properties may also be involved.

CHAPTER 1

INTRODUCTION

Acute ischaemic stroke is the most common neurological cause of death and disability in the world. In 1998 over 5 million persons around the world died from cerebrovascular disease, making stroke responsible for almost one in every ten deaths on the planet (WHO, 1999).

Stroke presents a potentially attractive target for neuroprotective drugs. It is a common and disabling disease, and a drug that had even a small effect on outcome would have significant public health benefits. The natural history of stroke is of sudden disease onset, with brain damage that evolves over subsequent hours and days. In consequence, treatments might be effectively given for this limited period. This contrasts with chronic neurodegenerative diseases where pathological processes are ongoing and therefore longer term treatment would be required. Finally, in some centres most patients are already admitted to hospital within a few hours of stroke onset (Harper et al., 1992) and so are in an environment where potentially dangerous treatments might be given under close medical supervision.

In this chapter I set the clinical context by discussing the epidemiology and classification of stroke. Then, I review the pathophysiology of acute ischaemic stroke, in particular the role of excitotoxicity, nitric oxide and free radical production, reperfusion injury and apoptosis. After reviewing the outcome of trials of different therapeutic strategies I discuss the neuroprotective properties of the macrolide immunosuppressant FK506 and outline the approach that I have taken to investigating the fundamental mechanism(s) subserving the neuroprotective effects of FK506.

EPIDEMIOLOGY AND CLASSIFICATION

In the Oxford Community Stroke Project, one year after a first stroke 23% of patients were dead and a further 28% were disabled (Bamford et al., 1991). Generalising these results to the UK as a whole, stroke can be expected to result in 40,000 deaths and 60,000 newly disabled persons each year. The average general practitioner can expect to see 6 patients with acute stroke per year, of whom one will die and two will become dependent on others. Stroke therefore represents a considerable burden on individuals, carers, the health service and on society as a whole.

The clinical manifestations of ischaemic stroke depend on the location and the extent of brain injury. These clinical features can be used to classify the stroke and give some predictions about likely outcomes. The Oxford Community Stroke Project Classification (Bamford et al., 1991) is a well validated system of classification (Anderson et al., 1994) which is based on clinical evaluation and which correlates well with neuroradiological findings at CT scanning (Wardlaw et al., 1996):-

Total anterior circulation syndromes (TACS) result from occlusion of an internal carotid or middle cerebral artery and lead to a triad of [1] contra- lateral weakness of the face, arm and leg with or without disturbance of sensation, [2] homonymous hemianopia, and [3] dysphasia and/ or neglect (dominant hemisphere) or visuo-spatial disturbance and/ or neglect (non- dominant hemisphere). Conscious level is often impaired early in the course of the illness. TACS are associated with a poor prognosis, with 56% of patients being dead at 6 months and only 4% of survivors being independent (Modified Rankin Handicap Score 0-2) (Bamford et al., 1991).

Partial anterior circulation syndromes result from a branch occlusion of the middle cerebral artery and lead to two out of three of the components of a TACS described above or dysphasia, visuospatial disturbance, or neglect occurring in isolation. The prognosis is markedly better, with 10% of patients dead at 6 months and 45% of survivors being independent (Bamford et al., 1991).

Lacunar syndromes occur following occlusion of small perforating vessels supplying

the brain stem, pons, thalamus, basal ganglia and internal capsule. The clinical consequences depend on the structure affected, but can include pure motor stroke, pure sensory stroke, mixed sensory- motor stroke and ataxic hemiparesis. The prognosis is good, with only 7% of patients dead at 6 months and 67% of survivors being independent (Bamford et al., 1991).

Posterior circulation syndromes affect the territory supplied by the vertebral arteries and can cause cerebellar signs, brain stem signs, homonymous hemianopia and/ or cortical blindness. The prognosis is intermediate, with 14% of patients dead at 6 months and 68% of survivors independent (Bamford et al., 1991).

PATHOPHYSIOLOGY OF HUMAN STROKE

Beyond the Circle of Willis, cerebral blood supply travels through end arteries with limited distal anastomosis. Occlusion of one such blood vessel therefore results in a rapid and almost complete interruption of blood flow to a volume of brain. This ischaemia deprives tissues of oxygen, glucose and other nutrients and leads to the accumulation of toxic metabolites that would otherwise be removed via the venous drainage to the systemic circulation.

The consequences of occlusions to cerebral end arteries depend on the duration of ischaemia and the extent of any residual flow. In primates, the amplitude of somatosensory evoked potentials falls dramatically when blood flow falls below 20mls per 100g brain tissue per minute (Symon, 1980), and such reductions in flow might result in, for instance, paralysis of a limb or loss of the power of speech. If flow is restored within a short period function returns to normal and the clinical syndrome is known as a transient ischaemic attack. However, sustained reductions in blood flow result pathologically in irreversible brain damage and clinically in stroke; Jones et al measured blood flow and subsequent infarction in awake monkeys and estimated thresholds for infarction of 3 hours at 12ml/ 100g brain/ min, 2 hours at 6ml/ 100g/ min and 1 hour at 2ml/ 100g/ min (Jones et al., 1981).

The cause of the blood vessel occlusion that leads to ischaemic stroke varies with patient age and with geographical and environmental factors. In the Western world, ischaemic stroke in middle- aged to elderly patients is commonly the result of extra-cranial (particularly carotid bifurcation) vascular disease with thrombo-embolism of platelet thrombi to the intra- cranial arterial arbourisation. Other sources of emboli include thrombotic or infected vegetations formed on damaged heart valves, intracardiac thrombus formation occurring as a result of cardiac rhythm abnormalities (e.g. atrial fibrillation) or of local wall motion abnormalities (e.g. left ventricular aneurysm following myocardial infarction), and paradoxical embolism of systemic deep venous thrombosis through a patent foramen ovale.

In younger patients other causes are relatively more common and include dissection of the carotid or vertebral arteries; inherited tendencies to thrombus formation such as deficiency of Protein C, Protein S or antithrombin III; and vasculitis affecting the intracranial vasculature. In the Far East, intra- cranial vascular disorders such as Moya-Moya disease are a more common cause of stroke than extra- cranial vascular disease, while in the developing world causes such as the vasculitis associated with syphilis infection are more common than elsewhere (Warlow et al., 1996).

EXPERIMENTAL MODELS OF STROKE

The study of the fundamental biological processes that subserve acute ischaemic stroke is based on the establishment of experimental paradigms using animal or cell culture models that are held to reflect certain features of human stroke. In addition to providing a means to study the mechanisms subserving pathophysiological processes, such models allow the testing of the efficacy of various agents that are imputed to have therapeutic properties. Clearly such models have their limitations, but they provide an important tool for the study of stroke.

Animal models of the focal ischaemia that occurs in stroke generally involve large vessel occlusions in the anterior cerebral circulation, thereby providing models

imitating total anterior circulation rather than lacunar syndromes. Particular benefits from the use of such animal models include the ability to determine precisely the time of onset of, and hence the duration of, ischaemia and to reinstate blood flow after variable defined periods of ischaemia; to reproducibly create infarcts of consistent size and distribution in a homogenous population of animals to test the efficacy of candidate drugs; to do so with close control of physiological variables such as body temperature, blood pressure and arterial oxygen saturation and avoiding the potentially confounding effects of diabetes, hypertension and aging; and the provision of post mortem material at various time points for histological and biochemical analysis.

However, it may be inappropriate to generalise from a homogenous population of young rats with standard sized infarcts to humans of all ages where there is huge variation in infarct size. Furthermore, such experiments require the use of significant numbers of laboratory animals, with the attendant ethical concerns.

While the cerebral circulation of primates closely reflects that in humans, resource and ethical concerns militate against their widescale use. The cerebral circulation of other large animals such as cats, dogs, pigs and sheep includes an extensive network of anastomotic arteries, veins and sinuses involving the terminal carotid artery termed the carotid rete mirablis which is not present in humans and this restricts their suitability in models of focal ischaemia. Among the rodents, gerbils do not possess vertebral arteries, and therefore rely on two carotid arteries for the entirety of their cerebral blood flow, and infarction can be induced relatively easily by the ligation of vessels in the neck.

Rats and humans both possess paired carotid and vertebral arteries that join within the cranium in a structure called the Circle of Willis from which the major intracerebral blood vessels arise. However, rats differ from humans in that their Circle of Willis is generally incomplete, with no anterior communicating artery, and the posterior communicating artery is larger relative to the other components. Rats also lack gyrification of the cortical mantle. However, in spite of these differences

the rat remains the animal most suited to the experimental modelling of human focal cerebral ischaemia.

In the experimental modeling of stroke it is important to distinguish between the effects of ischaemia alone and those of ischaemia followed be reperfusion. Experimental paradigms for the study of focal ischaemia may therefore be classified according to whether they cause permanent or temporary ischaemia. A number of methods have been described including (1) permanent occlusion of the intracranial middle cerebral artery by its surgical exposure and subsequent transsection, photocoagulation or diathermy (Tamura et al., 1981a; Tamura et al., 1981b); (2) permanent or reversible middle cerebral artery occlusion by the passage of an intralumenal thread of known caliber (Koizumi et al., 1986; Zea Longa et al., 1989); (3) variably reversible thromboembolic models where fibrin clot or inert microspheres are introduced to the carotid circulation and embolise distally (Hill et al., 1955; MCauley, 1995; Overgaard, 1994); and (4) permanent or reversible middle cerebral artery occlusion by extravascular stereotactic application of vasoconstrictive agents such as endothelin 1 (Sharkey et al., 1993; Sharkey and Butcher, 1995).

A number of factors may confound the results of such experiments. Small mammals such as rodents readily become hypothermic during operative procedures and hypothermia is itself neuroprotective (Buchan and Pulsinelli, 1990; Busto et al., 1989). The anaesthetic used must be chosen with care as some, including the dissociative anaesthetic ketamine and the GABA A agonist chloral hydrate, have potential intrinsic neuroprotective activity. The effect of putative neuroprotective agents on haemodynamic variables and cerebral blood flow should be determined, as observed effects may be secondary to changes in cerebral perfusion. Finally, the ability of drugs under investigation to penetrate the blood brain barrier and reach their putative site of action should be demonstrated.

Ionic disturbances

Neuronal function requires the active maintenance of transmembrane gradients in the concentrations of key ions including potassium and sodium. It has been estimated that almost two-thirds of neuronal ATP consumption is used in the maintenance of these ionic gradients (Alberts et al., 1994), which form the basis of the resting membrane potential and which in their controlled discharge allow membrane depolarisation and the action potential. The membrane bound enzyme sodium-potassium- ATPase is responsible for much of the maintenance and "recharging" of the membrane potential and consumes one molecule of ATP for every three sodium ions expelled. Neurons have little capacity for energy storage and rely on constant production of ATP, constant operation of the mitochondrial respiratory chain, and therefore constant supply of substrate, particularly oxygen and glucose.

In the ischaemic core energy failure leads to rapid anoxic depolarisation with extracellular [K+] rising to 70 mM within 1 - 3 minutes (Gido et al., 1997) and this is independent of NMDA receptor activity (Lauritzen and Hansen, 1992). In the ischaemic penumbra, where blood flow and ATP levels are less compromised, anoxic depolarisation does not occur. However, transient sporadic depolarisations (or spreading depressions) occur in waves of 3 to 5 minutes duration, are dependent on glutamate receptor activity (Iijima et al., 1992), and may originate from glutamate release in the ischaemic core.

Excitotoxicity and increased intracellular calcium

Glutamate is the most abundant neurotransmitter in the brain, and within 2 hours of experimental middle cerebral artery occlusion extracellular concentrations of glutamate rise by as much as 17- fold in ischaemic cortex and 27-fold in striatum (Butcher et al., 1990). While some of this glutamate originates from synaptic activity, it appears that a larger proportion accumulates as the result of failed neuronal and glial glutamate uptake that is in turn a consequence of impaired energy

status due to hypoxia (Wahl et al., 1994).

High extracellular concentrations of glutamate exert toxic effects on neurons through a number of pathways. Activation of the ionotropic NMDA and AMPA/kainate receptors results in sodium influx and subsequent loss of membrane potential. This adds to the energy crisis, as ATP is required to reinstate the resting membrane potential. Intracellular calcium concentrations rise markedly, probably due to a combination of entry through NMDA receptor channels, calcium- induced calcium release from the endoplasmic reticulum, and sodium- induced calcium release from mitochondria. The magnitude of the rise in intracellular calcium correlates with histological damage (Uematsu et al., 1989), and increased intracellular calcium results in diverse effects including activation of MAP kinases (Schwarzschild et al., 1999), immediate early genes (Xia et al., 1996), calpain and nitric oxide synthase.

Nitric oxide and free radical production

Neuronal nitric oxide synthase (nNOS) is a 160 kdal NADPH- and calmodulin-dependent enzyme which catalyses the conversion of arginine to citrulline and nitric oxide. It is inactive at basal calcium concentrations, but activated when calcium levels increase and, it is thought, by phosphorylation by kinases including cAMP dependent protein kinase, protein kinase C and calcium/ calmodulin dependent protein kinase (Bredt et al., 1991a); however, it seems that phosphorylation does not always result in increased nNOS activity (Okada, 1995; Okada, 1996). In healthy neuronal tissues NO has some functions as a messenger molecule, diffusing to surrounding cells and activating guanylate cyclase, leading to the accumulation of the second messenger cyclic GMP (Dawson et al., 1992). However, in ischaemic brain nNOS activity is also associated with the production of free radicals, in particular peroxynitrile, which are promiscuous electron donors and can damage many cellular structures including DNA and mitochondrial membranes.

In primary cortical cultures NOS inhibition reduces neuronal death (Dawson and Snyder, 1994), and cultures prepares from nNOS knockout animals are protected

from NMDA and oxygen/glucose deprivation induced death (Dawson et al., 1996). Furthermore, NOS donors mimic the effects of NMDA. In animal models of stroke, treatment with specific nNOS inhibitors such as 7-nitroindazole leads to reduced infarct size (Yoshida et al., 1994). Infarct size is also reduced following ischaemia in nNOS knockout animals (Hara et al., 1996; Huang et al., 1994). In addition, agents that are able to "scavenge", or inactivate, free radicals, are also protective in cell culture and animal models of stroke (Hall et al., 1996).

Free radicals may also be produced as a result of other processes including the oxidative metabolism of products of membrane breakdown (Katsuki and Okuda, 1995; Tegtmeier et al., 1990) and altered mitochondrial function (Piantadosi and Zhang, 1996).

Reperfusion injury

In focal cerebral ischaemia, experiments seek to replicate the sequence of events in human stroke. This might include not only the occlusion of a middle cerebral or internal carotid artery, but also the reopening of that vessel at a later time. In humans it is not possible to identify such reperfusion clinically, as there are seldom any immediate or dramatic changes in the patients condition. Zanette et al (Zanette et al., 1995) used transcranial Doppler ultrasonography to measure middle cerebral artery flow in 56 patients 6, 24, 48 hours, 7 days and 3 to 9 months after acute ischaemic stroke (Table 1.1). At 6 hours, 41% of patients had normal flow in the relevant middle cerebral artery, increasing to 45% at 24 hours, 57% at 48 hours, 71% at 7 days and 92% at 3 to 9 months. While no flow was detectable in 28% of patients at 6 hours, this fell to 22% at 24 hours, 17% at 48 hours, 14% at 7 days and 4% at 3 to 9 months.

That is, one half of stroke patients with initially abnormal MCA flow will regain normal flows within the first week. The proportion of patients with no flow declines most rapidly in the first 24 hours, and the proportion of patients with normal flow increases most rapidly between 24 and 48 hours (Table 1.1).

Table 1.1: Transcranial Doppler findings following acute ischaemic stroke

	% patients with no flow at transcranial Doppler examination	% patients with normal flow at transcranial Doppler examination
6 hrs	28	41
24 hrs	22	45
48 hrs	17	57
7 days	14	79
3 - 9 months	4	92
	Rate of resolution of "no flow"	Rate of restoration of "normal flow"
6 - 24 hrs	8	5.3
24 - 48 hrs	5	12
48 hrs - 7 day	0.6	2.8

Hemispheric transcranial doppler findings in 56 patients with carotid territory stroke. Data calculated from Zanette et al (Zanette et al., 1995).

Timing of reperfusion is held by some investigators to be important because of the potential for so called "reperfusion injury" (Aronowski et al., 1997), a phenomenon by which normalisation of oxygen delivery to metabolically impaired brain is held to lead to worsening tissue damage, perhaps through the production of free radicals.

If reperfusion injury were to represent a biologically significant phenomenon in human stroke, it would have an important bearing on the development of stroke treatments. Pathological processes implicated in reperfusion injury, notably free radical induced damage resulting from normalised oxygen delivery in the face of impaired oxygen and free radical detoxifying systems, might serve as important therapeutic targets, and by contrast treatments designed to normalise blood flow may be associated with increased reperfusion injury.

A number of strands of evidence are cited in support of existence of reperfusion injury:-

Progressive neuronal injury

Brain injury continues to worsen for hours and sometimes days after the restoration

of blood flow. In a rat four vessel occlusion forebrain ischaemia model a 30 minute occlusion resulted in neuronal damage (assessed at light microscopy) in neocortex and hippocampus which continued to increase between 24 and 72 hours after the onset of ischaemia (Pulsinelli et al., 1982). A similar progression of neuronal damage has been observed in rat cortex more than a week after a 30 minute filament - induced MCA occlusion (Nakano et al., 1990).

However, progressive neuronal damage may equally be due to past rather than contemporary insults. In 1982 Kirino et al described a sequence of changes in the CA_1 area of the hippocampus following brief (Wen et al., 1996) bilateral carotid artery occlusion in the Mongolian gerbil (Kirino, 1982). The phenomenon of delayed hippocampal damage following global cerebral ischaemia has also been observed at postmortem in humans dying 2 days to 18 months after a cardiorespiratory arrest from which they had initially recovered (Petito et al., 1987). More recently Chen et al have demonstrated increased expression of caspase 3 mRNA, cleavage of the caspase 3 substrate PARP and of caspase 3 itself, TUNEL positivity and DEVD cleavage activity in gerbil hippocampal CA_1 region following transient global ischaemia. Furthermore, neuronal survival was increased by the ventricular infusion of the selective caspase 3 antagonist z-DEVD-fmk (Chen et al., 1998).

There is therefore a substantial body of evidence to suggest that at least some of the damage that continues to occur after the reversal of a temporary ischaemic insult has features of apoptosis. Since apoptosis may continue after the initiating stimulus is withdrawn, it may be that such delayed neuronal injury is a consequence of the initial ischaemia rather than of subsequent reperfusion.

Damage in reperfused brains compared with permanent occlusion

There is conflicting evidence regarding the effects of permanent and temporary ischaemia on brain oedema and infarct volume. An early report of the effects of MCAO in the squirrel monkey suggested that while a 3 hour occlusion caused less morbidity or mortality than permanent occlusion, deaths following temporary occlusion occurred earlier than those following permanent occlusion. The authors

suggested that this might be due to the more rapid development of cerebral oedema following reperfusion (Sundt et al., 1969).

Reversible ischaemia in the Mongolian gerbil (6hrs left common carotid artery cliping followed by 3hrs of reperfusion) resulted in only a small increase in brain water content (83.5% wet weight v 82%) compared with permanent (9hrs occlusion) ischaemia (Ito et al., 1979). In support of the hypothesis that reperfusion is associated with increased damage, the same authors reported that passage of radiolabelled albumin through the blood brain barrier was increased in reversible compared to permanent occlusion, but since the tracer was introduced via the systemic venous circulation it is not possible to determine whether the observed differences were due to reduced hemispheric perfusion in the permanently occluded group or to increased blood brain barrier permeability in the reperfused group (Ito et al., 1979).

While the work of Memezawa and coworkers is often cited in this context (Memezawa et al., 1992), their study in the rat compared the effects of reversible MCAO at 7 days with a historical control group subjected to 24 hours of permanent MCAO. Their data do not therefore allow meaningful comparisons of the effects of occlusion with or without reperfusion.

Kaplan et al compared the effects in the rat of permanent versus reversible (1, 2, 3 or 4 hours, all animals sacrificed at 24hrs) MCAO. They found that the extent of oedema and infarction increased with increased duration of ischaemia up to 3 hours, after which there was no further increase (Kaplan et al., 1991). In none of the reperfused groups was edema or infarct volume greater than that observed with permanent occlusion.

Yang and Betz compared permanent and 3 hours filament- induced MCAO at 6 hours in the rat. The reperfused group had increased infarct size but there was no difference in cerebral oedema as measured by hemispheric water content. They also report increased blood brain barrier permeability, but as with the work of Ito et al cited above (Ito et al., 1979) they injected a radiotracer to the systemic venous

circulation and therefore it is not possible to determine whether their observed differences were due to reduced hemispheric perfusion in the permanently occluded group or to increased blood brain barrier permeability in the reperfused group (Yang and Betz, 1994).

Aronowski et al describe the effects of 24 hours of permanent or reperfused MCAO in the Long Evans rat induced by elevation of the MCA and clipping of the ipsilateral common carotid artery. They report that reperfusion occuring at between 2 and 5 hours was associated with a significant increase in infarct size compared with permanent ischaemia (Aronowski et al., 1997). The mean infarct volume reported for the permanently occluded animals, at $31mm^3$, is much lower than that found in other MCA occlusion models, and when experiments were repeated with a more severe insult (three vessel occlusion in the Long Evans rat) and with spontaneously hypertensive rats where infarct volumes were higher the deleterious effect of reperfusion was lost (Aronowski et al., 1997).

Interpretation of their results is further complicated because different experimental groups received different doses of chloral hydrate, an anaesthetic which may have intrinsic neuroprotective activity. 2,2,2-trichloroethanol, its active agent, inhibits NMDA- induced intracellular calcium accumulation in cultured cortical and mesencephalic neurons (Scheibler et al., 1999) and potentiates GABA- induced currents in hippocampal neurons (Lovinger et al., 1993) and in xenopus oocytes expressing $GABA_A$ receptors (Garrett and Gan, 1998).

The evidence regarding reperfusion injury is therefore mixed. There are no well-defined experimental paradigms which will reproducibly induce reperfusion injury, and in many paradigms reperfusion occurs without any apparent deleterious consequences. However, in some experimental situations it does appear that reperfusion may result in increased damage.

Apoptosis

First described in 1972 (Kerr et al., 1972), apoptosis is a process through which cells

die with resulting debris being removed with minimal disturbance to neighboring cells. As such it contrasts with necrosis, where rupture of cellular membranes leads to the indiscriminate release of intracellular contents to the potential hazard of surrounding cells. There are many biological situations in which such discrete cell death is advantageous. For instance, cells undergoing viral infection can be eliminated before viral replication is complete (Cuff and Ruby, 1996), and immune cells can be destroyed once infection had been eliminated (Boise et al., 1995). During development, cells that are redundant in the adult, or that have not developed in the correct position or with the correct connections, can be removed without disturbing properly placed and well connected adjacent cells (Chen and Zhao, 1998), and cells with damaged DNA can be eliminated before mutated cell cycle associated genes result in unrestricted cell division and cancer (Oren, 1994).

Features of apoptosis

In 1972 Wyllie and colleagues (Kerr et al., 1972) reported novel morphological features not typical of necrosis demonstration in tumour tissues examined at electron microscopy. They hypothesised that these features were the consequence of a regulated form of cell death, and they termed this process *apoptosis*, from the Greek *"to fall, as a leaf from the tree"*. Subsequently they and others have gone on to describe the process of apoptosis, or programmed cell death, in substantial detail. Apoptosis is a process by which cells die without rupture of cell membranes or extracellular release of intracellular contents. Its salient features include:

Activation of one or more cysteine- containing aspartate proteases (caspases)

The basic mechanisms of apoptosis appear to be conserved across diverse cell types and have been observed in species from the slime mould to humans. A proximal signalling pathway, often specific to the nature of the apoptotic stimulus, leads to the activation of one or more members of a family of proteases (caspases). These enzymes, of which 13 have been described to date, all contain an active site cysteine residue, cleave specific substrates at aspartic amino acids and reside in healthy cells as inactive proenzymes that are themselves activated by proteolysis into large and small subunits (Thornberry et al., 1997). Active caspases subsequently cleave diverse

cellular proteins including structural proteins and those responsible for repair processes. Extracts prepared from cells or tissues undergoing apoptosis show caspase activity against fluorescently- or colourimetrically- conjugated synthetic tetrapeptide substrates.

Fragmentation of macromolecules including proteins and DNA

Activated caspases cleave a number of structural proteins including lamin, actin, gelsolin and intermediate filaments which lead to dissassembly of cytoskeletal and nuclear architecture. In addition they cleave and inactivate enzymes involved in DNA repair such as poly- ADP ribose polymerase (PARP) and DNA dependent protein kinase. They cleave and activate other caspase family members and they activate the caspase- activated DNAse CAD (Enari et al., 1998) by cleaving the inhibtor protein ICAD (Sakahira et al., 1998). Activation of CAD leads to DNA cleavage at internucleosomal sites; at agarose gel electrophoresis this results in the "DNA ladder" caused by the presence of DNA fragments whose length is an integer multiple of the length of DNA associated with a single nucleosome, 180 base pairs (Wyllie, 1980). The ends of these DNA fragments are not blunt, and may be labeled using terminal deoxyUTP nick end labelling (TUNEL staining).

Changes in cell morphology

Early in the course of apoptotic cell death there is decreased cell volume, with cells become more spherical and, in tissue culture, becoming phase bright and detaching from the culture monolayer. In the nucleus there is chromatin condensation along with nuclear shrinkage and fragmentation. At later stages the cell may fragment into a number of smaller, membrane bound apoptotic bodies.

Change in the external composition of cells and apoptotic bodies

Healthy cells manifest an asymmetry in the membrane distribution of phosphatidylserine lipid moieties, with more than 99% being found on the inner part of the lipid bilayer. This distribution is actively maintained by enzymes including an ATP dependent aminophospholipid translocase (Bevers et al., 1996). During apoptosis, in some instances within 1 hour of stimulation, that asymmetry is lost

(Fadok et al., 1992a; Fadok et al., 1992b). This requires caspase activation, and is inhibited by tetrapeptide caspase inhibitors such as DEVD-fluoromethylketone (Martin et al., 1996). The appearance of phosphatidylserine on the external lipid bilayer is one of a number of membrane changes including changes in phospholipid packing (Fadok et al., 1992a), in surface charge (Savill et al., 1989), and in the carbohydrate profile (Duvall et al., 1985; Morris et al., 1984), at least some of which serve to target the cell for phagocytosis. Phosphatidylserine exposure results in changes in binding properties for molecules such as Annexin V, and increased Annexin V binding, detected immunohistochemically or at flow cytometry, has been used as the basis of quantitative assays of apoptosis (van Engeland et al., 1996).

Evidence for apoptosis in stroke

In a number of experimental systems moderate levels of chemical or physical insult result in death by apoptosis while higher levels of stress lead to necrosis (Ankarcrona et al., 1995;Bonfoco et al., 1995). It is likely that overwhelming noxious stimulation leads to such derangement of cellular energy status and homeostasis as to prevent completion of the apoptosis program. At intermediate insult intensities a mixed pattern of cell death may be apparent, with features of both apoptosis and necrosis, the balance of these depending on the extent to which apoptosis had been able to proceed before necrosis intervened.

The reduction in blood flow occurring as a result of occlusion of a vessel is not uniform throughout the affected volume. At its heart lies a densely ischaemic core where blood flow has been reduced to less than 15mls per 100mg brain per minute for a prolonged period and where damage is most intense, with the vast majority of neurons being destined to die. Between the ischaemic core and normal brain lies a region termed the ischaemic penumbra where blood flow has been reduced to intermediate levels and where neuronal death is less certain (Astrup et al., 1981). There is considerable evidence, reviewed below, to suggest that apoptosis may make a significant and potentially reversible contribution to neuronal death in stroke.

Evidence from post- mortem human tissue

There have been few morphological studies of post mortem material in human stroke. Guglielmo et al found increased TUNEL staining in penumbral neurons in patients dying two to three days after a stroke (Guglielmo et al., 1998). In 14 patients dying up to 6 months after a cardiorespiratory arrest Kaplan et al found features of delayed hippocampal damage (Kaplan et al., 1991) similar to the delayed neuronal death observed in rodents following transient global ischaemia; in rodents this process manifests many characteristic features of apoptosis but such factors were not examined by Kaplan et al.

Animal studies

Observational Studies

Following rat MCAO neurons exhibit oligonucleosomal DNA fragmentation (Linnik et al., 1993; Linnik et al., 1995a; Tominaga et al., 1993), TUNEL positivity (Li et al., 1995a; MacManus et al., 1994) and apoptotic nuclear morphology (Li et al., 1995b), and these changes appear to be more pronounced in neurons of the ischaemic penumbra (CharriautMarlangue et al., 1996).

Focal cerebral ischaemia leads to caspase activation as evidenced by increased DEVD cleavage activity (Fink et al., 1998; Namura et al., 1998) and the development of immunoreactivity to active caspase 3 (Namura et al., 1998; Velier et al., 1999) and 8 (Velier et al., 1999). Expression of caspase 3 is increased following focal cerebral ischaemia (Asahi et al., 1997), and the abundance of the apoptosis associated transcription factor p53 is increased following transient focal cerebral ischaemia (Chopp et al., 1992).

Transient global ischaemia leads to hippocampal apoptosis as evidenced by DNA fragmentation (Heron et al., 1993; MacManus et al., 1993; Gillardon et al., 1997), increased caspase 3 expression (Gillardon et al., 1997; Ni et al., 1998), caspase 3 activation (Gillardon et al., 1997) and increased DEVD cleavage activity (Chen et al., 1998; Gillardon et al., 1997). Furthermore, following global ischaemia the expression of the apoptosis repressor genes Bcl-2 and Bcl-xL was increased in cortex

and hippocampus (Chen et al., 1997).

Interventional Studies

TRANSGENIC ANIMALS

Infarct volume following middle cerebral artery occlusion is reduced in mice which lack p53 (Crumrine et al., 1994) and in those overexpressing Bcl-2 using either a transgenic approach (Martinou et al., 1994) or viral vector expression systems (Lawrence et al., 1996; Linnik et al., 1995b). It is also reduced in mice overexpressing the X chromosome linked inhibitor of apoptosis protein XIAP (Xu et al., 1999).

CASPASE INHIBITION

Neuronal damage is reduced following intracerebroventricular treatment with caspase inhibitors following focal (Cheng et al., 1998; Endres et al., 1998; Hara et al., 1997; Li et al., 2000; Loddick et al., 1996) or transient global (Chen et al., 1998; Himi et al., 1998) ischaemia.

Cell culture studies

Exposure of cultured primary cortical neurons to low concentrations of the excitotoxin NMDA leads to the development of morphological and biochemical features of apoptosis whereas high concentrations lead to necrosis (Ankarcrona et al., 1995); similarly under certain conditions nitric oxide donors can induce apoptosis in neuronal cell cultures (Bonfoco et al., 1995). NMDA- induced apoptosis in primary cortical culture is blocked by caspase inhibition (Tenneti et al., 1998), and in rat cerebellar granule cells glutamate toxicity is associated with release of cytochrome C from mitochondria, an important step in the apoptotic cascade (Atlante et al., 1999).

There is therefore a significant body of evidence to suggest that apoptosis plays an important role in neuronal cell death in both focal and transient global ischaemia. In some regions, particularly the ischaemic penumbra, apoptotic and necrotic death may proceed in adjacent cells. Indeed, individual cells might manifest features of both apoptosis and necrosis, the final outcome depending on whether the cell has sufficient resources to complete the apoptosis program before necrosis ensues.

In the search for an effective stroke treatment the question is not the precise definition of the mode of neuronal death, but rather the identification of death pathways which might be interrupted therapeutically. While there remains some debate as to the magnitude of the contribution of apoptosis to neuronal death in stroke, it is beyond question that apoptosis does play some role, and that in animal models inhibition of apoptosis results in significant reductions in neuronal damage. Against this background, the attribution of anti-apoptotic properties to a drug would provide a potential explanation of its neuroprotective properties.

TRIALS OF TREATMENT IN STROKE

In one of the first papers to address the potential benefits and hazards of restoring flow through a previously occluded middle cerebral artery the authors begin by stating that ...

"At the present time a patient with an acute middle cerebral artery occlusion represents an unanswered challenge for effective treatment. Conservative management has done little to alter the size of the resulting cerebral infarctions and the magnitude of related neurological deficits." (Sundt et al., 1969)

In the thirty years since that publication it might be argued that little has changed regarding pharmacological treatments for stroke.

Therapeutic strategies include treatments to achieve rapid reinstatement of blood flow; treatments designed to interfere with the cellular consequences of ischaemia and to limit its impact; and treatments and management strategies designed to minimise further damage and to maximise functional recovery.

Trials of thrombolysis in acute ischaemic stroke

Five large randomised controlled trials investigating the potential benefit of either streptokinase (Candelise et al., 1995; Donnan et al., 1996; MAST-E Study Group, 1996) or tissue plasminogen activator (Hacke et al., 1995; Hacke et al., 1998; The

National Institute of Neurological Disorders Stroke rt-PA stroke study group, 1995) as thrombolytic agents given within at most 6 hours of acute ischaemic stroke have reported in the last four years (Table 1.2). In two of these trials (Donnan et al., 1995; Hommel et al., 1995) recruitment to at least some sections of the trial was stopped early on the advice of the data monitoring committee because of significant adverse effects in the treatment group, notably excess mortality.

Those studies that did proceed to completion suggested that there might be a reduction in dependency amongst stroke survivors treated with thrombolysis which may occur at the expense of some increased mortality. In no study was significant benefit shown in an intention to treat analysis against a predefined combined end point of death or dependence at the end of the follow- up period. The incidence of symptomatic intracerebral haemorrhage was increased by as much as ten- fold in patients receiving thrombolysis.

Only in the NINDS study was a significant benefit seen on the predefined end point of disability alone. In this study randomization resulted in well balanced groups except for a small excess of lacunar, and hence good prognosis, strokes in the treatment group. Treatment with alteplase (0.9mg/kg) within three hours of stroke onset resulted in significant improvements in the number of patients with favourable outcomes whether this was determined using the Barthel Index, the Modified Rankine Handicap Score, the Glasgow outcome scale, the NIH Stroke Score or a combination of all scales. Death rates were 17% in the treatment group compared with 21% in the placebo group. The rate of intracranial haemorrhage was 12% in the treatment group and 5% in controls, although there was a more marked difference in the incidence of symptomatic intracranial haemorrhage (7% v 1%).

While tissue plasminogen activator has been licensed by the US Food and Drug Administration for use within 3 hours of acute ischaemic stroke, there are considerable practical difficulties in getting patients to hospital, clinically evaluated, CT scanned and treatment commenced within three hours of symptom onset. Indeed, the average number of patients randomised in each participating centre in the NINDS

Table 1.2: Trials of thrombolysis in acute ischaemic stroke

Trial	Agent	Time & Dose	No. Patients	Outcome Measures	Odds Ratio*
MAST-I Candelise et al 1995	Streptokinase 1.5MU	< 6 hrs	622	6 month death and disability	0.76 (0.48-1.21)
MAST-E MAST-E Study Group 1996	Streptokinase 1.5 MU	< 6 hrs	310	6 month death and disability	0.86 (0.49-1.51)
ASK Donnan et al 1996	Streptokinase 1.5 MU	0 - 4 hrs 0 - 3 hrs 3 - 4 hrs	340 70 270	3 month death and disability	1.16 (0.76-1.78) 0.66 (0.28-1.58) 1.22 (0.80-1.86)
ECASS Hacke et al 1995	rt-PA 1.1 mg/kg	< 6 hrs	620	3 month disability 1 month mortality	Not given 1.20 (0.98-1.46)
NINDS NINDS 1995	rt-PA 0.9 mg/kg	0 - 90 mins	302	BI MRHS GOS NIHSS	0.6 (0.3-0.8) 0.6 (0.4-1.0) 0.6 (0.4-1.0) 0.5 (0.3-0.8)
		90 - 180 mins	322	BI MRHS GOS NIHSS	0.6 (0.4-0.9) 0.4 (0.3-0.7) 0.5 (0.3-0.8) 0.5 (0.3-0.8)
ECASS II Hacke et al 1998	rt-PA 0.9 mg/kg	0 - 3 hrs 3 - 6 hrs	158 635	3 month disability	0.8 (0.4-1.7) 0.8 (0.6-1.2)

Odds ratio < 1 favours treatment, > 1 favours placebo:
BI, Barthel Index; MRHS, Modified Rankin Handicap Score; GOS, Glasgow Outcome Scale; NIHSS, National Institute of Health Stroke Scale:

trial was only three per year (The National Institute of Neurological Disorders Stroke rt-PA stroke study group, 1995), and enrollment within 3 hours in the ASK study was so slow that the study was terminated before the recruitment target was met (Donnan et al., 1996). A recent meta- analysis of the results from all identifiable trials of thrombolysis in acute ischaemic stroke (Wardlaw et al., 1999) concluded that "the data so far are scant, and quite insufficient to make any definite conclusion about the benefit or otherwise of thrombolysis to treat acute ischaemic stroke".

In spite of these concerns, thrombolysis given within 3 hours of stroke onset according to the NINDS protocol has been successfully adopted in the treatment of at least a proportion of strokes in both urban and rural settings. In Houston, Texas 3% of stroke patients presenting to three hospitals received thrombolysis on average 157 minutes after symptom onset and had outcomes broadly similar to those of the treatment group in Part II of the NINDS study (Chiu et al., 1998). Similarly, over 6% of patients presenting to 20 mostly rural hospitals in Illinois received thrombolysis an average of 147 minutes after symptom onset and had outcomes if anything slightly better than those of the NINDS study (Wang et al., 2000). Both of these studies are uncontrolled, and so it is not possible to make a robust estimate of the magnitude of any benefit accruing to patients. However, they do demonstrate that for around 5% of stroke patients thrombolysis is a practicable, relatively safe and potentially efficacious treatment.

Trials of putative neuroprotective agents in acute ischaemic stroke

An alternative therapeutic strategy has been to attempt to arrest or reverse the pathophysiological consequences of ischaemia rather than the ischaemia itself. While a large number of drugs have been shown to be effective in limiting infarct size and reducing behavioural deficits in animal models of stroke efficacy has yet to be demonstrated in human stroke. Possible reasons for this have been extensively reviewed and include include heterogeneity of infarct size and time since stroke; comorbidity in a predominantly elderly population; the use of functional measures of outcome rather than assessment of lesion size; physiological differences in the neuronal response to and tolerance of ischaemia between humans and rodents; and

difficulties in attaining appropriate drug concentrations in blood and brain because of haemodynamic compromise or concerns over side effects.

A number of agents that have been shown to have activity in animal stroke models have been tested in large-scale clinical trials. In some cases these trials have been suspended prior to completion, and so data is not available. In others, no effect was found (eg lubeluzole (Diener, 1998)). In some, initial studies suggested that particular subgroups of patients might benefit, and subsequent trials are ongoing to test these hypotheses (for example clomethiazole, a GABA-mimetic (Wahlgren et al., 1999), and piracetam, which may have AMPAkine activity (De Deyn et al., 1997)),

The efficacy of a number of other putative neuroprotective drugs has been investigated in somewhat smaller studies. Taken together these trials, along with those discussed above, indicate that no compound yet tested leads to a dramatic reduction in death and disability in unselected patients with stroke.

Other treatments in acute ischaemic stroke

The International Stroke Trial (International Stroke Trial Collaborative Group, 1997) examined the effect of starting treatment with aspirin (300mg per day) and/ or heparin (5,000 or 12,500 i.u. twice daily) in 19,435 patients within 48 hours of the onset of acute ischaemic stroke. Heparin at either dose had no effect on 14- day morality or on death or dependency at 6 months. While patients on heparin had fewer recurrent ischaemic strokes this was offset by an increased incidence of haemorrhagic stroke and they also had increased incidence of extracranial bleeding and requirement for transfusion. In contrast, the aspirin group showed a significant reduction of 1.1% in death or recurrent stroke occurring within 14 days; they had a non- significant 1.3% reduction in the number of patients dead or dependent at six months (61.2% versus 62.5%) which reached significance after adjusting for baseline stroke severity.

The Chinese Acute Stroke Trial (CAST Collaborative Group, 1997) examined the effect of treatment with aspirin (160mg) started within 48 hrs of acute ischaemic

stroke and demonstrated a significant reduction in 4 week in- hospital mortality.

Taken together these two large trials demonstrate that early treatment with aspirin leads to a small (0.9%) but significant ($p = 0.001$) reduction in the proportion of patients rendered dead or disabled (CAST Collaborative Group, 1997).

A recent meta- analysis of 20 controlled trials of stroke unit care demonstrated a 17% reduction in death, a 23% reduction in death or institutional care and a 25% reduction in death or dependency at 6 months. The benefits of stroke units were most marked in those over 75, males, and those with severe strokes (Stroke Unit Trialists' Collaboration, 1999).

Summary

Aspirin and stroke units improve outcome following stroke. Early thrombolysis is beneficial in some patients, but appears to be practicable in less than 10% of patients. In spite of a major research effort, no agent that has been shown to have neuroprotective properties in animal models of ischaemia has been demonstrated to have activity in clinical trials. Stroke therefore continues to represent a major challenge to the neuroscience community.

FK506 (TACROLIMUS)

FK506 is a macrolide immunosuppressant isolated from Streptomyces tsukubaensis strain 9993 in 1984 (Ochiai et al., 1987). Its structure is shown in Figure 1.1. FK506 first received attention as an inhibitor of interleukin 2 (IL-2) production *in vivo* in the mouse, and in this effect it was 30 times more potent than cyclosporine. FK506 was subsequently reported to inhibit IL-2 receptor expression in human T cells in mixed lymphocyte culture; in this case FK506 was 30 to 100 fold more potent than cyclosporine. In 1987 FK506 treatment following heterotopic cardiac allotransplantation in the rat was shown to result in permanent allograft survival in most animals, compared with mean graft survival in the control group of less than one week (Ochiai et al., 1987). FK506 has gone on to widespread clinical use as an immunosuppressant following kidney and liver transplantation.

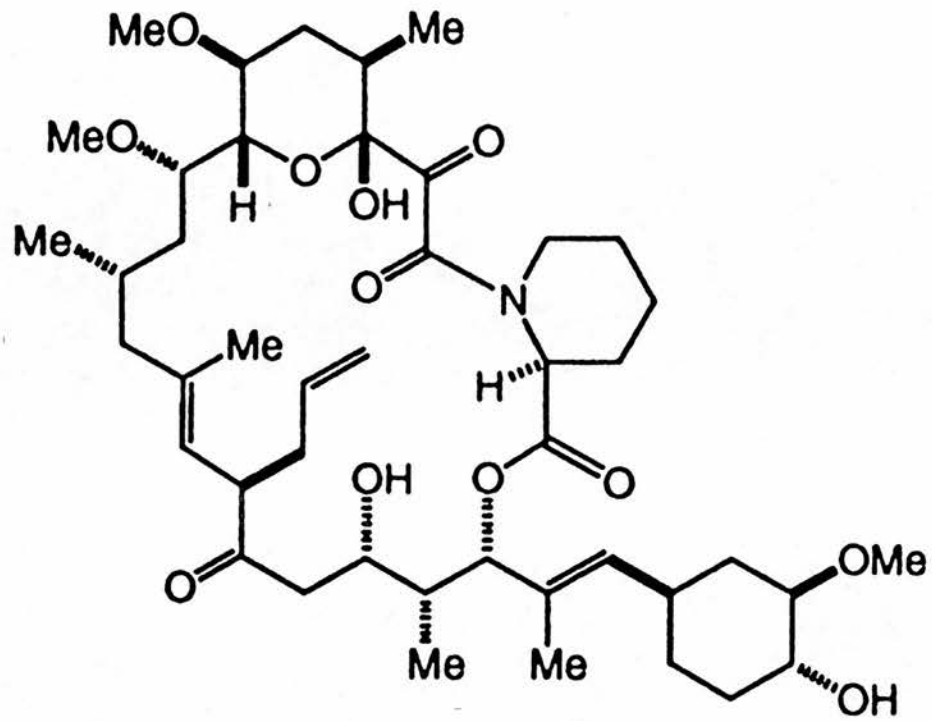


Figure 1.1: Structure of FK506

At the cellular level, FK506 interacts with a subfamily of immunophilins termed the "FK506 binding proteins" (FKBPs), which exhibit peptidyl-prolyl cis-trans isomerase activity (Harding et al., 1989; Siekierka et al., 1989), and are widely distributed in brain and other tissues (Dawson et al., 1994; Steiner et al., 1992). The immunosuppressive action of FK506 requires inhibition of protein phosphatase 2B (calcineurin; E.C. 3.1.3.16; (Clipstone and Crabtree, 1992; O'Keefe et al., 1992)) by a complex of FK506 and a 12kDa FKBP (FKBP12; (Kissinger et al., 1995; Liu et al., 1991)). Nuclear Factor of Activated T cells (NFAT) is a transcription factor whose targets include the gene encoding IL-2. Normally resident in the cytoplasm, NFAT is dephosphorylated and translocates to the nucleus following stimuli including ionomycin, and this translocation is associated with increased DNA binding activity (Shaw et al., 1995). NFAT dephosphorylation is mediated by calcineurin and inhibited by FK506 and cyclosporine (Loh et al., 1996; Shaw et al., 1995). The immunosuppressive properties of FK506 are due in large part to this

inhibition of IL-2 production in activated T cells.

In addition to these affects on T cell activation, FK506 inhibits activation induced apoptosis in T cell hybridomas (Bierer et al., 1990), an effect thought to be mediated through reduced expression of Fas ligand (Brunner et al., 1996).

FK506 also protects tissues in animal models of cardiac (Nishinaka et al., 1993), liver (Sakr et al., 1993) and intestinal (Kubes et al., 1991) ischaemia, although the mechanism of these effects is not clear. In the nervous system, FK506 has no effect on striatal excitotoxicity following intraperitoneal injection of NMDA, AMPA or quinolinate (Butcher et al., 1997). However, it is a potent neuroprotectant in animal models of focal (Bochelen et al., 1999; Sharkey and Butcher, 1994) and transient global (Drake et al., 1996; Tokime et al., 1996) cerebral ischaemia. In focal cerebral ischaemia at least this effect is shared by other calcineurin inhibitors such as cyclosporin (Sharkey and Butcher, 1994) and SDZ ASM 981 (Bochelen et al., 1999) and is blocked by rapamycin, a drug which binds FKBP12 but does not inhibit calcineurin, implying that inhibition of calcineurin is required for neuroprotective efficacy. However, the precise mechanism(s) underlying this protective effect are not clear.

While some have attributed the neuroprotective actions of FK506 to a reduction in calcineurin- mediated dephosphorylation and activation of neuronal nitric oxide synthase (Dawson et al., 1993) there remains considerable debate regarding the effects of phosphorylation on the activity of nNOS and on the relevance of any such effects to the neuroprotective actions of FK506. Other candidate mechanisms include a trophic effect of FK506 (Gold et al., 1999a; Steiner et al., 1997), or an effect on one of the many other putative FK506 targets including TGF-ß (Wang et al., 1994), IP_3 (Cameron et al., 1995) ryanodine (Brillantes et al., 1994) and steroid hormone (Beckman, 1991; Kosano et al., 1998; Milad et al., 1995; Silverstein et al., 1997; Yem et al., 1992) receptors; and the RAFT/MTOR kinases which belong to the ataxia telangiectasia mutuated (ATM)/ PI3 kinase family (Alarcon et al., 1996).

PURPOSE OF RESEARCH

The purpose of this research was to explore potential mechanisms of the neuroprotective effect of FK506. First, I have examined the effect of FK506 on NMDA stimulated cGMP accumulation in cerebellar prisms derived from neonatal rats. Subsequently I have described the characteristics of SHSY-5Y neuroblastoma cells under different culture conditions and their terminal differentiation, and I go on to examine the effects of FK506 on death induced by the protein kinase C inhibitor staurosporine, by the calcium ionophore ionomycin and by serum withdrawal in terminally differentiated SHSY-5Y cells.

CHAPTER 2

MATERIALS AND METHODS

SOURCES OF MATERIALS USED

1,1,2- triflurotrichloroethane	BDH Laboratory Supplies
13 mm diam. glass coverslips	BDH Laboratory Supplies
180 cm2 tissue culture plates	NUNC
2- mercaptoethanol	Sigma Chemical Company
24 well tissue culture plates	NUNC
3-[cyclohexylamino]-1-propanesulphonic acid	Sigma Chemical Company
35 mm diameter tissue culture dishes	NUNC
75 cm^2 tissue culture plates	NUNC
A1.1 T cell hybridoma cell line	gift from Doug Green, La Jolla, California
acetic acid	BDH Laboratory Supplies
acetyl-DEVD- aldehyde	Bachem
acetyl-DEVD-aminomethylcoumarin	Bachem

acrylamide	Sigma Chemical Company
all- trans retinoic acid	Sigma Chemical Company
ammonium persulphate	Sigma Chemical Company
anti- calcineurin A monoclonal antibody	Transduction Laboratories
anti- calcineurin B monoclonal antibody	Upstate Biotechnology Incorporated
anti- caspase 3 polyclonal antibody	gift from Don Nicolson, Merck Frosst Centre for Therapeutic Research, Quebec, Canada
anti- FKBP12 (3F4-70) monoclonal antibody	Fujisawa Pharmaceutical Company Ltd.
anti- MAP2 monoclonal antibody	Sigma Chemical Company
anti- p21waf monoclonal antibody	Pharmingen
anti- PARP monoclonal antibody	gift from Guy Poirier, Centre de Recherche du CHUL, Quebec, Canada
anti- phosphoserine polyclonal antibody 16B4	Alexis Neurochemicals
anti- tubulin amonoclonal antibody	Sigma Chemical Company
aprotinin	Sigma Chemical Company
avidin-biotin Elite™ mouse IgG ABC™ kit	Vector Laboratories
BCA™ protein assay kit	Pierce

bis-acrylamide	Sigma Chemical Company
bovine serum albumin	Sigma Chemical Company
Bromophenol Blue	Sigma Chemical Company
calcium and magnesium free Hanks balanced salt solution	Gibco
Calcein AM™	Molecular Probes
Complete Protease Inhibitor Cocktail™ tablets	Boehringer Mannheim
Coomassie Brilliant Blue™	Sigma Chemical Company
cyclic GMP radioimmunoassay kit	Amersham Life Sciences
DAB™ peroxidase substrate kit	Vector Laboratories
dexamethasone	Sigma Chemical Company
dimethylsulphoxide	Sigma Chemical Company
disodium hydrogen orthophosphate anhydrous	BDH Laboratory Supplies
dithiothrietol	Sigma Chemical Company
donkey anti-rabbit HRP linked secondary antibody	Amersham Life Sciences
Dulbecco's minimal essential medium	Gibco

Ecl+Plus™ chemilumenescence reagents	Amersham Life Sciences
Emulsifier-Safe scintillant	Packard Instrument Company
ethylene glycol-bis (ß-aminoethyl ether) N,N,N',N' tetraacetic acid	Sigma Chemical Company
ethylenediaminetetraacetic acid	Sigma Chemical Company
FK506	Fujisawa Pharmaceutical Company Ltd.
Fluoroscan Ascent fluorescent plate reader	Labsystems
foetal calf serum	Gibco
glucose	Sigma Chemical Company
glutaraldehyde	Sigma Chemical Company
glycerol	Sigma Chemical Company
glycine	Sigma Chemical Company
Ham's F12 medium	Gibco
Ham's F14 medium	Gibco
Hanks balanced salt solution	Gibco
horse serum	Gibco

hydrochloric acid	Sigma Chemical Company
hydrogen peroxide	Sigma Chemical Company
isopropanol	BDH Laboratory Supplies
L-nitro arginine methyl ester	Sigma Chemical Company
laminin	Sigma Chemical Company
LDH assay kit	Promega
leupeptin	Sigma Chemical Company
L-glutamine	Gibco
L-thyroxine	Sigma Chemical Company
lysine hydrochloride	Sigma Chemical Company
magnesium chloride	Sigma Chemical Company
magnesium sulphate	Sigma Chemical Company
McIlwaine tissue chopper	Mickle Laboratory Engineering Co. Ltd.
MK-801	Research Biochemicals International
modified Fuchs- Rosenthal haematocytometer	Fisher Scientific UK Ltd.

MTS Assay	Promega
N- methyl D-aspartate	Sigma Chemical Company
N,N,N',N', tetramethyethylene diamine	Sigma Chemical Company
N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid	Sigma Chemical Company
nitrocellulose electrophoresis membranes	Amersham Life Sciences
non fat dried mild	Bio Rad Laboratories
orthophosphoric acid	BDH Laboratory Supplies
paraformaldehyde	Fisher Scientific UK Ltd.
PD10 columns	Amersham Pharmacia Biotech
penicillin	Sigma Chemical Company
pepstatin	Sigma Chemical Company
phenylmethylsulphonyl flouride	Sigma Chemical Company
polyornithine	Sigma Chemical Company
polyoxyethylene (20) sorbitan monolaurate	Sigma Chemical Company
polyvinylidine difluoride (Immobilon™) electrophoresis membranes	Sigma Chemical Company

potassium chloride	BDH Laboratory Supplies
potassium dihydrogen orthophosphate	BDH Laboratory Supplies
potassium hydroxide	BDH Laboratory Supplies
progesterone	Sigma Chemical Company
putrescine	Sigma Chemical Company
recombinant human nerve growth factor	R&D Systems
sheep anti- mouse HRP linked secondary antibody	Amersham Life Sciences
SHSY-5Y cells (passage 8)	European Collection of Animal Cell Cultures
SHSY-5Y cells (passage 72)	gift from Prof. Nahorski, University of Leicester, UK
SigmaPlot 4.0 graphical software package	Jandel Scientific
sodium bicarbonate	BDH Laboratory Supplies
sodium cacodylate	BDH Laboratory Supplies
sodium chloride	Sigma Chemical Company
sodium dihydrogen orthophosphate dihydrate	BDH Laboratory Supplies
sodium dodecyl sulphate	Sigma Chemical Company

sodium fluoride	Sigma Chemical Company
sodium hydroxide	Sigma Chemical Company
sodium nitroprusside	Sigma Chemical Company
sodium periodate	Sigma Chemical Company
sodium selenite	Sigma Chemical Company
streptomycin	Sigma Chemical Company
sucrose	Sigma Chemical Company
trichloroacetic acid	BDH Laboratory Supplies
triiodothyronine	Sigma Chemical Company
tri-n-octylamine	BDH Laboratory Supplies
tris [hydroxymethyl] aminomethane	Sigma Chemical Company
Triton X-100	Kool-light Ltd Haversshill Sussex England
trypan blue	Gibco
trypsin	Gibco
urea	Sigma Chemical Company

CYCLIC GMP PRODUCTION IN NEONATAL RAT CEREBELLAR PRISMS

Tissue Preparation

8 day old Sprague Dawley rats of either sex were killed by decapitation and their cerebella rapidly dissected. These were transferred to pre-warmed (37°C) and pre-gassed (95% O_2/5% CO_2) Krebs-bicarbonate buffer (118mM NaCl, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 11.1mM glucose, 25mM $NaHCO_3$, and 1.2mM $CaCl_2$) and chopped on a McIlwain tissue chopper into 400μm^2 prisms. The prisms were transferred to a conical flask in a shaking water bath containing pre-warmed (37°C) and pre-gassed (95% O_2/5% CO_2) Krebs-bicarbonate buffer. The buffer was changed every 15 minutes for an hour; FK506 was included after the second buffer change where indicated.

Drug Exposure

50µl aliquots of gravity packed prisms were transferred to flat-bottomed tubes in the shaking water bath after the fourth buffer change. Prisms were exposed to NMDA or sodium nitroprusside (SNP), in the presence or absence of MK801, L-nitro arginine methyl ester (L-NAME) or FK506 in a final volume of 300µl for 5 minutes except where indicated. The reaction was terminated by the addition of 300µl ice cold 1M trichloroacetic acid, and the samples were left to stand on ice.

cGMP estimation

After 20 minutes the samples were vortex mixed and 500µl of the supernatant was added to 125µl of ethylenediaminetetraacetic acid (EDTA) (10mM, pH 7.0) and 500µl of a freshly made 1:1 mixture of 1,1,2-triflurotrichloroethane and tri-n-octylamine to remove unwanted impurities to the organic phase. Samples were vortex mixed and centrifuged at 10,000 g for 3 minutes and 400µl of the aqueous phase was added to 100µl of 60mM $NaHCO_3$ prior to storage at 4°C. Concentration

of cGMP was measured by radioimmunoassay according to the manufacturer's instructions. For each experiment, two 50µl aliquots of gravity packed prisms were taken for protein estimation. These samples were homogenised in 100µl phosphate buffered saline (PBS) and protein content was measured using the Bradford protein assay (Bradford, 1976).

Western Blotting

For determination of FKBP12 and calcineurin prisms were homogenised in Complete™ protease inhibitor solution according to the manufacturers instructions. An equal volume of 2x Laemmli sample buffer (final concentrations, sodium dodecyl sulphate, 2%; glycerol, 10%; dithiothrietol, 1mM; 2-mercaptoethanol, 5%; Tris-HCl [pH 6.8], 62.5mM; and Bromophenol Blue, 0.001%) was then added, and samples were heated to 80°C for 10 minutes. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis was performed using a 12.5% gel, and proteins were transferred to Immobilon-P™ polyvinylidene difluoride membranes overnight at 70mA. Membranes were blocked in PBS with 5% non-fat dry milk and 0.1% Tween20 for 30 minutes prior to exposure to antibodies against FKBP12 (1/200), calcineurin A (1/500) or calcineurin B (1/1000) for 2 hours. The membrane was washed for 4 x 5 minutes in the blocking solution before incubation with a horse radish peroxidase-conjugated sheep anti-mouse secondary antibody (1:1000) for one hour. These antibody dilutions were determined in preliminary experiments to represent the optimal balance between high specific and low non- specific binding. The membrane was washed for 3 x 10 minutes in blocking solution and for 30 minutes in PBS prior to detection of antigen by chemiluminescence using Ecl+Plus™.

For phosphosereine residue detection prisms were exposed to drug for 5 minutes as above then snap frozen on dry ice. They were homogenised in PBS and an equal volume of 2x Laemmli sample buffer was added as above. Samples were centrifuged at 10,000 rpm for five minutes to remove debris. Protein concentrations were estimated by Coomaassie staining against standard concentrations and adjusted by

further dilution with Laemmli sample buffer to 1 mg/ ml. Samples were heated to 90°C for 10 minutes. 10 μg of protein was added to each lane of a 10% gel and sodium dodecyl sulphate - polyacrylamide gel electrophoresis performed. Proteins were transferred to Immobilon-P polyvinylidene difluoride membranes overnight at 70mA. Membranes were blocked for 1 hour in Tris buffered saline pH 7.4 with 0.5% BSA, 0.2% Tween20 and 10mM NaF prior to exposure to antibody 16B4 (0.1 μg/ ml) in blocking solution. Membranes were washed 5 times for five minutes in TBS (pH 7.4) containing 0.1% Tween20 and incubated for 1 hour with an HRP-conjugated sheep anti-mouse secondary antibody (1:1000). These antibody dilutions were determined in preliminary experiments to represent the optimal balance between high specific and low non- specific binding. Membranes were again washed 5 times for five minutes in TBS (pH 7.4) containing 0.1% Tween20 prior to rinsing in TBS pH 7.4 and signal detection using ECL+Plus. Membranes were then stripped by incubation in 0.2M Glycine pH2.0, 0.1% SDS, 0.1% Tween20 at room temperature for 60 minutes and reprobed with a primary antibody directed against tubulin (1/1000) to correct for differences in protein loading. The resulting immunoblots were digitally captured and exported as .tif files to the AIDA software package, where the optical density of phosphoserine immunopositivity was normalised to tubulin immunopositivity to give the abundance of phosphorylated serine residues relative to the abundance of tubulin and by inference to total protein. Since calcineurin acts as a serine/ threonine protein phosphatase, changes in the activity of calcineurin will be reflected in changes in the relative abundance of phosphorylated serine residues.

Data analysis

Data represent the means of at least three independent experiments. Where a number of treatments were being compared to control, for instance in cell viability assays or effects of a number of drugs on cGMP concentration, statistical significance was assessed using one way analysis of variance. Where ANOVA indicated that a significant difference between groups existed then Dunnett's post hoc test was used. Where a single comparison was being made, for instance Ac-DEVD-amc cleavage

activity in serum withdrawn cultures versus control, or the effect of FK506 on phosphoserine immunoreactivity in cerebellar extracts, then the students' t-test was used. .

For the calculation of EC_{50} values for the cerebellar cGMP response to NMDA data were fitted to the formula $y = y_0 + a/ (1+\exp (- ((x-x_0)/b))$ using SigmaPlot 4.0, where x represents the concentration of NMDA, y represents the concentration of cGMP and y_0, a, b and x_0 are constants.

GENERATION OF TERMINALLY DIFFERENTIATED NEURON- LIKE CELLS

Cell culture

Cells were plated at a density of 10^6 cells per $175cm^2$ flask and grown in an incubator at 37°C in an atmosphere of 5% CO_2 and 95% air. They were grown in a medium consisting of Dulbecco's minimum essential medium (DMEM) containing 2mM L-Glutamine, 100iu/ml penicillin, 100 mg/ml streptomycin (DMEM/G/P/S) and 10% foetal calf serum (FCS)(Control medium). A tabulated description of the composition of the various media used is given in Table 2.1. Medium was changed after four days and cells were passaged after 7 days by detachment in Hank's balanced salt solution (HBSS) containing 0.1% EDTA, counting in a modified Fuchs- Rosenthal haematocytometer and replating.

Effect of differentiation on cell number

For determination of the effects of differentiation on cell number 2.5 x 10^6 cells at low or high passage were seeded to 80 cm^2 flasks in Control medium. After 24 hours medium was replaced with fresh medium of the same composition or with DMEM/G/P/S containing 1% FCS and 10μM retinoic acid (Differentiating medium),

and the medium was thereafter changed every 48 hours. Retinoic acid (RA) was prepared as a 10mM stock solution of all- trans retinoic acid in dimethylsulphoxide (DMSO)

Table 2.1: Composition of designated media

Basic Composition	Foetal Calf Serum	Retinoic Acid	Designation
DMEM + L-Glutamine + penicillin + streptomycin	10%	none	Control
		10μM	Retinoic acid
	1%	none	Serum reduction
		10μM	Differentiating
	none	10μM	Serum withdrawal

and stored in the dark at $-70^{O}C$ until required. Unused stocks were discarded after two weeks.

1, 4 and 8 days after plating (0, 3 or 7 days after the induction of differentiation) cells were detached by incubation in HBSS/ 0.1% EDTA and Trypan Blue excluding cells were counted in a modified Fuchs- Rosenthal haematocytometer.

Morphology and Immunocytochemistry

For morphological studies and immunocytochemistry cells were seeded at 50,000 cells per well on 13 mm diameter glass coverslips in 24 well plates in DMEM/G/P/S containing either 10% FCS (Control medium), 1% FCS (Serum reduction medium), 10% FCS and 10μM RA (Retinoic Acid medium) or 1% FCS and 10μM RA (Differentiating medium). Medium was replaced every 48 hours. At 1 or 8 days after plating cells were washed in PBS (pH 7.4) and fixed in a paraformaldehyde-lyseine-periodate fixative containing 60mM lyseine hydrochloride, 32.5mM Na_2HPO_4, 7.5mM $NaH_2PO_4.2H_2O$, 20mg/ml paraformaldehyde and 2mg/ml sodium periodate for 30 minutes. Cells were again washed in PBS, permeabilised for 5 minutes in 0.25% Triton- X, rinsed again in PBS and stored in PBS at $4^{0}C$.

For immunocytochemistry fixed cells were incubated with 0.6% hydrogen peroxide in methanol for 5 minutes, rinsed in water and incubated in PBS containing 0.2% bovine serum albumin (BSA) for 5 minutes. Non specific binding was blocked by incubating cells in PBS containing 10% BSA for 30 minutes prior to incubation with mouse anti- MAP2 monoclonal antibody or mouse anti- $p21^{waf}$ monoclonal antibody in PBS containing 3% BSA for two hours. Cells were subsequently washed three times for five minutes each in PBS with 0.2% BSA prior to sequential incubation with components of the avidin-biotin Elite mouse IgG ABC kit according to the manufacturer's instructions. Immunoreactivity was visualised using a DAB peroxidase substrate according to the manufacturer's instructions. Initial immunostaining was performed with a range of primary antibody dilutions to identify the optimal balance between specific and non-specific binding, and the final dilutions used were 1/1000 for the anti- MAP2 antibody and 1/200 for the anti- $p21^{waf}$ antibody. For each experiment for each condition sister preparations of fixed cells were processed with the omission of primary antibody to confirm the specificity of the observed immunoreactivity. Fixed cells were examined and photographed under phase contrast or light microscopy as appropriate.

CELL SURVIVAL EXPERIMENTS

SHSY-5Y cells

SHSY-5Y cells at passage 8 to 16 were seeded at 50,000 cells in 500µl medium per well in 24 well plates and differentiated as described above. After 7 days the effects of exposure to drugs or insults of interest was determined by washing cells 3 times in DMEM then incubating with the drug of interest dissolved in Differentiating medium or in Serum free medium (Differentiating medium lacking FCS). Cell survival was measured determined by measuring fractional LDH release, the MTS reduction assay or the Calcein AM™ assay.

LDH assay

50µl of culture medium was removed in duplicate from each well and processed for estimation of prelysis LDH concentration. The volume was readjusted to 500µl through the addition of 90µl of the appropriate medium and 10µl of 10% Triton X-100 to lyse remaining cells. After 15 minutes incubation 50µl of medium was removed in duplicate and processed for estimation of postlysis LDH concentration. Background signal was measured independently for prelysis (medium alone) and postlysis (49:1 mix of medium and 10% Triton X-100) samples. LDH concentration was measured by incubating the 50µl samples with 50µl of the reaction mixture in the dark for 30 minutes, after which time the reaction was stopped according to the manufacturer's instructions and absorbance at 560nm read in a colourimetric plate reader. The percent cell survival may be derived as follows:

$$\% \text{ Death} = \frac{\text{Prelysis[LDH]}}{\text{Total[LDH]}} \times 100 \quad (1)$$

$$\text{Total[LDH]} = \text{Prelysis[LDH]} + \text{Newly released[LDH]} \quad (2)$$

Because the addition of 100µl of fluid will dilute LDH present before cell lysis occurs by a factor of 400/500, the Postlysis[LDH] cannot be taken to represent the sum of Prelysis[LDH] and Newly released[LDH]. Rather,

$$\text{Measured postlysis[LDH]} = \text{Newly released[LDH]} + \{400/500 \times \text{Prelysis[LDH]}\}$$

Therefore

$$\text{Newly released[LDH]} = \text{Measured postlysis[LDH]} - \{400/500 \times \text{Prelysis[LDH]}\} \quad (3)$$

Combining (3) with (2)

$$\text{Total[LDH]} = \text{Measured Postlysis[LDH]} + \{0.2 \times \text{Prelysis[LDH]}\} \quad (4)$$

Combining (4) with (1),

$$\% \text{ Death} = \frac{\text{Prelysis [LDH]}}{\text{Measured Postlysis [LDH]} + \{0.2 \times \text{Prelysis [LDH]}\}} \times 100$$

While this correction has little impact at low levels of cell death, where death is 100% the uncorrected calculation estimates death to be 120%. The correction is valid provided that the starting volume of medium in which the cells were bathed is 500µl. While evaporation of medium does occur even in humidified tissue culture incubators, measurement of volume of medium remaining after 24 hours (the elapsed time since the last change of medium for these cells under most experimental conditions) showed an average volume reduction of less than 20µl per well. This is of no practical consequence, and would have resulted in maximum overestimation of death of less than one percent.

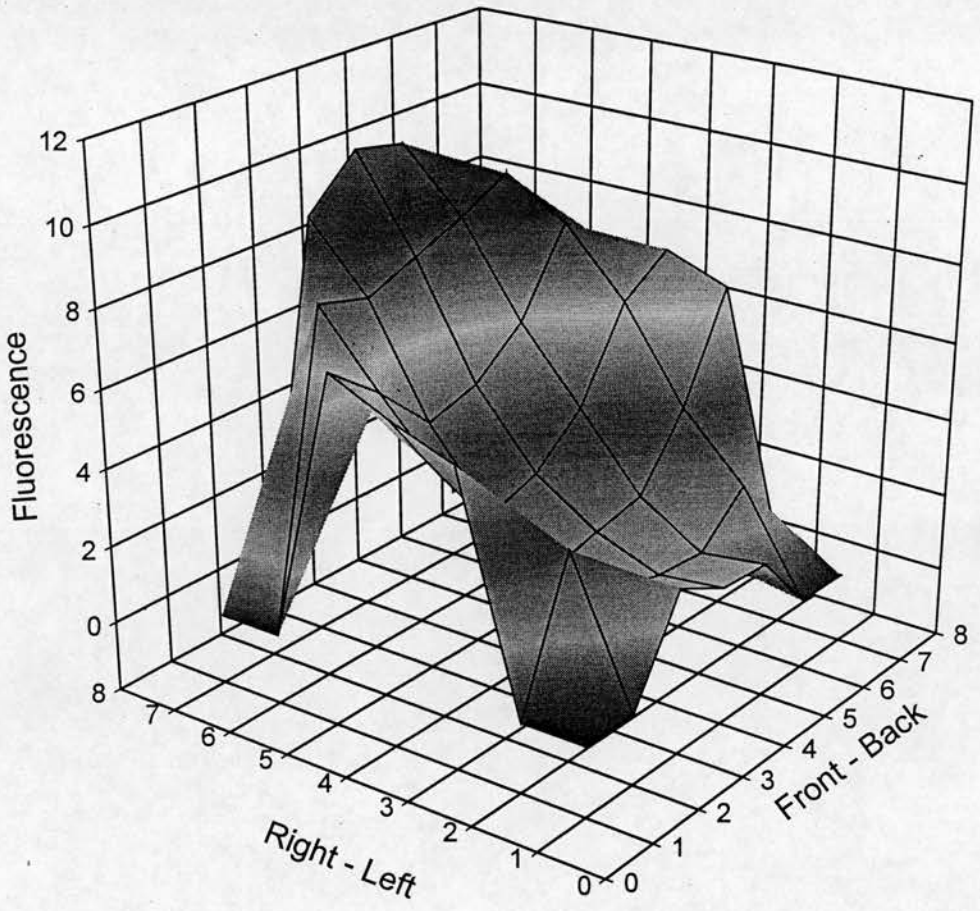
MTS assay

100µl of the MTS reagent was added to wells containing cells and to blank wells containing medium alone 3 hours before the end of the period of drug exposure. At the end of the drug exposure period 100µl aliquots in duplicate were removed and absorbance at 490 nM measured in a colourimetric plate reader.

Calcein AM assay

The Calcein AM assay is based on the unhindered passage of non-fluorescent Calcein AM into living cells, where cytoplasmic esterases cleave the AM group resulting in the development of fluorescence, which can then be quantified on a fluorescent plate reader. Preliminary experiments using Calcein AM as a measure of survival showed substantial variability in signal. In differentiated but otherwise untreated SHSY-5Y cells, measuring fluorescent signal from 37 discrete positions within the well showed great variability in the signal in different parts of the well (Figure 2.1; n=4 wells). These differences might reflect differences in the distribution

Figure 2.1: Effect of well position on Calcein AM signal



SHSY-5Y cells were seeded to 24 well plates at 50,000 cells per well and grown in Differentiation medium for 7 days. cell were then washed in PBS and exposed to PBS containing 1μM Calcein AM™ for 30 minutes. The well area was divided into 37 squares and intracellular retention of the esterase cleavage product Calcein was determined for each square by measuring emission at 520nM (excitation = 490nm) over an acquisition time of 60ms.

of cells within the well, optical characteristics of the tissue culture plastic, or a combination of the two. This, along with the variability seen in the Calcein AM signal, argued against the use of Calcein AM to estimate survival in SHSY-5Y cells.

Nuclear Morphology following serum withdrawal

Passage 8 SHSY-5Y cells were plated at 5 x 10^6 cells per 175cm^2 flask and differentiated for 7 days. They were washed 3 times in DMEM then exposed to Differentiating medium or Serum withdrawal medium for 24, 48 or 72 hours. Cells were detached in HBSS containing 0.1% EDTA and washed in warm cadolyate buffer (0.1M pH 7.4) before fixing in 3% glutaraldehyde in cadolyate buffer and processing in thick sections by toluidine blue staining by staff of the electron microscopy facility at the Royal Dick Veterinary College.

Ac-DEVD cleavage activity

Treated cells were harvested by scraping with a rubber policeman, washed in ice cold PBS (pH 7.4) and collected by centrifugation at 1000g for 15 minutes. Cells were lysed in a buffer containing 10mM N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid (HEPES) KOH pH 7.4, 50mM KCl, 2mM $MgCl_2$, 5mM ethylene glycol-bis (ß -aminoethyl ether) N,N,N',N' tetraacetic acid (EGTA), 0.5% 3-[cyclohexylamino]-1-propanesulphonic acid (CHAPS), 1mM phenylmethysulphonyl flouride, 10µg/ml pepstatin A, 10µg/ml leupeptin, 10µg/ml aprotinin and 1mM dithiothrietol (DTT) for 30 minutes on ice, subjected to one freeze/thaw cycle in liquid nitrogen and centrifuged at 2000g for 30 minutes. Protein concentration in the supernatant was measured after 1:10 dilution in distilled water using the BCA method according to the manufacturer's instructions and was adjusted to 2mg/ml by dilution in a buffer containing 25mM HEPES-KOH pH 7.4, 50mM NaCl, 5mM EGTA, 0.1% CHAPS, 10mM DTT, 5mM EDTA and 10% sucrose. An extract prepared by Dr. Timothy Allsopp from A1.1 cells undergoing apoptosis following dexamethasone treatment served as positive control.

10µl of extract was incubated at 37°C with Ac-DEVD-amc (final concentration 25µM) in the presence of absence of inhibitor (Ac-DEVD-aldehyde; final

concentration 187.5nM). Fluorescence at 460nm was measured every 90s for 90 minutes, and DEVD cleavage activity was determined by fitting a straight line to the linear portion of the fluorescence accumulation curve. Results are expressed as units of fluorescence per mg protein per minute and represent the means of at least three independent determinations.

Western blotting

Treated cells were harvested by scraping with a rubber policeman, washed in ice cold PBS (pH 7.4) and collected by centrifugation at 1000g for 15 minutes. Cells were resuspended in a buffer containing 2% sodium dodecyl sulphate, 10% glycerol, 6M urea, 5% 2-mercaptoethanol, 62.5mM Tris-HCl [pH 6.8] and 0.001% Bromophenol Blue before sonication for 20s on ice. Protein content was estimated by Coomassie staining and 15µg of protein per lane was loaded to 8% (PARP) or 15% (Caspase 3) gels for SDS-PAGE. Electrophoresis was performed at 40mA prior to transfer to nitrocellulose (PARP) or polyvinylidene difluoride (Caspase 3) membranes at 60mV. For PARP, membranes were blocked in PBS with 5% non-fat dried milk and 0.1% Tween (PBSMT) for 1 hour prior to incubation with a mouse anti-PARP monoclonal antibody (C2-10, 1/10,000, gift from Guy Poirier, Centre de Recherche du CHUL, Quebec, Canada) overnight. The membrane was washed five times for 5 minutes in PBSMT prior to incubation with sheep anti-mouse HRP linked secondary antibody (1/2000) for 30 minutes. These antibody dilutions were determined in preliminary experiments to represent the optimal balance between high specific and low non- specific binding. The membrane was again washed five times for five minutes in PBSMT and then for 30 minutes in PBS. Signal was detected by chemiluminescence (ECL+Plus™).

For Caspase 3 immunoreactivity, membranes were blocked in tris buffered saline pH 8.0 (TBS) with 5% non-fat dried milk and 0.1% Tween for 1 hour prior washing twice for five minutes in TBS/ 0.1% Tween and subsequent incubation with a rabbit anti-Caspase 3 polyclonal antibody (1/2,000, gift from Don Nicolson, Merck Frosst Centre for Therapeutic Research, Quebec, Canada). The membrane was washed three times for 5 minutes in TBS/ 0.1% Tween prior to incubation with donkey anti-rabbit

HRP linked secondary antibody (1/2,500) for 1 hour. These antibody dilutions were determined in preliminary experiments to represent the optimal balance between high specific and low non- specific binding. The membrane was washed three times for five minutes in TBS/ 0.3% Tween then three times for five minutes in TBS/ 0.1% Tween prior to detection of signal by chemiluminescence (Ecl+plus™).

Trigeminal Sensory Ganglion Cell Culture

To investigate the effects of FK506 in a previously described model of trophic factor withdrawal induced apoptosis a series of experiments was performed by Dr. Timothy Allsopp. These experiments were designed to complement the work on SHSY-5Y cells and so are reproduced here with due acknowledgement. The methodology used was as follows.

Mouse embryos were removed from time mated pregnant females and staged at embryonic day 14 according to the criteria of Theiler (Theiler, 1972). At this stage in vivo trigeminal sensory neurons are undergoing naturally- occurring cell death that is suppressed by target- derived nerve growth factor (NGF). Ganglia were aseptically dissected using sharpened tungsten needles and incubated for 7 min at 37^0C with 0.05% trypsin in calcium/ magnesium free Hanks balanced salt solution. After removal of the trypsin solution cells were washed in Ham's F12 medium containing 5% heat inactivated horse serum and were separated by gentle trituration through a fire polished siliconised Pasteur pipette to give a single cell suspension. 300 to 700 cells were plated in 35mm diameter culture dishes which had been precoated with polyornithine (0.5 mg/ml) and laminin (20 µg/ml). Neurons were grown at 37°C, 5% CO_2 in Ham's F14 supplemented with 2mM glutamine, 0.35% bovine serum albumin, 60ng/ml progesterone, 16µg/ml putrescine, 400ng/ml L-thyroxine, 38ng/ml sodium selenite, 340ng/ml triiodothyronine, 60mg/ml penicillin, 100mg/ml streptomycin and 4ng/ml recombinant human NGF. These conditions resulted in cultures composed of over 90% neurons.

After 24 hours the number of neurons in a 12mm x 12mm grid in the centre of each dish were counted. Neurons were then washed in NGF-free medium and then grown

with or without NGF in the presence of varying concentrations of FK506. For estimates of cell viability the number of viable neurons remaining in the same 12mm x 12mm grid were counted every 24 hours and the results expressed as a percentage of the initial count. Neurons were considered viable if they possessed a phase bright cell soma and intact neurites more than two cell body diameters in length.

In vitro stability of FK506 in solution

In order to ensure the continuing bioavailability of FK506 in tissue culture plates concentrations of FK506 in culture medium were measured by radioligand binding assay (kindly performed by Dr Abby Charters). Briefly, FK506 was added to Differentiation medium at concentrations ranging from 1nM to 1μM and these solutions placed in triplicate in wells of a 24 well plate for 24 hours in the tissue culture incubator. 50μl aliquots of medium or of freshly prepared FK506 were added to a reaction buffer containing 50mM Tris-HCl, pH 7.4, 172pM [^{3}H]FK506, 40μg/ml cytosolic protein, and 2mg/ml BSA and incubated at room temperature for 60 minutes. Non specific binding was determined in the presence of 1μM FK506. Bound ligand was separated from free by gel filtration on PD-10 columns and eluted with 50mM NaCl. 1ml eluent was mixed with 20ml Emulsifier-Safe scintillant and counted in a scintillation counter. A straight line was fitted to a plot of log(average DPM) against log(concentration known FK506) and from the formula describing this line the concentration of FK506 remaining at 24 hours was estimated.

Table 2.2: Stability of FK506 in tissue culture environment

[FK506] added	Measured [FK506] at 24 hours
1 μM	172 nM
100 nM	33 nM
10 nM	1.8 nM
1 nM	950 pM

The results are given in Table 2.2, from which it can be seen that concentrations had

fallen by four fifths at 24 hours. Based on these results it was judged that for exposures to FK506 of 24 hours or less a single application of FK506 was sufficient, but that for longer exposures it would be necessary to apply fresh FK506 every 24 hours.

CHAPTER 3

EFFECT OF FK506 ON NITRIC OXIDE SYNTHASE- MEDIATED CYCLIC GMP PRODUCTION IN NEONATAL RAT CEREBELLAR PRISMS

INTRODUCTION

The stereotactically guided application of the potent vasoconstrictor endothelin-1 to the proximal middle cerebral artery of rats consistently results in the development of large cortical and striatal infarcts (Sharkey et al., 1993; Sharkey and Butcher, 1995). Administration of the macrolide immunosuppressant FK506 up to 60 minutes after the onset of vasoconstriction results in a reduction in the volume of cortical infarction of at least 46% (Sharkey and Butcher, 1994) and improved performance on tests of skilled motor tasks (Sharkey et al., 1996). These protective effects have also been demonstrated in models of global ischaemia (Drake et al., 1996; Tokime et al., 1996). FK506 also has protective effects in animal models of cardiac (Nishinaka et al., 1993), liver (Sakr et al., 1993) and intestinal (Kubes et al., 1991) ischaemia. The mechanism by which this protection occurs is presently unclear.

At the cellular level, FK506 interacts with a subfamily of immunophilins termed "FK506 binding proteins" (FKBPs), which exhibit peptidyl-prolyl cis-trans isomerase activity (Harding et al., 1989; Siekierka et al., 1989), and are widely distributed in brain and other tissues (Dawson et al., 1994; Steiner et al., 1992). In focal cerebral ischaemia at least it appears that the interaction with FKBPs is required for neuroprotection, as displacement of FK506 from FKBPs by rapamycin blocks the protective effect. Furthermore, the neuroprotective properties of FK506 are shared by SDZ ASM 981, a drug which binds FKBP12 and inhibits calcineurin (Bochelen et al., 1999), and by cyclosporin (Sharkey and Butcher, 1994), which

inhibits calcineurin by a different mechanism.

The immunosuppresive action of FK506 requires inhibition of protein phosphatase 2B (calcineurin; E.C. 3.1.3.16) by a complex of FK506 and a 12kDa FKBP (FKBP12) (Kissinger et al., 1995; Liu et al., 1991). In addition, FKBP12 interacts with several other proteins including the skeletal muscle ryanodine receptor (Wagenknecht et al., 1997), the inositol triphosphate receptor (IP_3) (Cameron et al., 1995; Ohi et al., 1999), and the type 1 transforming growth factor-ß receptor (Wang et al., 1994); FKBP56 is associated with steroid receptor complexes (Yem et al., 1992). Effects of FK506 on the function of these proteins may be direct, via inhibition of the intrinsic isomerase activity of the relevant immunophilin, or indirect, via disruption of FKBP-mediated anchoring of calcineurin to a target protein. For example the effect of FK506 on calcium flux through the IP_3 receptor is mediated by displacement of calcineurin from a protein complex that includes FKBP12 and the IP_3 receptor (Cameron et al., 1995).

FK506 inhibits excitotoxic cell death in vitro (Dawson et al., 1993), but not in vivo (Butcher et al., 1997). Both excitotoxin- and free radical- induced damage may contribute to neuronal death in stroke; indeed, excitotoxic stimulation of NMDA receptors results in activation of NOS and production of NO free radicals (Bredt and Snyder, 1989). It is now clear, both from studies with specific inhibitors of nNOS such as 7 -nitroindazole (Dalkara et al., 1994) and from experiments using animals with targeted deletions in genes encoding NOS isoforms (Huang et al., 1994; Samdani et al., 1997), that nNOS activation contributes to neurotoxicity in cerebral ischaemia and that its inhibition is neuroprotective. Since NOS activity may be regulated by phosphorylation (Bredt et al., 1992; Dawson et al., 1993), FK506 might exert its neuroprotective effect by increasing NOS phosphorylation (via inhibition of calcineurin), leading to inhibition of NOS activity and a consequent reduction in NO production (Dawson et al., 1993).

Other potential mechanisms subserving the neuroprotective effect of FK506 include an effect on apoptosis following stroke. There is considerable evidence, reviewed

above, to indicate an important contribution of apoptosis to neuronal death in cerebral ischaemia. It is therefore of interest that FK506 inhibits apoptotic cell death in lymphocytes (Bierer et al., 1990) and in ischaemic brain FK506 reduces expression of Fas ligand and TNF related apoptosis inducing ligand (TRAIL) (MartinVillalba et al., 1999). Also, FK506 has been observed to have neurotrophic effects (Snyder and Sabatini, 1995; Steiner et al., 1997; Gold et al., 1999b), and these might sustain neurons deprived of endogenous survival influences following ischaemia.

In this chapter I describe a series of experiments designed to test the hypothesis that the neuroprotective effect of FK506 is mediated through inhibition of NOS. nNOS is activated in response to NMDA receptor stimulation and catalyses the conversion of arginine to citrulline and NO. NO thus produced diffuses to surrounding cells and activates guanylate cyclase, leading to the accumulation of the second messenger cyclic GMP (Dawson et al., 1992). Here I describe the effects of FK506 on NMDA stimulated, nNOS-mediated accumulation of cGMP in a well characterised system, the neonatal rat cerebellar prism (Garthwaite et al., 1988; Garthwaite and Balazs, 1978).

RESULTS

Optimisation of methodology

1. Measurement of NOS activity

Since cGMP has a longer half life than NO and NO is freely diffusible the level of cGMP provides a more stable indication of NOS activity than the measurement of NO. Furthermore, in preliminary experiments both the Griess reaction and a commercially available nitrate detection assay were insensitive to stimulation of neonatal rat cerebellar prisms with supramaximal concentrations of either NMDA or the NO donor sodium nitroprusside (SNP).

2. Choice of tissue

While the cerebellum may be damaged following ischaemia in posterior circulation

Syndromes (see page 20), ischaemia of the cerebral hemispheres in anterior circulation syndromes is a more common cause of stroke. Furthermore, most animal work models forebrain ischaemia and it would therefore be appropriate to study the cGMP response in cortical or hippocampal prisms. Finally, because most strokes affect adults rather than neonates, it would be most appropriate to study the response in adult tissues. However, prisms prepared from adult cortex and hippocampus showed no detectable cGMP response to supramaximal concentrations of NMDA or SNP. Neonatal cortex was also unresponsive, and the small volume of tissue recovered from the harvesting of neonatal hippocampi militated against using this tissue because of the large numbers of animals that would be required.

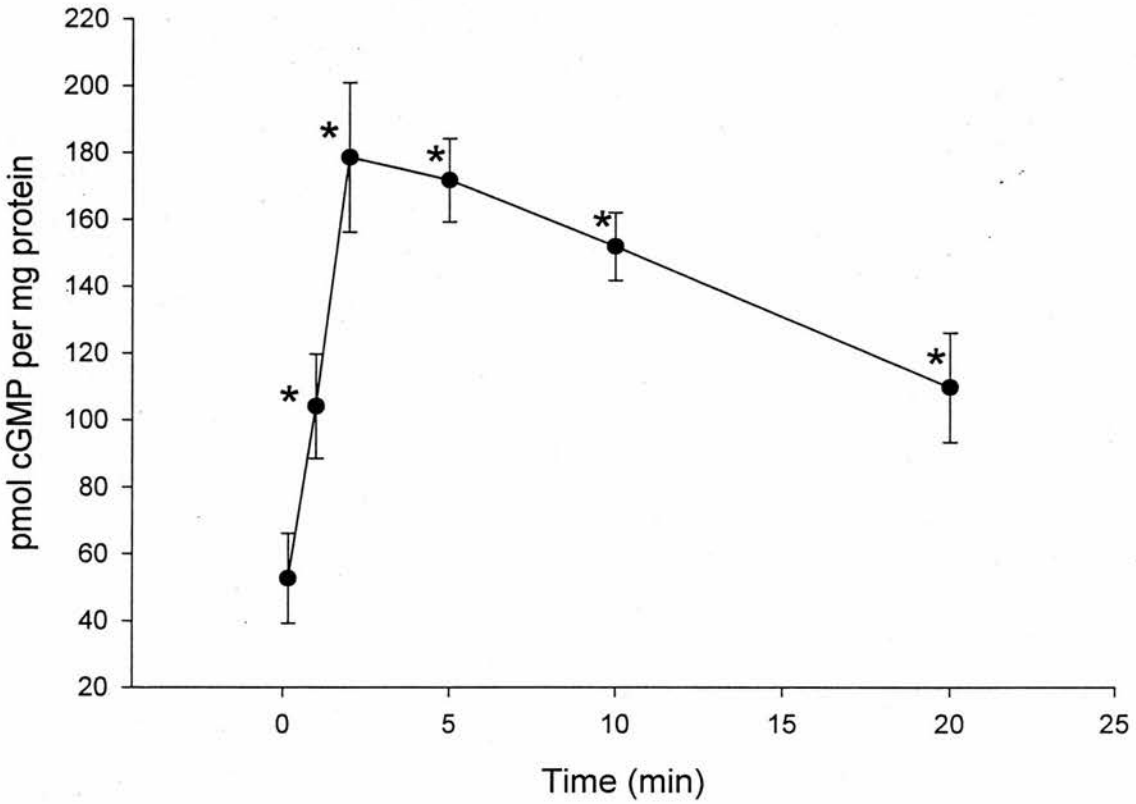
In the cerebellum, the supersensitivity of cGMP to NMDA is a transient phenomenon first seen around 5 days, observed maximally in tissue from 8 day old pups and much reduced by 21 days (Garthwaite and Balazs, 1978). I therefore used prisms prepared from the cerebella of 8-day-old rats. This is a tissue in which a robust cGMP response has been described, from which nNOS was originally sequenced (Bredt et al., 1991a) and which contains high levels of nNOS mRNA and protein (Bredt et al., 1991b; Sessa et al., 1993).

Response of neonatal rat cerebellar prisms

In neonatal rat cerebellar prisms NMDA induced a rapid accumulation of cGMP (Fig 3.1). This effect reached a maximum at 2 minutes ($p<0.05$). All subsequent experiments were carried out with a five-minute incubation. The response was concentration dependent (Fig 3.2) with an EC_{50} of 16μM and maximum cGMP production of 240 +/-15 pmol per mg protein (mean +/- s.e.m.) in response to 100μM NMDA. NMDA- stimulated cGMP production was inhibited by the NMDA receptor antagonist MK801 (10μM) ($p<0.05$) and by the NOS inhibitor L-NAME (10 μM) ($p<0.05$). Neither of these agents affected SNP-stimulated cGMP production (Fig 3.3).

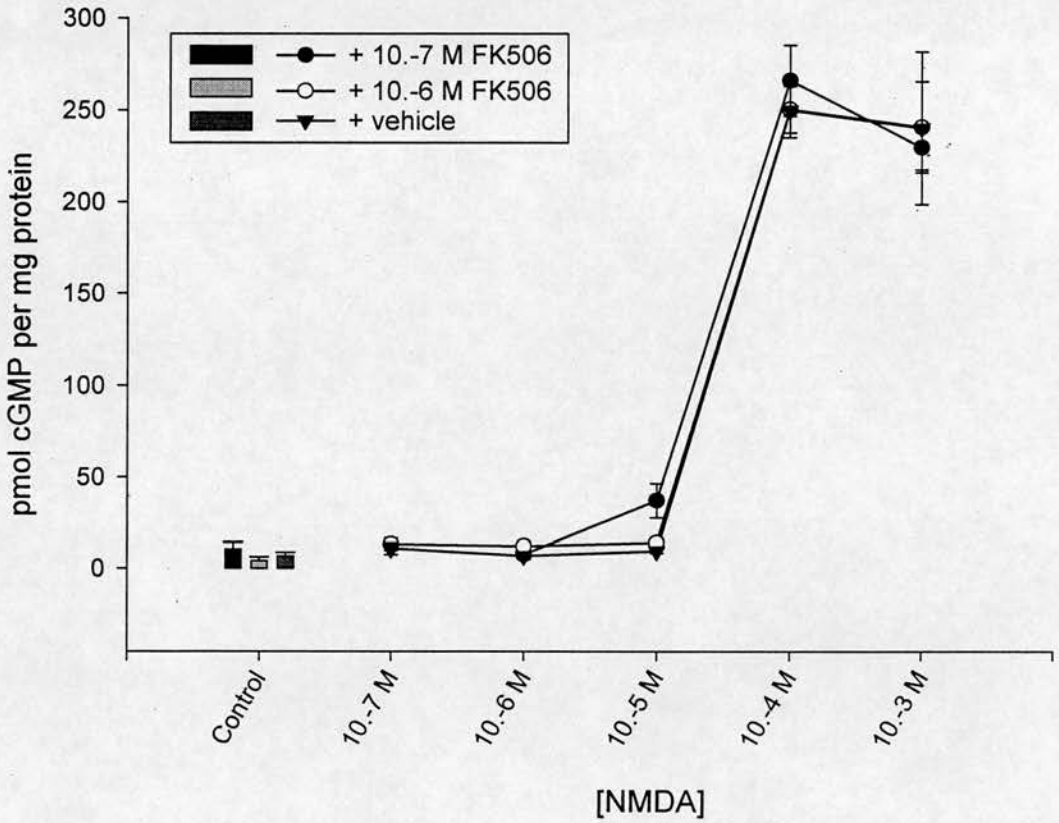
FK506 had no affect on NMDA- induced cGMP accumulation when added simultaneously (data not shown), and pre-incubation with FK506 for 30 minutes was

Figure 3.1: Timecourse of cGMP accumulation.



50µl of packed cerebellar prisms were exposed to 100µM NMDA in a final volume of 300µl for the times indicated. The reaction was terminated by addition of 1M trichloroacetic acid and cGMP was extracted and quantified by radioimmunoassay. cGMP is expressed as pmol/mg protein and each point represents the mean ± s.e.m. of at least 5 determinations. *$p<0.05$ versus 10 seconds sample (one way ANOVA with Dunnett's method for multiple comparisons).

Figure 3.2: Effect of FK506 on NMDA-induced cGMP accumulation.



Following a 30 minute incubation with vehicle (0.1% ethanol), 100nM FK506 or 1μM FK506 samples were exposed to varying concentrations of NMDA for five minutes and cGMP accumulation quantified as previously described. Data points represent the mean ± s.e.m. of at least 6 determinations.

also ineffective; under these conditions the EC_{50} and maximum cGMP production were 22μM and 265 ± 19 pmol per mg protein respectively for 100nM FK506 and 18μM and 247 ± 12 pmol per mg protein for 1μM FK506 (Fig 3.2).

To show that FK506 could inhibit calcineurin under these conditions I first sought to demonstrate that the relevant proteins are present. Western blotting of samples from neonatal cerebellar prisms demonstrated immunoreactivity at appropriate molecular weights for FKBP12, calcineurin A and calcineurin B using specific antibodies (Fig 3.4a).

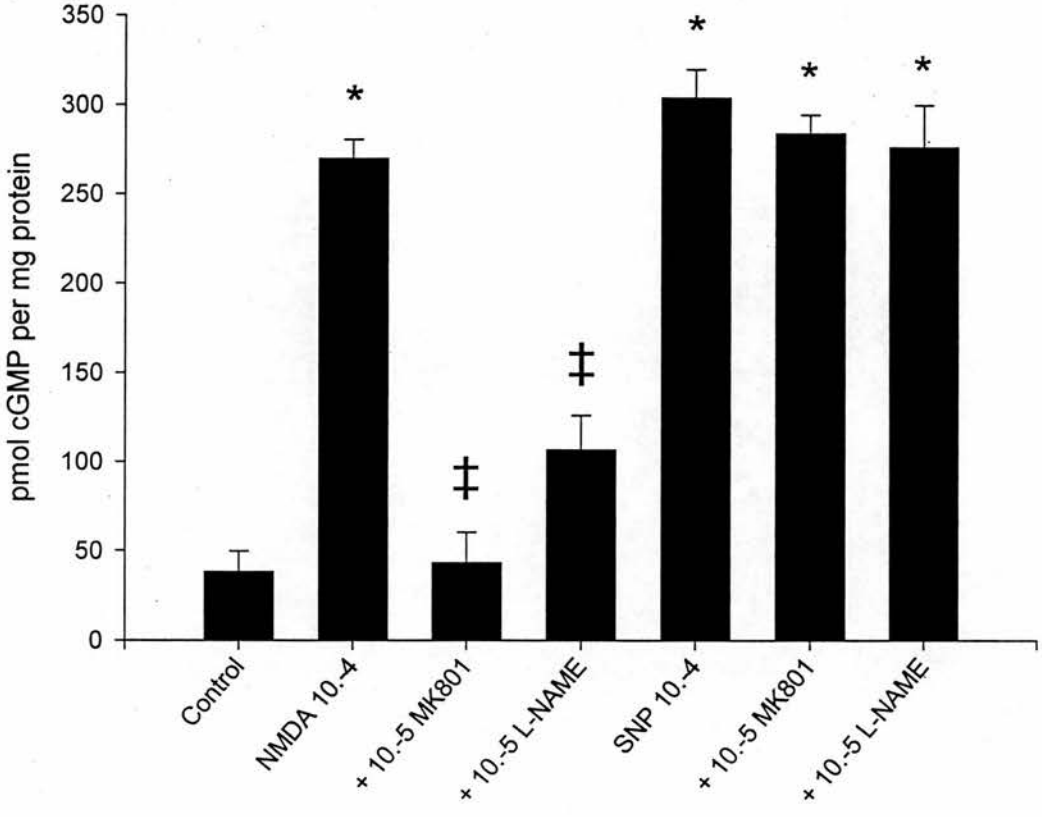
To determine whether FK506 can inhibit calcineurin activity in cerebellar prisms I measured the abundance of phosphoserine residues in protein extracts of NMDA-stimulated prisms. Calcineurin acts as a serine phosphatase, and its stimulation, for instance following treatment with NMDA, will lead to reduced levels of serine phosphorylation. Inhibition of this stimulated activity, for instance by FK506, should lead to increased serine phosphorylation.

Because the cGMP response to NMDA was determined following a 30-minute preincubation with FK506 or vehicle, prisms were preincubated with FK506 for 30 minutes prior to NMDA exposure and subsequent determination of phosphoserine residues. Using an anti- phosphoserine antibody Western blotting demonstrated the presence of a number of immunopositive proteins of varying molecular weight following treatment with 1mM NMDA. However, when prisms had been preincubated with 100nM FK506 for 30 minutes prior to NMDA exposure relative phosphoserine immunopositivity was increased by 26 +/- 4% (mean +/- s.d., n=3) over that seen with NMDA alone (Fig 3.4b,c), confirming inhibition of calcineurin activity.

DISCUSSION

The results presented in this chapter demonstrate that NMDA stimulated cGMP accumulation is inhibited by the NMDA receptor antagonist MK801 and the NOS inhibitor L-NAME but not by FK506.

Figure 3.3: Effect of MK801 and L-NAME on NMDA- and SNP-stimulated cGMP accumulation.



Samples were incubated for five minutes in the presence or absence of NMDA (100μM), SNP (100μM), MK801 (10μM) and L-NAME (10μM) before cGMP quantification as previously described. Data points represent the mean ± s.e.m. of at least 4 determinations. *$p<0.05$ versus control, ‡$p<0.05$ versus NMDA alone (one way ANOVA with Dunnett's method for multiple comparisons).

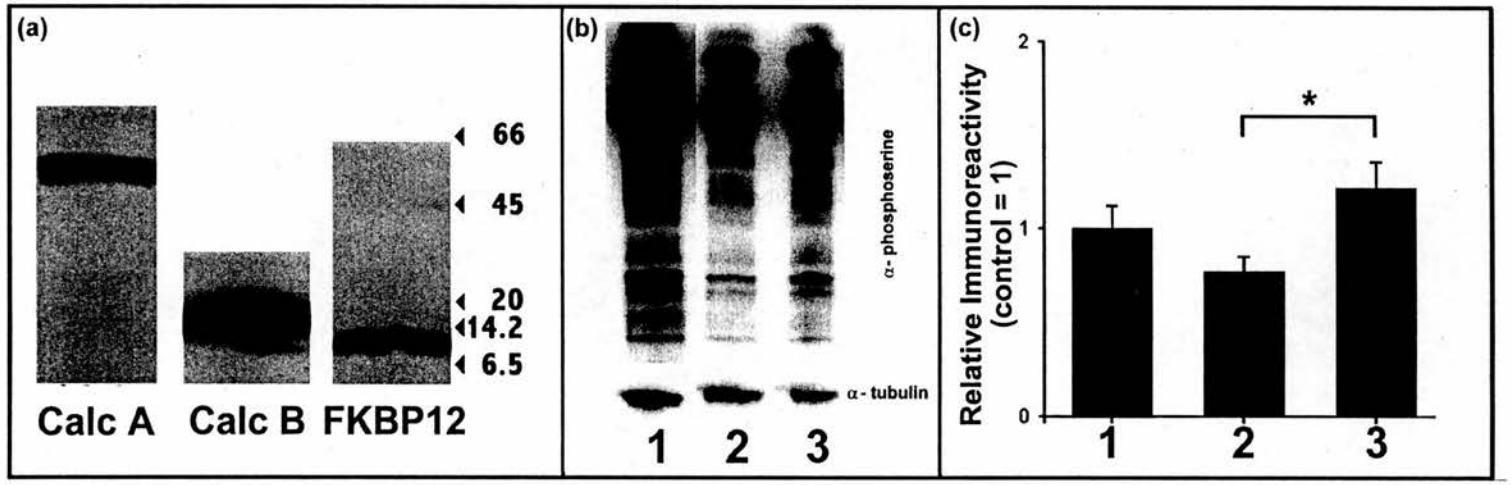
A potential criticism of this interpretation is that cerebellar prisms from neonatal rats might not express the full repertoire of proteins required to transduce FK506 mediated calcineurin inhibition. To meet this criticism I have demonstrated the presence of FKBP12 and both A and B subunits of calcineurin by Western blotting.

A second criticism might be that even given the necessary components, FK506 might not inhibit calcineurin activity at the concentrations tested in this preparation over this timescale. FK506 is known to inhibit calcineurin in vitro, so simply demonstrating calcineurin inhibition in extracts prepared from cerebellar prisms does not demonstrate inhibition of activity in the prisms themselves; conditions might be created during preparation of samples for assays of calcineurin activity that allowed FK506 to inhibit calcineurin under assay conditions when it was without activity before the extraction. For instance, FKBP12 and calcineurin might exist in separate cellular compartments prior to extraction, and be brought together following tissue homogenisation. For these reasons, measuring the effect of FK506 on calcineurin activity as measured in for instance the RII kinase assay (Perrino et al., 1992) is not helpful.

To demonstrate FK506 inhibition of calcineurin following a 30 minute incubation I have measured the abundance of immunoreactive phosphoserine residues at Western blotting and demonstrated that serine phosphorylation in NMDA treated prisms is increased following pre-incubation with FK506 consistent with calcineurin inhibition. The speed with which these samples were processed minimises any likelihood of artefactual changes in phosphorylation occurring during sample preparation.

The present data demonstrate that in neonatal rat cerebellar prisms cGMP accumulates through the activity of NOS; that this accumulation is inhibited by NOS inhibition; and that a concentration of FK506 that increases serine phosphorylation of cellular proteins has no effect on NMDA stimulated cGMP accumulation. While it is possible that FK506 is altering protein serine phosphorylation through some mechanism other than inhibition of calcineurin, direct effects on other phosphatases

Figure 3.4: Proteins and protein phosphorylation in cerebellar prisms



(a) Western blotting of protein extracts from neonatal rat cerebellum. Protein was extracted as described in Materials and Methods and western blotting performed using antibodies against calcineurin A, calcineurin B or FKBP12. Molecular weights (kdal) were estimated from coloured molecular weight standards. (b) unstimlated cerebellar prisms (1), and prisms preincubated for 30 minutes with vehicle (2) or 100nM FK506 (3) prior to 5 minute exposure to 1mM NMDA (2,3), were snap frozen in dry ice, homegenised in ice cold PBS and resuspended in Laemmli sample buffer prior to western blotting with anti- phosphoserine antibody. (c) densitometry of images from (b) (three independent blots) normalised to abundance of tubulin confirms increased anti- phosphoserine immunopositivity in presence of FK506 ($n=3$, $p<0.01$, t-test).

or kinases have not been described at these concentrations of FK506, and the most parsimonious interpretation of the data is that FK506, while inhibiting calcineurin activity, has no effect on NOS activity.

Phosphorylation of NOS was not measured directly because the effects of phosphorylation on NOS activity are not clear. In a genetically modified human kidney cell line overexpressing nNOS, where the nNOS sequence had been derived from rat cerebellum, phosphorylation of NOS results in reduced activity (Dawson et al., 1993). Similarly, NOS activity is reduced by activation of protein kinase C (PKC) (Bredt et al., 1992) and protein kinase A and G (Dinerman et al., 1994). PKC depletion or inhibition increases basal and stimulated NOS activity in cultured cerebellar granule cells (Riccio et al., 1996), and NMDA-stimulated cGMP production is reduced by PKC activation in mouse cortical cultures (Carroll et al., 1996).

However, PKC-activation increases cGMP production in response to metabotropic glutamate receptor stimulation in adult rat cerebellar slices (Okada, 1995) and NMDA stimulated NOS activity in cultured mouse striatal neurons is dependent on PKC (Marin et al., 1992). While CaM kinase II-mediated phosphorylation decreases NOS activity in whole brain extracts, phosphorylation by PKC increases activity (Nakane et al., 1991). In nNOS purified from cerebellar tissue PKC increases the maximal in vitro activity while in cerebellar slices it increases the calcium sensitivity but has no effect on maximal nNOS activity. This increased calcium sensitivity occurs without an increase in nNOS phosphorylation, implying the phosphorylation of an intermediary protein (Okada, 1996). The effects of phosphorylation on NOS activity are therefore not clear, and consequently reduced NOS activity cannot be inferred from increased NOS phosphorylation.

In addition to these context- dependent differences in the effects of NOS phosphorylation, the cGMP response of cerebellar prisms to NMDA changes with age. Prisms from animals 5-21 days old demonstrate a pronounced cGMP response that is not seen with prisms from older animals. Not all of this age related decline in

responsiveness can be attributed to disruption of neuronal arborisation during tissue preparation, since at intermediate ages there is reduced responsiveness in the face of preservation of structure (Garthwaite and Balázs., 1978), and differences between neonatal and adult tissue in the distribution of cGMP production may be more important in this regard (De Vente et al., 1990). In addition, a two component inhibition of NMDA-stimulated cGMP production in neonatal rat cerebellar prisms has been observed with L-N^G-nitroarginine (East and Garthwaite, 1990), implying the co-existence of multiple regulatory pathways.

While the data presented here demonstrate that FK506 has no effect on NOS activity in neonatal cerebellar prisms, they cannot be taken to prove the lack of such an effect in adult cortex. Not only are there age related differences in cGMP responsiveness, but also the activity of phosphorylated NOS varies depending on the tissue in which it is tested. Without further evidence it would therefore be unjustified to generalise from the current data to infer a lack of effect of FK506 on NOS activity in adult rats subjected to focal cerebral (predominantly cortical) ischaemia.

However, such further evidence does exist. In 1993, Dawson et al demonstrated a protective effect of FK506 on NMDA toxicity in primary cortical culture that was associated with reduced NOS activity as evidenced by reduced cGMP production. They attributed this effect to reduced NOS activity caused by FK506- mediated calcineurin inhibition (Dawson et al., 1993). However, the same authors have more recently reported that the protective effect of FK506 is still observed in cultures completely devoid of nNOS derived from transgenic animals with a deletion of the nNOS gene (Dawson et al., 1996). Furthermore, the neuroprotective effect of FK506 following gerbil transient global ischaemia (Tokime et al., 1996) or rat middle cerebral artery occlusion (Toung et al., 1999) is not a consequence of nNOS inhibition.

In conclusion, evidence from primary cortical culture and animal models along with this evidence from cerebellar prisms provide strong evidence that the neuroprotective effect of FK506 is not a consequence of NOS inhibition. To address the issue of which alternative mechanisms might be involved I have adopted a cell culture based

approach, and the characterisation of terminal differentiation in the SHSY-5Y neuroblastoma cell line is described in the next chapter.

CHAPTER 4

CHARACTERISATION OF THE TERMINAL DIFFERENTIATION OF SHSY-5Y CELLS

In this chapter I report the characterisation of the properties of SHSY-5Y cells at low and high passage in response to serum reduction, retinoic acid or combined serum reduction and retinoic acid. In low passage SHSY-5Y cells combined treatment leads to terminal differentiation to a neuron- like phenotype, as manifest by a stabilisation of population number, the development of rounded phase bright cell bodies and of immunopositivity for the neuronal marker MAP-2 and the G_o associated protein $p21^{waf}$. At higher passages this process of differentiation is incomplete.

INTRODUCTION

SHSY-5Y cells

SHSY-5Y cells have been used for more than two decades in studies of the neuronal response to diverse stimuli. However, in spite of their widespread application there is no clear consensus on their optimal use. Many different differentiation regimes have been reported, and often no reference is made in such reports to the passage number at which the cells are used or even to the differentiation status.

Over 25 years ago the SK-N-SH cell line was derived from a bone marrow aspirate of a 4 year old girl with metastatic neuroblastoma (Biedler et al., 1973), and its subcloning to form the SHSY-5Y cell line was reported in 1978 (Biedler et al, 1978). Undifferentiated cells form homogenous neuroblast- like populations and demonstrate levels of dopamine ß- hydroxylase and butyryl cholesterase activity similar to those seen in extracts of neural tissue (Biedler et al., 1978; Ross et al., 1983).

As a tumour cell line derived from human tissue SHSY-5Y cells have a number of potential advantages over other in vitro and in vivo approaches. Compared with primary culture and notwithstanding the recent growth in the availability of transgenic animals, tumour cell lines provide greater opportunity to influence patterns of gene expression. Sub cloned cell lines manifest, at least at early passages, more substantial genetic identity between preparations than do cells in primary cortical culture. Furthermore, the capacity of tumour cell lines for population expansion allows a ready supply of material without the need for animal sacrifice. Compared with animal models of disease cell lines are less expensive, are easier to manipulate for biochemical or molecular analyses, and avoid ethical concerns about the use of animals in scientific procedures. A further advantage enjoyed by SHSY-5Y cells is that, being derived from a human tumour, they enjoy a compliment of human, rather than rat or mouse, genes and proteins.

Novel proteins may be introduced to such cell lines using viral transfection techniques, and because population expansion occurs cells bearing transcripts can be selected, for instance by co-transfection with genes bearing resistance to antibiotics such as neomycin. Similarly, cells may be grown for prolonged periods in the presence of antisense oligodeoxynucleotides, or DNA encoding antisense sequences may be introduced, leading to reductions in the expression of target proteins.

Undifferentiated SHSY-5Y cells have been used to study the effects of diverse insults including staurosporine (Posmantur et al., 1997), beta amyloid peptide (Li et al., 1996), and 1-methyl 4-phenylpyridinium (MPP+) (Itano and Nomura, 1995), but the extent to which the results of these investigations are relevant to terminally differentiated adult neurons is not clear. Treatment with retinoic acid (Pahlman et al., 1984), nerve growth factor (Jensen, 1987; Perez-Polo et al., 1979), phorbol ester (Spinelli et al., 1982) or dibutyrl cyclic AMP (Perez-Polo et al., 1979) has been reported to result in the terminal differentiation of SHSY-5Y cells as evidenced by neurite outgrowth and enhanced electrical excitability. However, many studies do not record the passage number at which cells were used, and the effects of passage

number on the differentiation process are unclear.

The capacity to grow and divide which is central to tumour cell lines is not a feature of mature adult CNS neurons. Indeed, at least in undifferentiated tumour cells, regulation of cell survival is by definition disordered. Any inferences made from the study of cell death in such systems must therefore be qualified, and supportive evidence sought from other disease models.

Preliminary experiments

Because of the advantages outlined above, in particular the human derivation of the SHSY-5Y line and the potential to use an antisense strategy to reduce the expression of putative targets of FK506, I set out to test the efficacy of FK506 in preventing SHSY-5Y cell death in response to a number of stimuli. Other workers in the laboratory had demonstrated no effect on death caused by exposure to glutamate or to NMDA. Therefore the insults chosen were ionomycin, staurosporine, and serum withdrawal.

Preliminary experiments to examine the toxic effects of ionomycin and staurosporine were carried out on cells at passage 74 to 80 derived from stocks frozen at passage 72. The differentiating regime used at this time was exposure to 10μM retinoic acid in medium containing 10% serum. Laboratory practice was to make up a 10mM solution of retinoic acid in alcohol, to store this protected from light in the fridge for up to six months and to treat cells once in a seven-day differentiating period.

However, it became apparent that after 7 days there was considerable variability in cell morphology and number, with many cells appearing more like fibroblasts than neurons. This was reflected in marked variability in the cellular response to a given insult, and resulted in considerable difficulty in robustly reproducing any given result.

Stability of retinoic acid

One possible reason for the variation was differences in the activity of added retinoic acid. Following discussion with the technical support section of the Sigma Chemical Company laboratory practice in the preparation of retinoic acid was changed such that it was subsequently made up in DMSO at 10mM and stored in aliquots at -70°C for a maximum of one month before use. Because of concerns about its stability in aqueous solution medium containing retinoic acid was replaced every 48 hours during differentiation rather than only once.

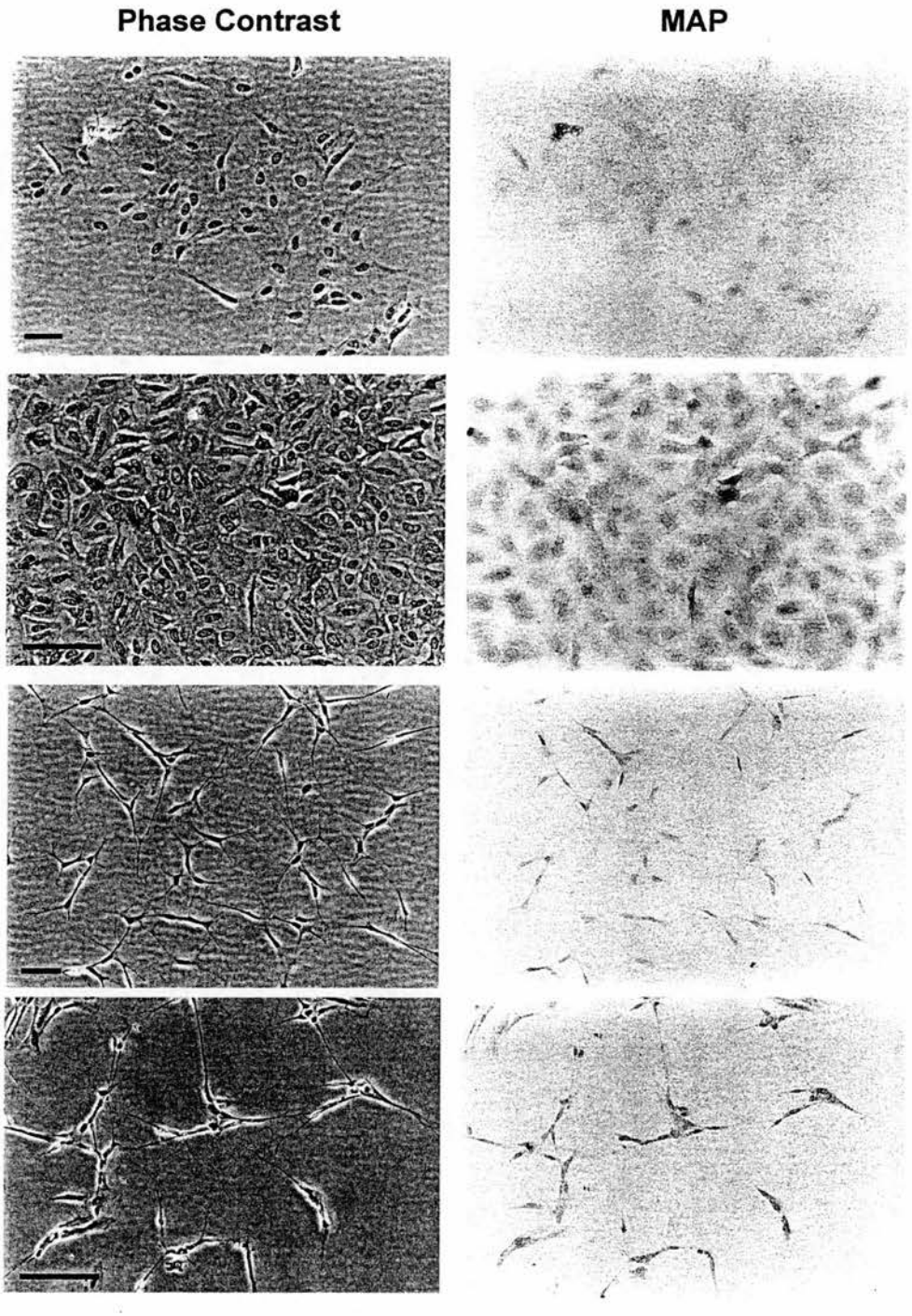
High passage

A second potential explanation was that at high passage the homogeneity of the SHSY-5Y population might be compromised, with culture conditions selecting emergent clones with shorter cell cycles, resistance to the population- stabilising effects of differentiating agents, and more primitive morphologies. Indeed, there is some evidence to support this view, with the demonstration that while at passage 3 clusters of SHSY-5Y cells have exclusively neuroblast morphologies, by passage 19 non- neuroblast (epithelial or mixed) morphologies were seen in 4.9% of such clusters (Ross et al., 1983).

To test the homogeneity of passage 82 SHSY-5Y cells 1,500 cells per well were plated to 6 well plates and grown under normal, non- differentiating conditions. After seven days the cells were fixed and stained for MAP2 as described in Chapter 2. Under these conditions cells are sufficiently widely distributed for those cells arising from a single plated cell - that is cells of clonal origin - to be clearly identifiable in well-localised clusters within which there was a high degree of morphological homology.

In 100 such clusters the morphology was neuron- like in 27%, epithelial in 37%, intermediate in 20% and mixed in 16%. Photomicrographs of such clusters are shown in Figure 4.1. In (a) the morphology is in large part epithelial, although a few cells show phase bright spindle shaped cell bodies, some of which show more MAP2

Figure 4.1: Phase contrast images and MAP2 immunopositivity in p82 SHSY-5Y clusters at 7 D.I.V.



Bar = 50μm.

immunopositivity than neighbouring cells. In (b), which is at higher power, cells are more uniformly epithelial, forming a carpet of cells with only one pyramidal neuron- like MAP2 positive cell. In (c) the cells are more typically neuron- like, being widely distributed and demonstrating rounded to phase bright cell bodies with fine neuritic processes and MAP2 positivity at immunostaining. (d) shows a similar cluster at higher power, and in addition to the features in (c) also shows the presence of numerous connections between adjacent cells.

These findings provided compelling evidence that at passage 82 SHSY-5Y cells cannot be considered to be a clonal cell line. I therefore identified a source of low passage SHSY-5Y cells (the European Collection of Animal Cell Cultures, ECACC number 94030304) and compared the behaviour of cells at high and low passage.

Differentiating regimen

A third explanation might be that the differentiating regime was not optimal. In a number of tumour cell lines, differentiation is achieved by a combination of a differentiating agent and a reduction in the amount of serum added to the culture medium. I therefore examined the effects of retinoic acid and of reducing the serum concentration from 10% to 1%.

Experimental approach

The extent of terminal differentiation was determined quantitatively on the basis of cell number and qualitatively by effects on cell morphology and the abundance of the neuronal marker MAP2 and the G_0 associated protein $p21^{waf}$. MAP2 was chosen as it is ubiquitously expressed in neurons but not in other cell types, and so would serve as a marked for a neuron- like phenotype. Terminally differentiated SHSY-5Y cells should exit the cell cycle and demonstrate increased abundance of proteins associated with G_0, and for this reason $p21^{waf}$ immunopositivity was determined.

In this chapter I demonstrate that treatment of undifferentiated cells at passage 13 to 15 with 10μM retinoic acid and serum reduction from 10% to 1% for seven days

results in stabilisation of population number, a more neuron- like morphology and increased immunopositivity for MAP2 and $p21^{waf}$. However, cells at passage 74 to 77 subjected to the same treatment resume population expansion after three days. These results provide a method for the terminal differentiation of low passage SHSY-5Y cells.

RESULTS

Population number

Undifferentiated cells

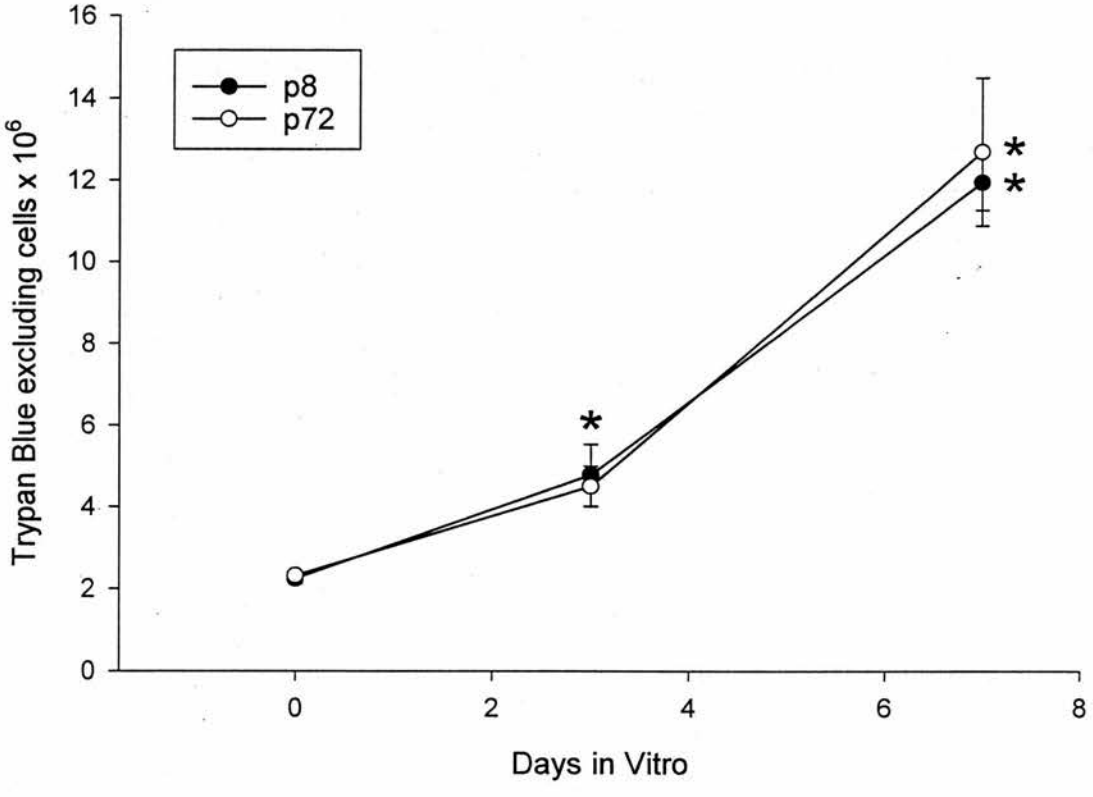
Population number for cells grown with 10% FCS increased rapidly, and the rate of increase was similar for cells at both low and high passage. The number of Trypan Blue excluding cells doubled after three days in culture and was increased five fold after 7 days (Fig 4.2).

Differentiating conditions

In the presence of 1% serum and 10μM retinoic acid cultures at low passage showed no significant increase in population number at three or seven days. In contrast, while cell number in high passage cultures was unchanged at three days, by seven days it was significantly higher than control (6.45m +/- 0.24m versus 2.32m +/- 0.11m, mean +/- s.e.m., $p<0.05$, one way ANOVA with Dunnett's method for multiple comparisons) (Fig. 4.3).

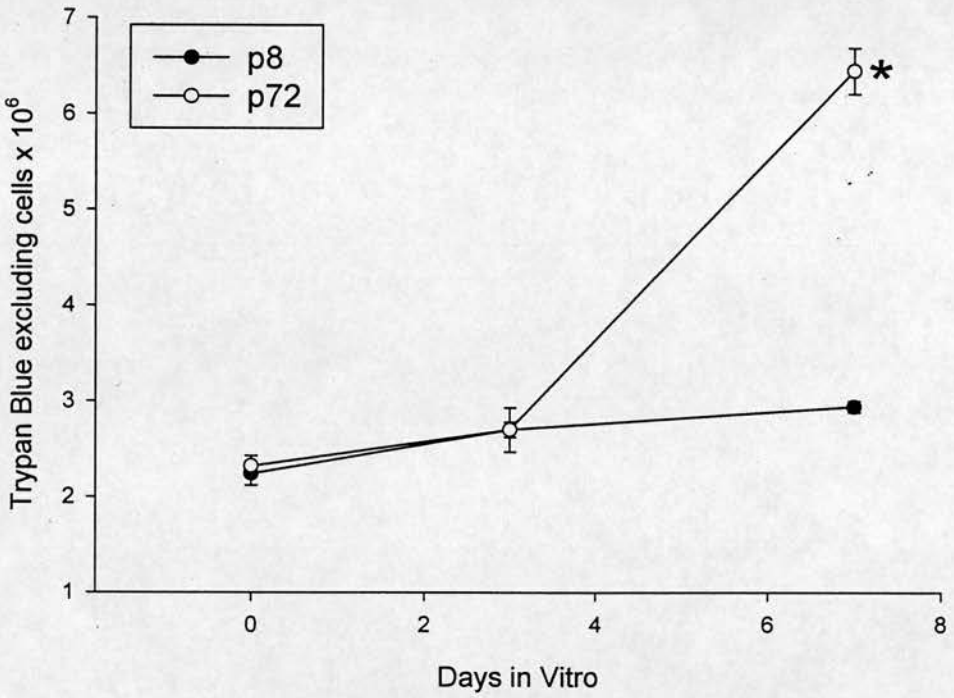
These data are brought together in Figure 4.4, demonstrating that once population expansion begins in high passage cells under differentiating conditions, the rate of expansion is similar to that seen under non-differentiating conditions. It appears that high passage cells become almost completely resistant to the restraining influence of differentiating conditions on the rate of cell division.

Figure 4.2: Population expansion under non- differentiating conditions



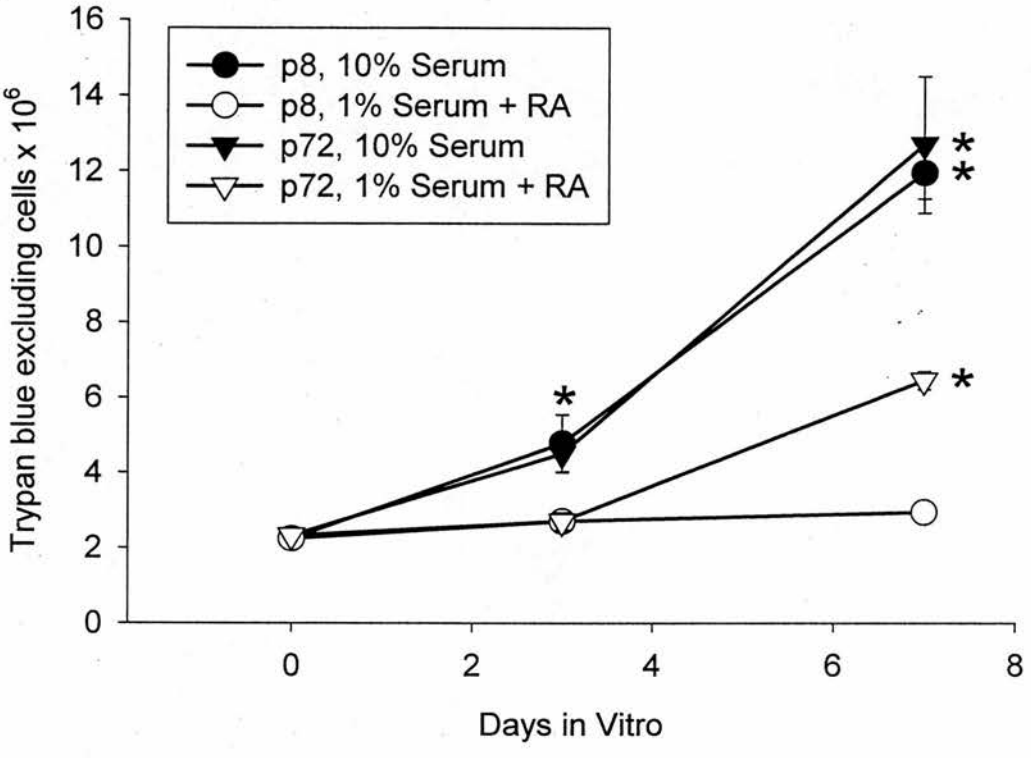
Increase in number of harvested Trypan Blue excluding SHSY-5Y cells at passage 8 (filled circles) or 72 (open circles) under non - differentiating conditions. Results represent means of three independent experiments, *$p<0.05$ versus day 0, one way ANOVA with Dunnett's post hoc test.

Figure 4.3: Population expansion under differentiating conditions



Increase in number of harvested Trypan Blue excluding SHSY-5Y cells at passage 8 (filled circles) or 72 (open circles) grown in 1% serum and 10μM retinoic acid. Results represent means of three independent experiments, *$p<0.05$ versus day 0, one way ANOVA with Dunnett's post hoc test.

Figure 4.4: Population expansion under differentiating and non- differentiating conditions



Combined data from Figs 2 and 3. Increase in number of harvested Trypan Blue excluding SHSY-5Y cells at passage 8 (circles) or 72 (inverted triangles) under non-differentiating (10% serum, filled symbols) of differentiating (1% serum and 10μM retinoic acid, open symbols) conditions. Results represent means of three independent experiments, *$p<0.05$ versus day 0, one way ANOVA with Dunnett's post hoc test.

High passage cells

Cell morphology

Figure 4.5 compares the morphologies of high passage cells treated for seven days with serum reduction and retinoic acid alone or in combination. Both treatments lead to a reduction in cell density, and this is more marked when they are given in combination. This reduction in density is associated with the development of rounded, phase bright cell bodies and neuritic processes seen most clearly with combined treatment. However, even under these conditions many cells retain a more primitive, undifferentiated appearance.

$p21^{waf}$ immunopositivity

With the exception of occasional cells no treatment was associated with $p21^{waf}$ immunopositivity above background levels (Fig 4.6). A higher magnification view of cells subjected to combined treatment is shown in Fig. 4.7, and again no immunopositivity is apparent.

MAP2 immunopositivity

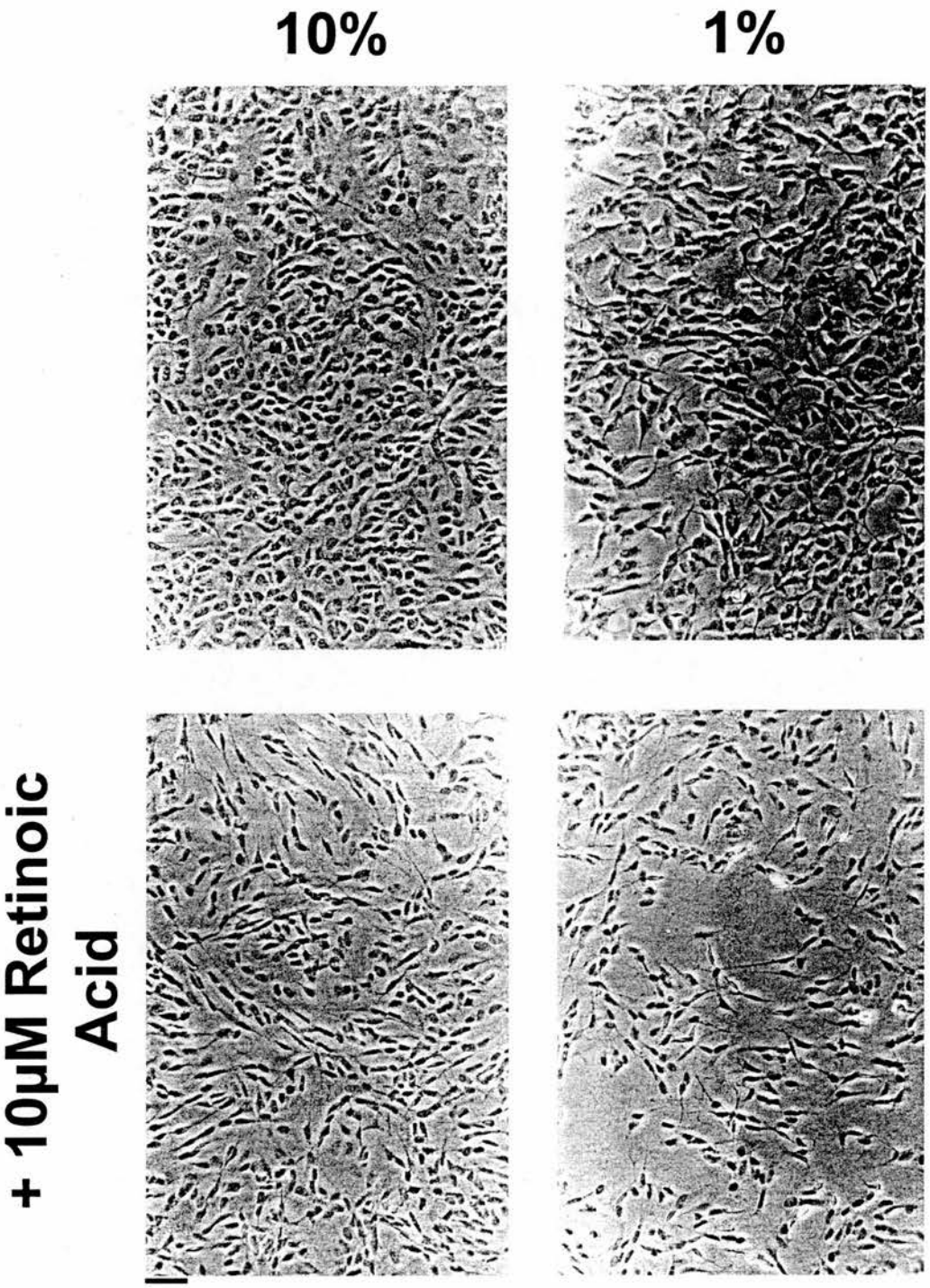
Serum reduction and retinoic acid treatments were each associated with a minor increase in MAP2 immunopositivity (Fig. 4.8), and with combined treatment this effect was more pronounced. At higher magnification (Fig. 4.9) it can be seen that MAP2 immunoreactivity is localised to cells manifesting neuron- like morphologies, and that within such cells it has a peri-nuclear and axonal distribution.

Low passage cells

Cell morphology

Figure 4.10 compares the morphologies of low passage cells treated for seven days with serum reduction and retinoic acid alone or in combination. Both treatments lead to a reduction in cell density, and this is considerably more marked when they are

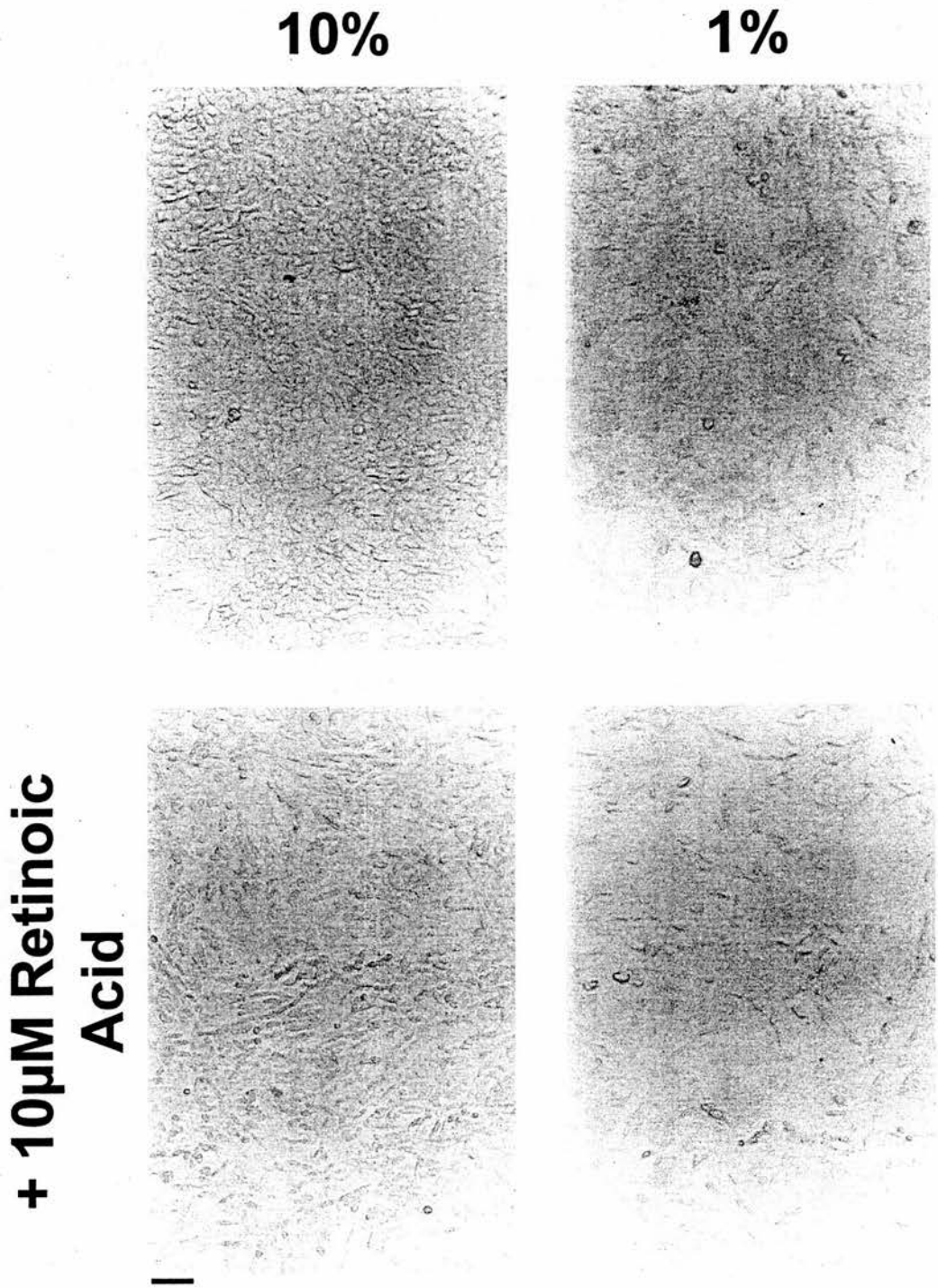
Figure 4.5: Effect of 7 days treatment with serum reduction, 10μM retinoic acid or both on phase contrast appearance of high passage SHSY-5Y cells.



Bar = 50μm.

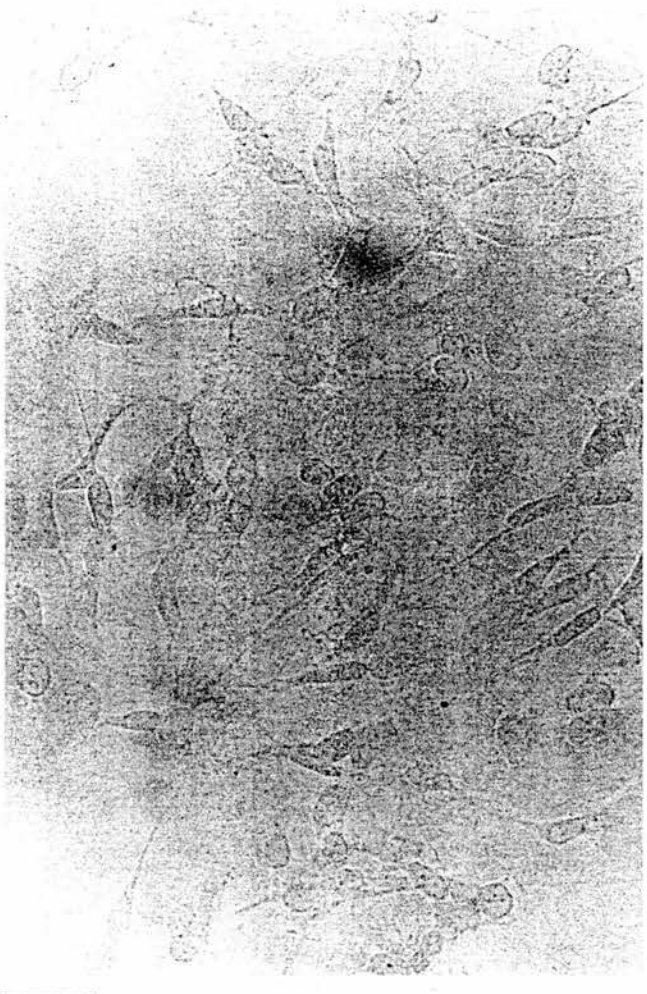
Figure 4.6: Effect of 7 days treatment with serum reduction, 10μM retinoic

Figure 4.6: Effect of 7 days treatment with serum reduction, 10μM retinoic acid or both on $p21^{waf}$ immunopositivity in high passage SHSY-5Y cells.



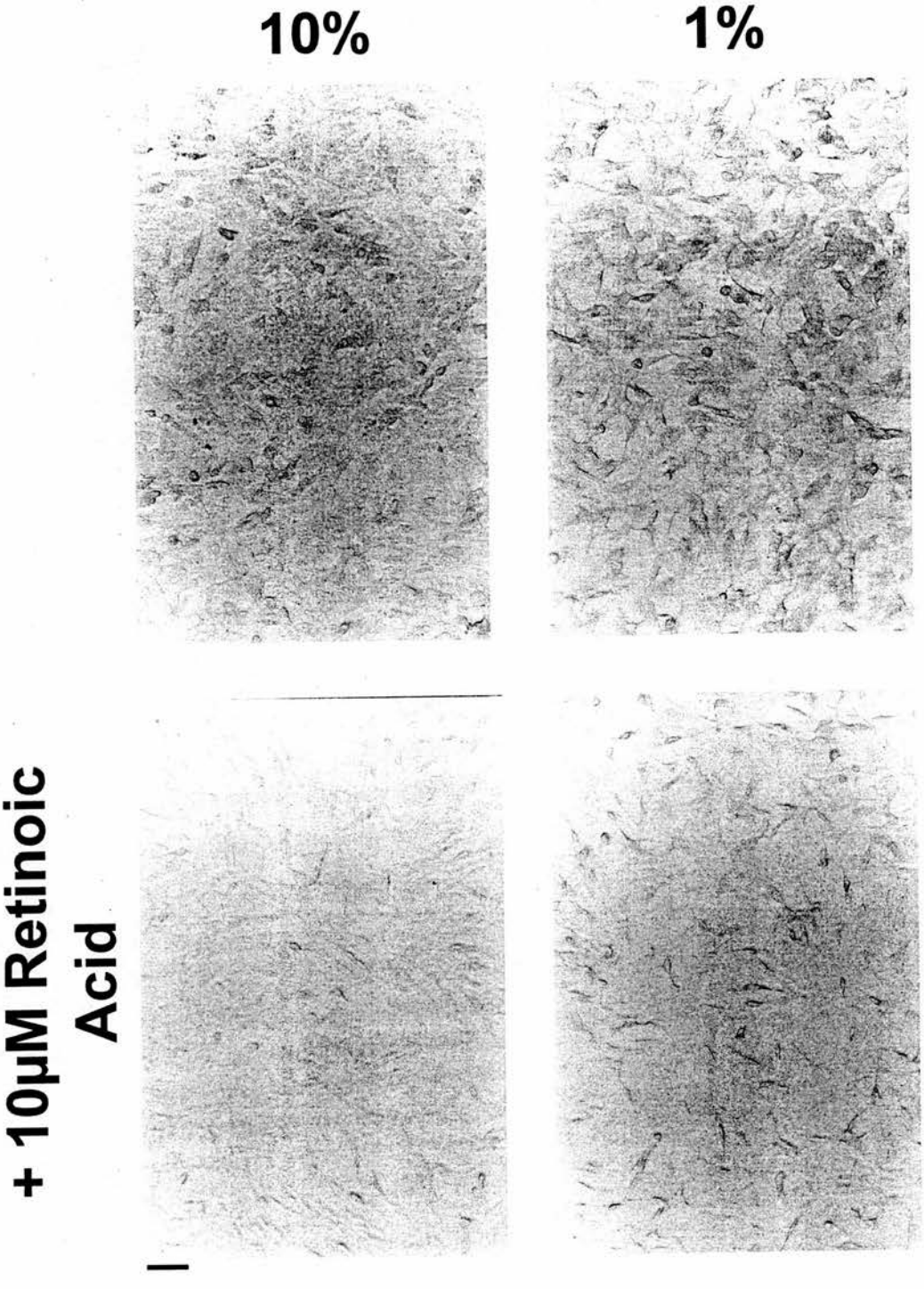
Bar = 50μm.

Figure 4.7: Effect of 7 days treatment with serum reduction and 10μM retinoic acid on $p21^{waf}$ immunopositivity in high passage SHSY-5Y cells.



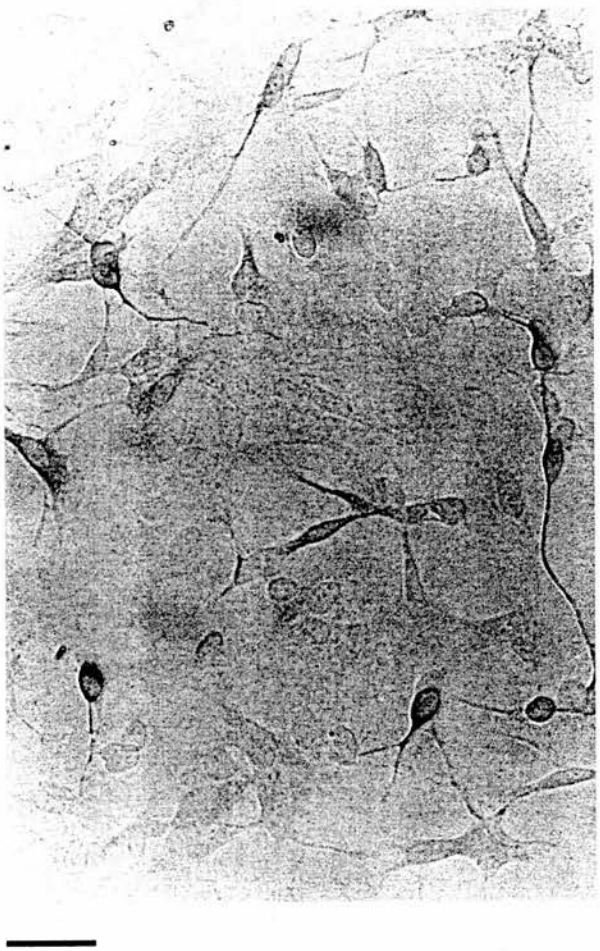
Bar = 12.5μm.

Figure 4.8: Effect of 7 days treatment with serum reduction, 10μM retinoic acid or both on MAP2 immunopositivity in high passage SHSY-5Y cells.



Bar = 50μm.

Figure 4.9: Effect of 7 days treatment with serum reduction and 10μM retinoic acid on MAP2 immunopositivity in high passage SHSY-5Y cells.



Bar = 12.5μm.

given in combination. This reduction in density is associated with the development of rounded, phase bright cell bodies and neuritic processes seen most clearly with combined treatment, although some cells retain a more primitive appearance.

$p21^{waf}$ immunopositivity

Under control conditions, scattered cells are $p21^{waf}$ positive (Fig. 4.11). Serum reduction, but not retinoic acid, leads to increased immunopositivity, and in combination this difference is pronounced. At higher magnification (Fig. 4.12) it is clear that this immunopositivity is distributed throughout neuron- like cells, but is most pronounced in the nucleus. However, it is also clear that epithelial cells remain even under these conditions, and these do not stain for $p21^{waf}$.

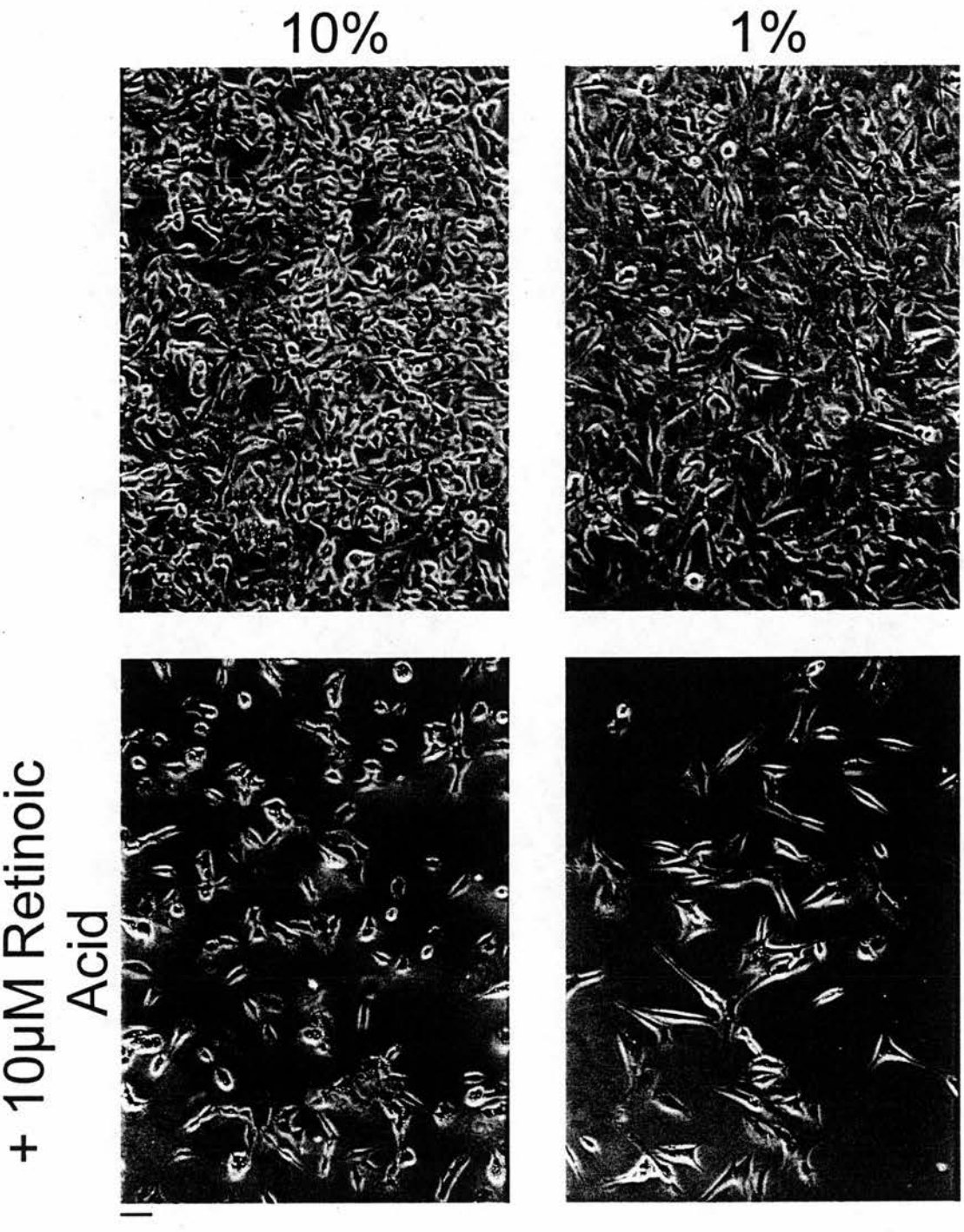
MAP2 immunopositivity

Under control conditions a large proportion of cells are MAP2 positive, although there is also a considerable contribution from epithelial, immunonegative cells (Fig. 4.13). Serum reduction but not retinoic acid led to a qualitative increase in the proportion of MAP2 positive cells, and in combination serum reduction and retinoic acid lead to MAP2 immunopositivity in cells with neuron- like morphologies (seen at higher power in Fig. 4.14). However, there remain cells with epithelial morphologies that do not stain for MAP2.

DISCUSSION

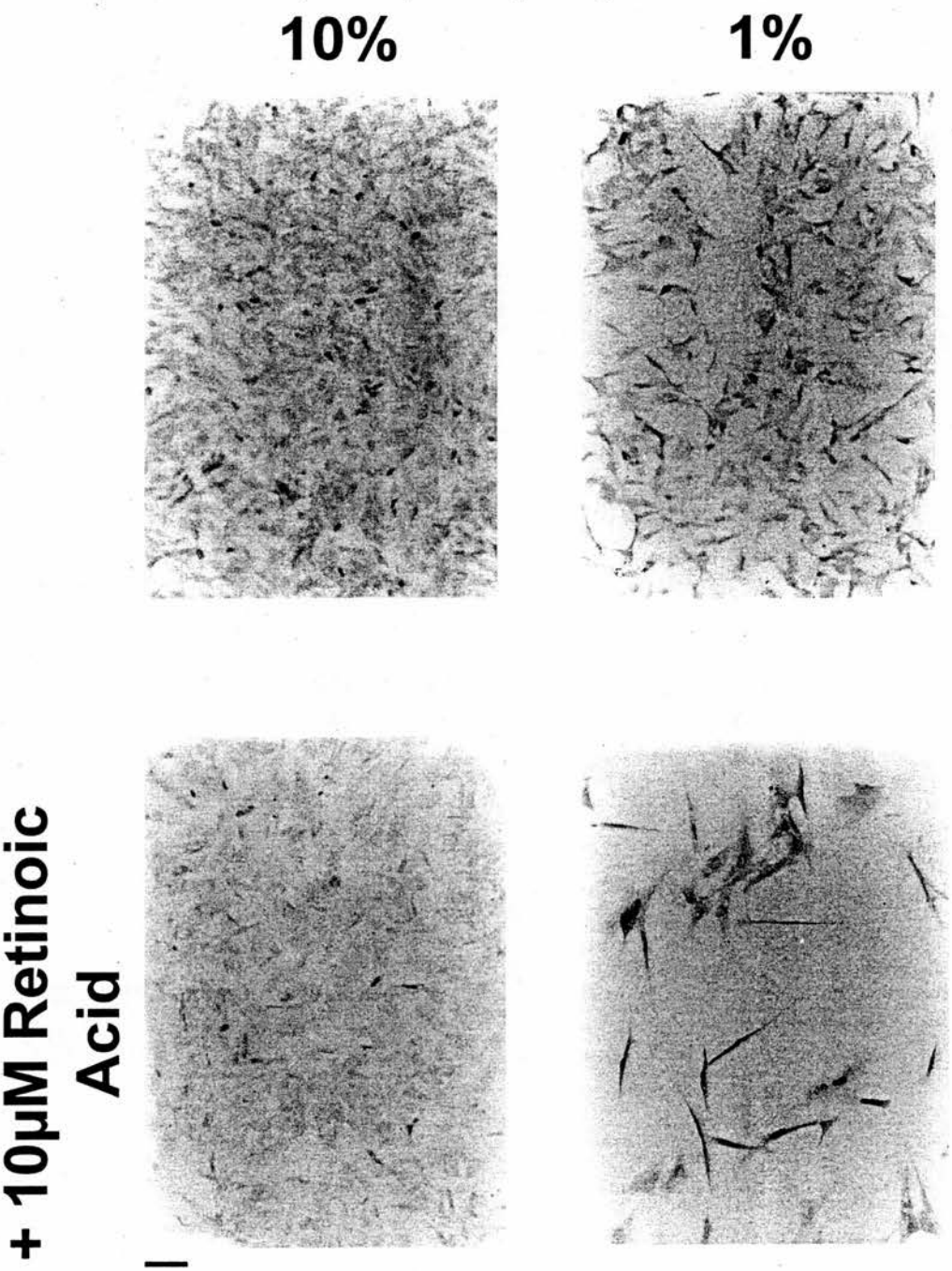
These results demonstrate that treatment of low passage SHSY-5Y neuroblastoma cells with serum reduction and retinoic acid for seven days results in them assuming a phenotype more typical of terminally differentiated adult neurons. This change is manifest as a stabilisation of population number, the development of rounded phase bright cell bodies set against a dense neuritic network and immunopositivity for MAP2 and $p21^{waf}$. However, even under these conditions a proportion of cells remain which have epithelial morphology and which do not stain for $p21^{waf}$ or for MAP2. In contrast, while high passage cultures show some reduction in population growth in response to the combination of retinoic acid and serum reduction this effect is not sustained, and while they develop some immunopositivity for MAP2

Figure 4.10: Effect of 7 days treatment with serum reduction, 10μM retinoic acid or both on phase contrast appearance of low passage SHSY-5Y cells.



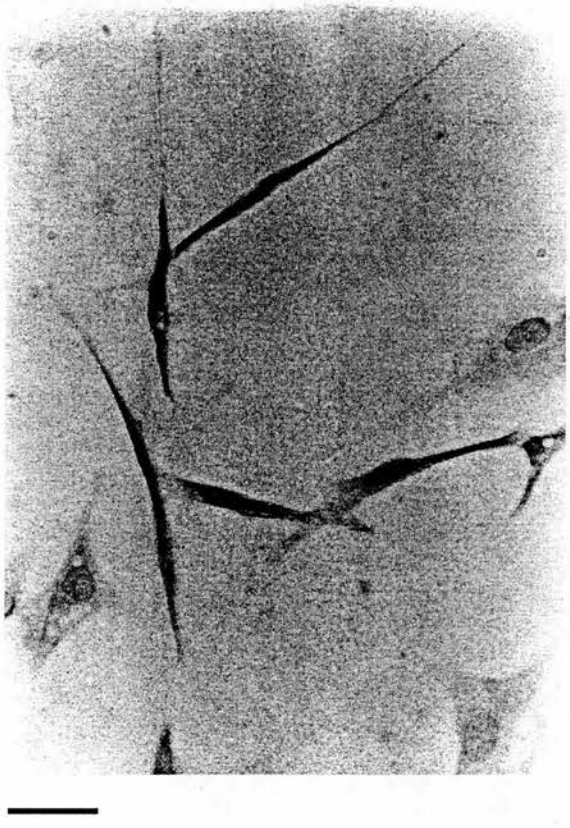
Bar = 25μm.

Figure 4.11: Effect of 7 days treatment with serum reduction, 10μM retinoic acid or both on $p21^{waf}$ immunopositivity in low passage SHSY-5Y cells.



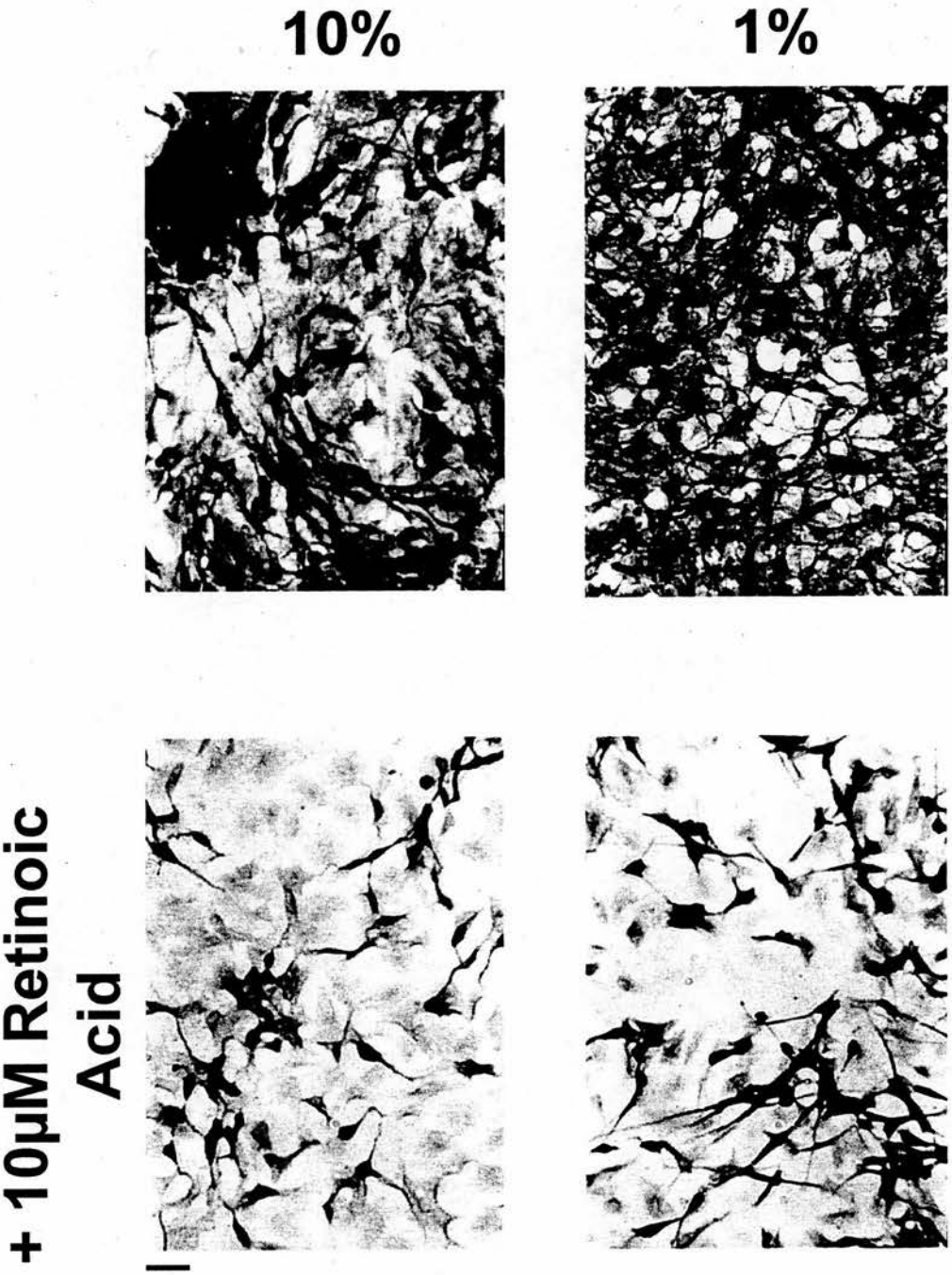
Bar = 50μm.

Figure 4.12: Effect of 7 days treatment with serum reduction and 10μM retinoic acid on $p21^{waf}$ immunopositivity in low passage SHSY-5Y cells.



Bar = 12.5μm.

Figure 4.13: Effect of 7 days treatment with serum reduction, 10μM retinoic acid or both on MAP2 immunopositivity in low passage SHSY-5Y cells.



Bar = 50μm.

Figure 4.14: Effect of 7 days treatment with serum reduction and 10μM retinoic acid on MAP2 immunopositivity in low passage SHSY-5Y cells.

Bar = 12.5μm.

they do not do so for $p21^{waf}$.

$p21^{waf}$ belongs to a family of proteins that interact with and inhibit cyclin - cyclin dependent kinase complexes, resulting in cells withdrawing from the cell cycle. The gene for $p21^{waf}$ contains a functional retinoic acid response element in its promoter region (Liu et al., 1996). However, in the current experiments the effect of serum reduction alone was more marked than that of retinoic acid alone. One possible interpretation is that increased $p21^{waf}$ abundance may be a general feature of differentiation independent of the differentiating stimulus. This view is supported by the increased $p21^{waf}$ abundance observed in SHSY-5Y cells differentiated with aphidocolin and nerve growth factor (Poluha et al., 1996). Indeed application of this differentiating stimulus to SHSY-5Y cells where $p21^{waf}$ expression had been blocked using antisense techniques resulted in cell death with nuclear changes typical of apoptosis, implying that $p21^{waf}$ expression is required for cell survival during differentiation (Poluha et al., 1996).

High passage SHSY-5Y cells are clearly less responsive to serum reduction and retinoic acid than those at low passage. This may be related to increased genomic instability at higher passages leading to the emergence of clones resistant to terminal differentiation. In their original paper Biedler et al (Biedler et al., 1973) reported that the modal number of chromosomes in the parent SK-N-SH cell line was 47 (range 45 to 48), the most common additional chromosome being a long, structurally abnormal submetacentric marker of unknown origin. Subsequently Ross et al (Ross et al., 1983) demonstrated changes in the distribution of different colony types with passage number. After 3 passages each of 1,000 colonies examined demonstrated neuroblastic morphology, but after a further 16 passages 37 of 1,000 colonies manifested epithelial morphology and 12 a mixed morphology. The observation reported here that only 27% of colonies at passage 82 had a predominantly neuron like morphology is consistent with the emergence of yet more heterogeneity, and divergence from neuroblast- like properties, at passages higher than those examined by Ross et al.

These findings demonstrate that following treatment with retinoic acid and serum reduction low passage SHSY-5Y cells develop features of terminally differentiated neurons. However, not all cells manifest all such features, and indeed some manifest none of them. On this basis low passage terminally differentiated SHSY-5Y cells should be used with caution. While they have many of the advantages described above for cell lines and are human in origin, they cannot be considered to represent a uniform population of neuron- like cells, and therefore some dilution of any effect may be anticipated.

At higher passages the effect of retinoic acid and serum reduction is much less pronounced. SHSY-5Y cells should not be used at high passage, and reports of the use of SHSY-5Y cells should specify the passage number.

Terminally differentiated SHSY-5Y cells provide an opportunity to study the death of a neuron- like cell in response to diverse stimuli. Such death in response to the calcium ionophore ionomycin, the protein kinase C inhibitor staurosporine and to serum withdrawal is described in the next chapter.

CHAPTER 5

RESPONSE OF SHSY-5Y CELLS TO IONOMYCIN, STAUROSPORINE AND SERUM WITHDRAWAL

INTRODUCTION

One approach to the investigation of the mechanism(s) subserving the neuroprotective actions of FK506 in animal models of focal cerebral ischaemia is to identify cell culture systems in which cells die in response to a relevant stimulus and in which FK506 prevents or inhibits that death. The importance of proteins purported to mediate the protective effect of FK506 might then be assessed either through the use of pharmacological inhibitors of such proteins, of primary cultures derived from transgenic animals lacking genes encoding those proteins or of cell lines using antisense techniques to reduce the expression of those proteins.

The terminal differentiation of the SHSY-5Y neuroblastoma cell line was described in the previous chapter. These cells have some properties that might be considered useful in the investigation of the neuroprotective effect of FK506. As tumour cells they provide an effectively unlimited source of cells that can be grown for prolonged periods in the presence of antisense oligodeoxynucleotides, allowing reductions in the level of proteins. Alternatively, they might be transfected with foreign DNA to over express proteins of interest, or DNA encoding antisense sequences to genes of interest, or DNA encoding mutated proteins with dominant- negative activity. Using cotransfection with antibiotic resistance genes it would be possible to select cells in which foreign DNA had been successfully incorporated, and then using retinoic acid and serum reduction to terminally differentiate these cells to a mature neuron- like phenotype. Furthermore, as a human cell line they would be, in some respects, more representative of cellular responses following human neuronal cell injury than

models using tissue derived from other animals.

Against this background I have examined the effect of ionomycin, staurosporine, serum withdrawal on terminally differentiated SHSY-5Y cells.

Ionomycin is a calcium ionophore, exposure to which causes a rapid increase in intracellular calcium. In T lymphocytes ionomycin causes activation of NFAT1 and T lymphocyte activation. This effect is blocked by FK506 (Shaw et al., 1995). In HL-60 human promyelocytic cells, ionomycin causes apoptosis (Park et al., 1996) and in neuronal primary cortical cultures ionomycin reduces survival (Durkin et al., 1996).

Staurosporine is a broad-spectrum protein kinase C inhibitor which also has inhibitory effects on other protein kinases and which in addition has recently been shown to increase the activity of a previously uncharacterised c-Jun NH_2 terminal kinase (Yao et al., 1997). In a wide range of cell types including neurons exposure to staurosporine rapidly leads to a cell death with morphological and biochemical features of apoptosis (Wiesner and Dawson, 1996; Weil et al., 1996; Jacobson et al., 1996; Krohn et al., 1998). The precise mechanism through which this occurs is not known. Given the evidence discussed in Chapter 1 suggesting an important role for apoptosis in the neuronal cell death in stroke I have examined staurosporine-induced death in SHSY-5Y cells and the effect on this of FK506.

In many neuronal cell culture systems withdrawal of serum leads to apoptotic cell death (Miller and Johnson, 1996; Umegaki et al., 1996; Kim et al., 1999; Howard et al., 1993). In undifferentiated SHSY-5Y cells, serum withdrawal causes apoptotic cell death (Posmantur et al., 1997), and I have examined the consequences of serum withdrawal in terminally differentiated SHSY-5Y cells and the impact on this of FK506.

Exposure to excitotoxins is often used in cell culture models of stroke, and indeed FK506 has been shown to have protective effects in primary neuronal culture in

response to such stimuli (Dawson et al., 1993). However, previous work in our laboratory had shown no effect of FK506 on glutamate- or NMDA- induced death in SHSY-5Y cells, and so the effect of FK506 on excitotoxic death in SHSY-5Y cells was not tested.

RESULTS

Ionomycin

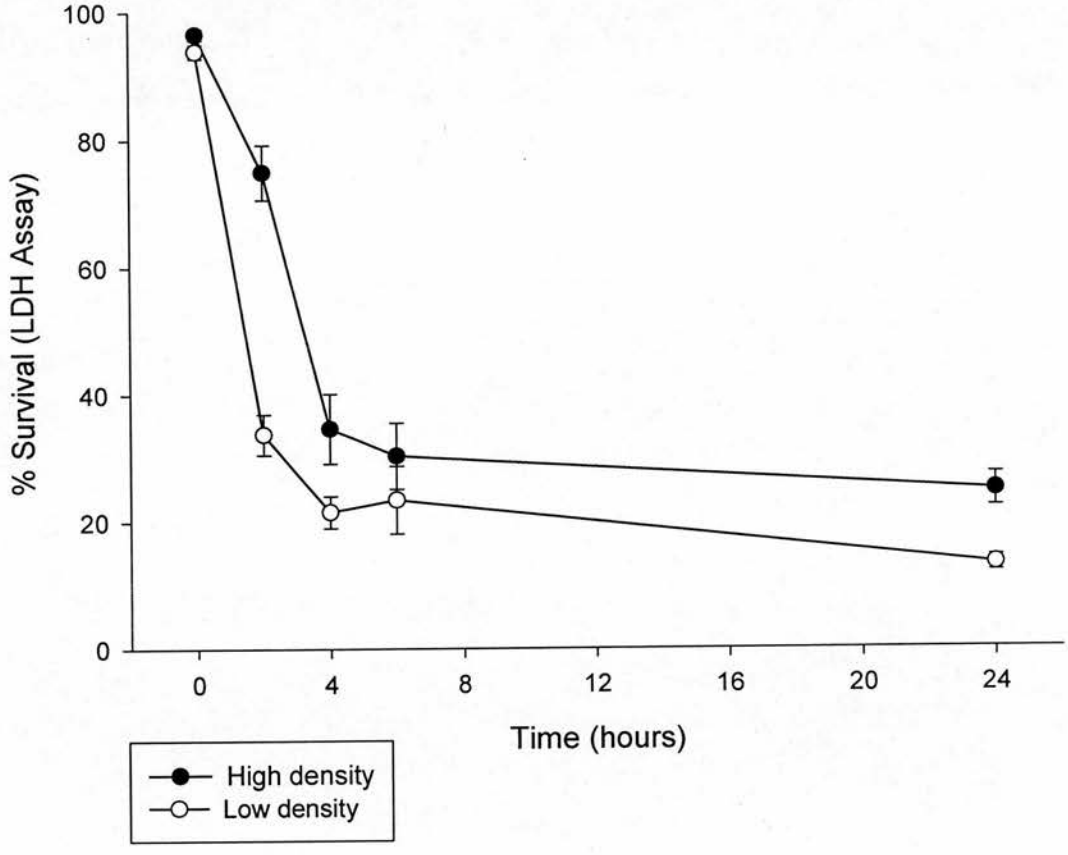
Time course

Exposure to ionomycin at a concentration of 1μM caused a rapid reduction in survival as measured using release of lactate dehydrogenase to the surrounding medium. This effect was most pronounced in cells grown at low density (50,000 cells per well), where near- maximal death was observed within 2 hours of ionomycin exposure. However, even in cells grown at high density (150,000 cells per well) death was rapid, with near maximal death occurring within 4 hours (Fig. 5.1).

Concentration dependence

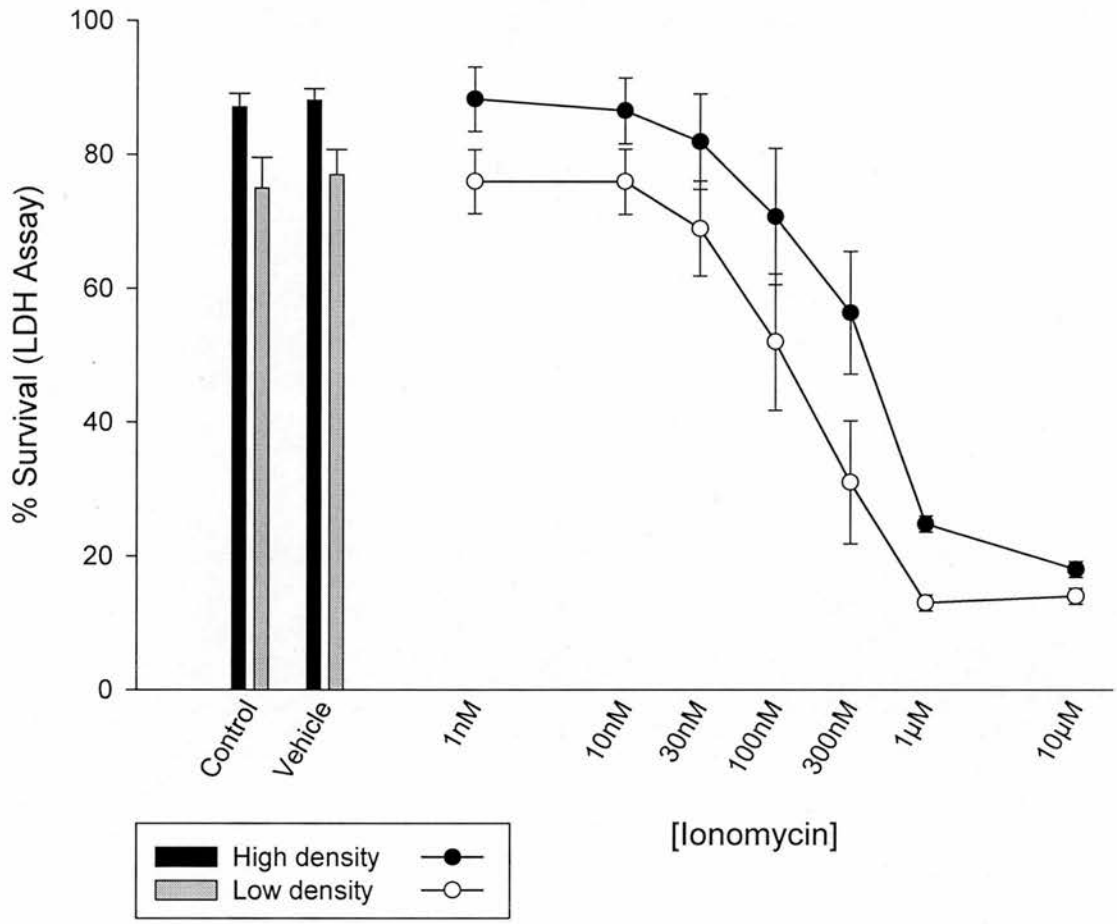
This death was concentration dependent at both low- and high- density. LDH release at 24 hours is shown in Figure 5.2. Interestingly, under control conditions proportionate LDH release at 24 hours was higher in cells grown at low density, and the concentration- response curve for ionomycin for low density cells was shifted downwards with respect to high density cells, implying that cells at low density are more susceptible to the toxic effects of medium change (control conditions) and ionomycin. The EC_{50} was estimated by curve fitting to be 200nM at low density (r^2 = 0.996), and 450nM at high density (r^2 = 0.991) (Fig 5.3). However, when the data was normalized such that survival under control conditions was defined as 100% survival (Fig 5.4), there was no significant difference between the concentration-response curves at the two densities (F=3.171, p=0.077, two way ANOVA).

Figure 5.1: Timecourse of death in response to ionomycin



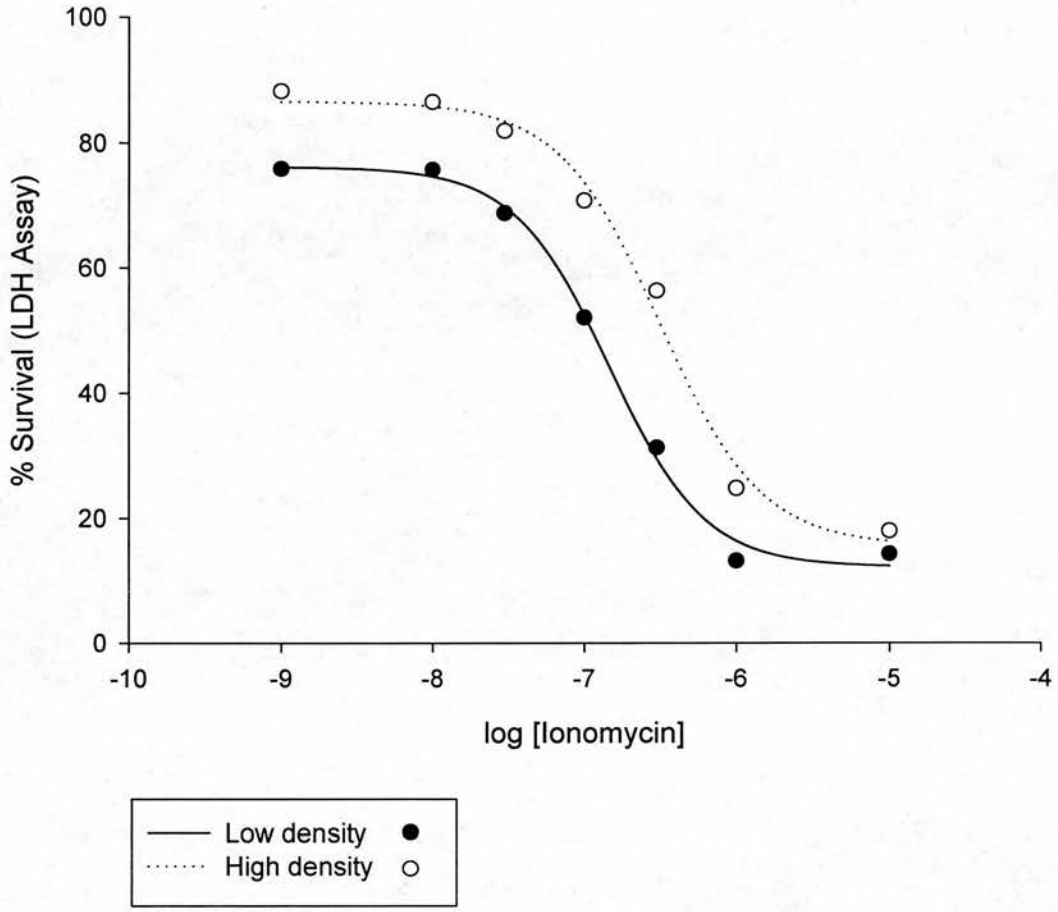
Survival of terminally differentiated SHSY-5Y cells at passage 12 to 14 at high (filled circles: 150,000 cells per well) and low (open circles: 50,000 cells per well) density measured using the LDH assay 0, 2 4, 6 and 24 hours following exposure to 1μM ionomycin (mean ± s.e.m.: n=8 per point).

Figure 5.2: Concentration- response curves for ionomycin



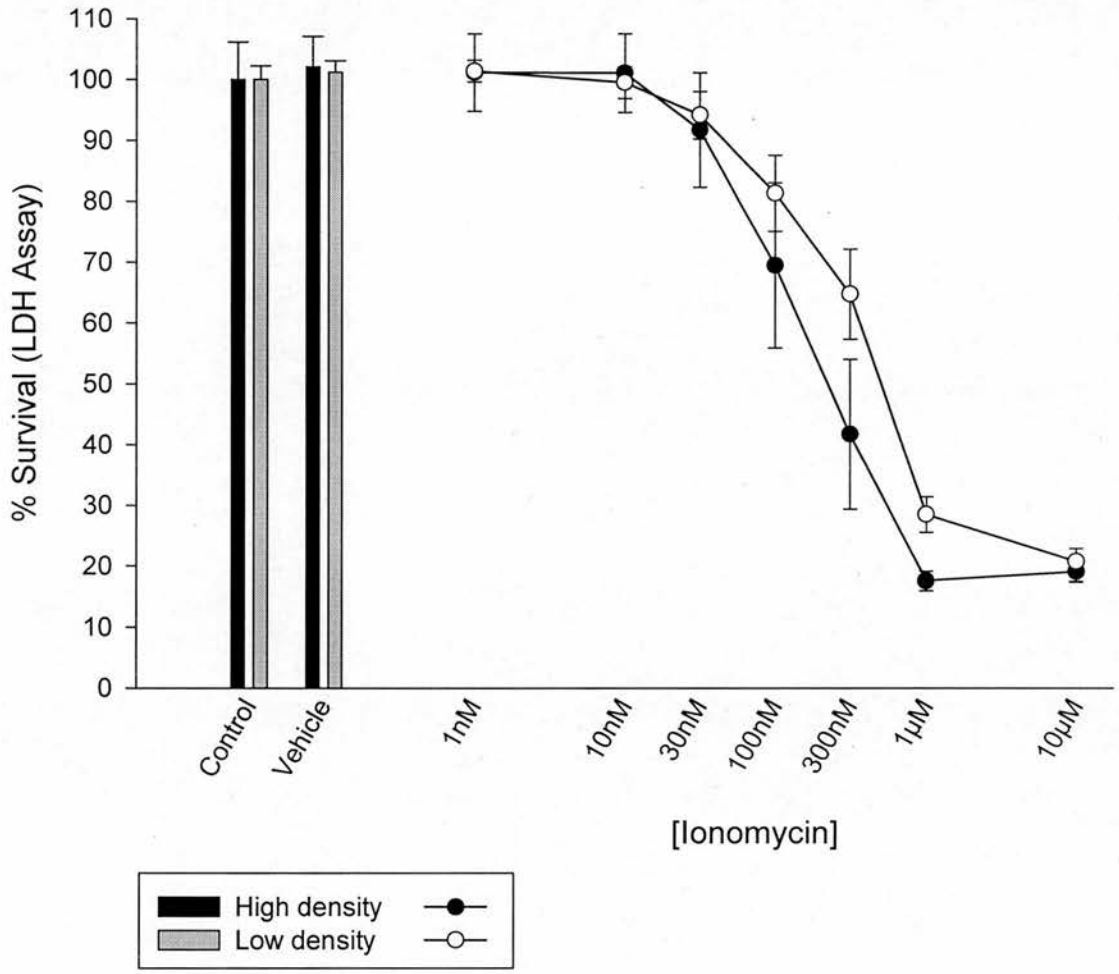
Concentration- response curves for LDH release following 24 hours exposure to ionomycin in terminally differentiated SHSY-5Y cells at passage 12 to 14 at high (filled circles: 150,000 cells per well) and low (open circles: 50,000 cells per well) density (mean ± s.e.m.: n=8 per point).

Figure 5.3: Curve fitting to ionomycin concentration- response data



Fitting of ionomycin concentration- response data from Figure 2 to the formula Survival (y) = y_0 + (a/(1+e^((x_0-log[ionomycin])/b) where a, b, x_0 and y_0 are constants. The correlation coefficient r^2 for the line- fit was 0.996 for low density (closed circles) and 0.991 for high density (open circles) cultures.

Figure 5.4: Normalised ionomycin response



Data from Figure 2 normalised to Control survival = 100%. Note that the concentration- response curves become closer compared with figure 2, and the data for low (closed circles) and high (open circles) density cultures are not significantly different (mean ± s.e.m.: Two way ANOVA; n=8, F=3.171, p=0.077).

Comparison of different endpoints

Both the Calcein AM™ and LDH assays measure the integrity of the cell membrane, whereas the MTS assay is an index of mitochondrial respiratory activity, measuring the conversion of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyphenoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] to a coloured formazan product. While both mitochondrial function and cell membrane integrity are lost as cells die, the timecourse of these changes may be different, and at any one time they may have proceeded to a different extent. In particular, loss of cell membrane integrity is often held to be an early event in necrotic cell death and a late event in apoptosis.

I therefore compared the MTS, LDH and Calcein AM assays after exposure to ionomycin for 24 hours. Figure 5.5 shows that across these assays the response to increasing concentrations of ionomycin is broadly similar, and the normalised curves are superimposed.

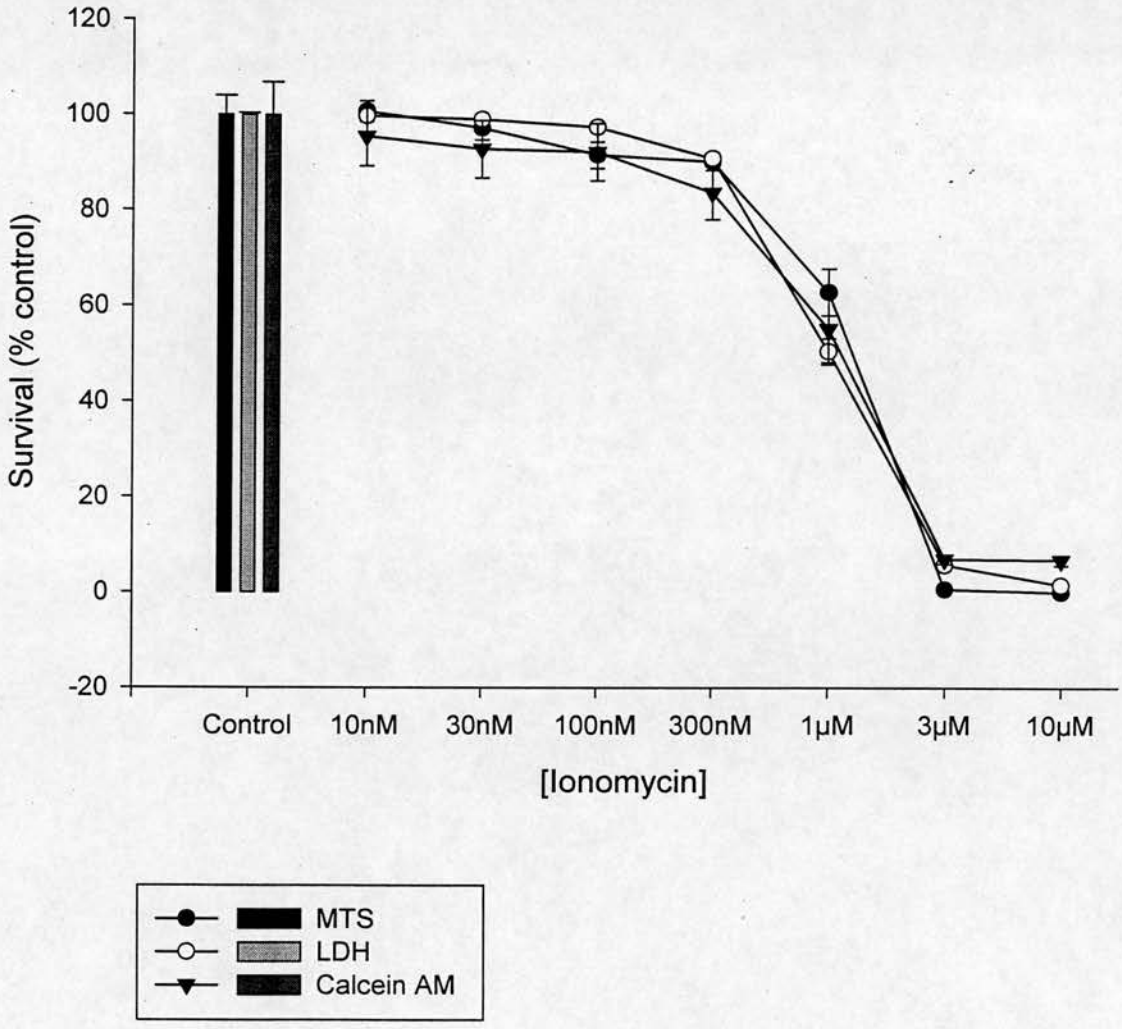
Characterisation of ionomycin- induced death

In primary cortical culture ionomycin causes neuronal death with some features of apoptosis, but the response of SHSY-5Y cells has not been characterised. The features of ionomycin induced SHSY-5Y death were therefore examined, with particular regard to the presence or absence of features of apoptosis.

Caspase activity

Apoptosis requires the efficient disassembly of the cell, co-ordinated by the activity of one or more members of the caspase family. Using an antibody that detects the processed subunits of caspase 3 the large p17 subunit was not detected, and there was no proteolysis of one known caspase 3 substrate, poly (ADP-ribose) polymerase (PARP). In extracts from cells exposed to ionomycin, caspase 3-like activity (measured using a fluorogenic Group II caspase peptide substrate (Ac-Asp-Glu-Val-Asp-; Ac-DEVD-AMC; Thornberry et al, 1997) was not significantly increased above control levels (Table 5.1). However, activity was present in extracts of A1.1 T

Figure 5.5: Comparison of different endpoints for ionomycin- induced death



Concentration- response curves for ionomycin- induced death in terminally differentiated SHSY-5Y cells at passage 13 to 18 at high density (150,000 cells per well) measured using the MTS (closed circles), LDH (open circles) and Calcein AM (inverted triangles) assay systems. For each assay survival is normalised to Control survival = 100%. Note that the curves are superimposed one on the others (mean ± s.e.m.: n=12 per point).

cell hybridomas that had been induced to undergo apoptosis following exposure to dexamethasone.

Table 5.1: Effect of ionomycin on DEVD cleavage activity

Negative Control	Ionomycin 300nM	Positive Control Dexamethasone treated A1.1 cells
0.144 ± 0.016	0.243 ± 0.073	1.091 ± 0.064

mean ± s.d; relative fluorescent units per mg protein per minute; Data from three independent experiments

Other features of apoptosis

Oligonucleosomal DNA fragmentation was not present, nor were the nuclear changes found in apoptosis; neither Hoescht 33258 fluorescence or light microscopy of toluidine blue stained thick sections demonstrated nuclear fragmentation or crescentic chromatin condensation.

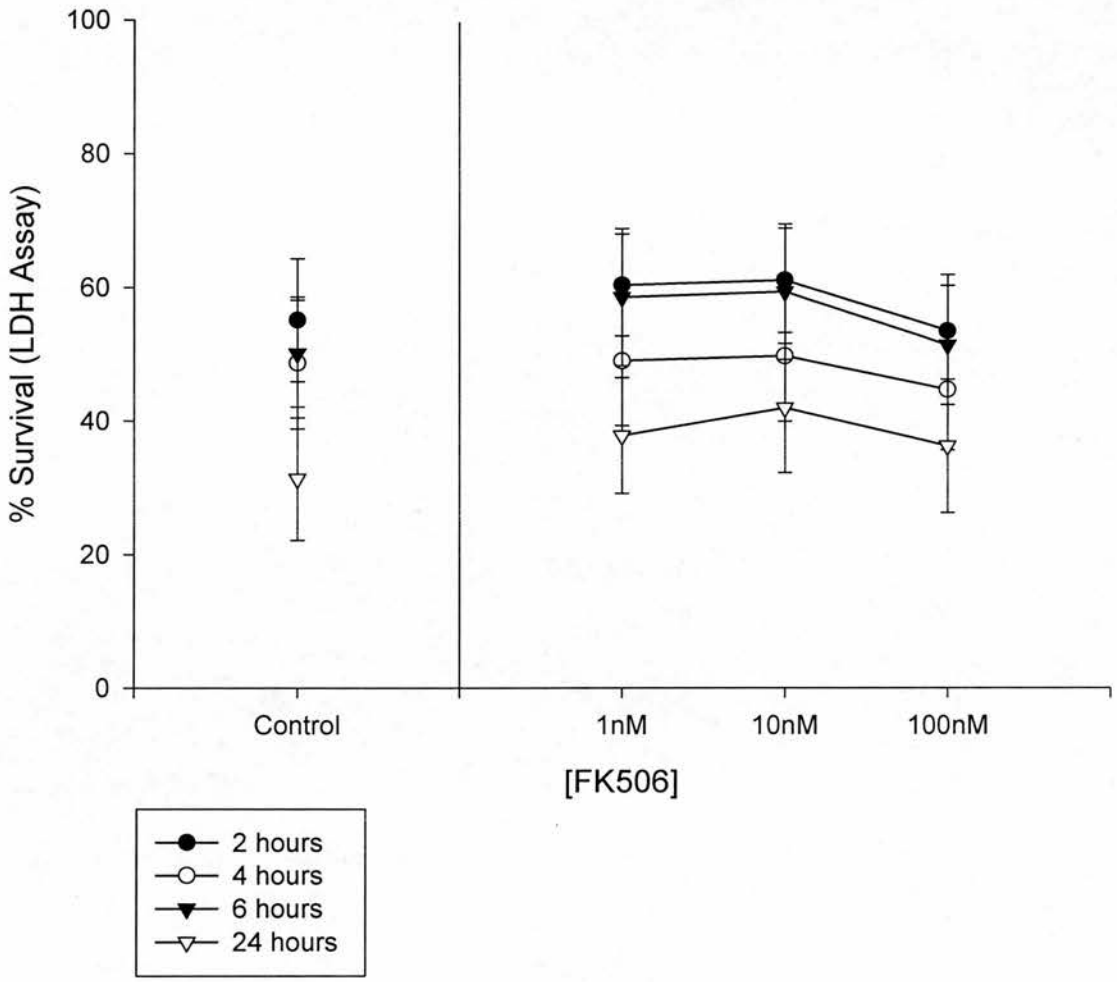
Effect of FK506

Figures 5.6 and 5.7 show survival in cells exposed to 300nM ionomycin either alone or in combination with three different concentrations of FK506 for 2, 4, 6 or 24 hours at low (Fig. 5.6) or high (Fig. 5.7) density. At no combination of duration of exposure, concentration of FK506 or cell density was a protective effect of FK506 seen, and indeed at the highest concentration, particularly in high density cultures, there is a trend towards a toxic effect of FK506. In these experiments, cells were incubated with FK506 for 24 hours before ionomycin was added, but the results were comparable if FK506 was added at the same time as ionomycin.

Discussion

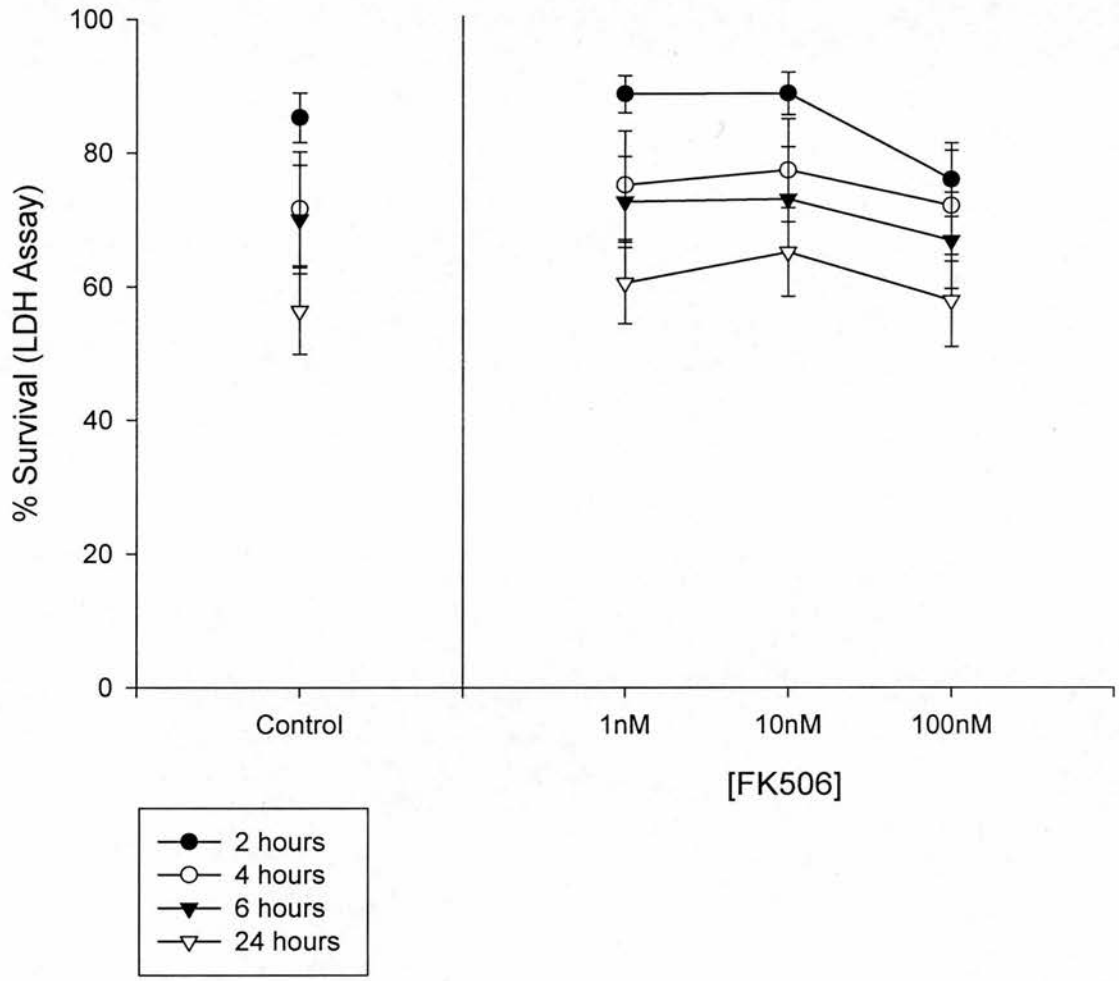
Ionomycin causes death in SHSY-5Y cells at similar concentrations as reported for primary cortical cultures (Takei et al., 1995; Datta et al., 1997a). The timecourse of death in SHSY-5Y cells is very rapid, with near maximal death occurring in low-

Figure 5.6: Effect of FK506 on ionomycin- induced death in low density cultures



Survival in low density cultures cells exposed to 300nM ionomycin for 2 (closed circles), 4 (open circles), 6 (closed triangles) or 24 (open triangles) hours in the presence of increasing concentrations of FK506. FK506 was without effect (mean ± s.e.m.: n= 8 per point).

Figure 5.7: Effect of FK506 on ionomycin- induced death in high density cultures



Survival in high density cultures cells exposed to 300nM ionomycin for 2 (closed circles), 4 (open circles), 6 (closed triangles) or 24 (open triangles) hours in the presence of increasing concentrations of FK506. FK506 was without effect (mean ± s.e.m.: n= 8 per point).

density cultures within two hours. While apoptosis can proceed to completion over such short timescales, it usually takes longer.

Furthermore, the equivalence of concentration- response curves using endpoints measuring mitochondrial function and membrane integrity implies that membrane integrity is not preferentially preserved, as occurs in apoptosis. Finally, there was no caspase activation, DNA fragmentation or nuclear changes of apoptosis.

Taken together, these observations demonstrate SHSY-5Y cells exposed to ionomycin do not die by apoptosis. This is in contrast to primary cortical culture, where exposure to relatively high (1μM) concentrations of ionomycin have been reported to lead to the development of changes in nuclear morphology typical of apoptosis (Hatanaka et al., 1996) and of oligonucleosomal DNA fragmentation (Takei et al., 1995).

Cell survival is clearly affected by the density at which cells are grown, under both control conditions and following ionomycin exposure. The reduction in survival under control conditions seen in the LDH assay may reflect differences in the concentration of survival factors elaborated by the cells themselves, with cells at high density having higher concentrations of such survival factors. The shift of the concentration- response curve following ionomycin exposure may simply be a consequence of this general effect of density on survival, as when the data were normalized (Control survival = 100%) no significant difference was observed.

While FK506 effectively inhibits ionomycin- and calcium- mediated events including cell death in lymphocytes, it was without effect on SHSY-5Y cells. This was the case across a range of concentrations of FK506 against a concentration of ionomycin causing approximately 60% cell death. FK506 was also unable to delay death, as it was without effect at 2, 4, 6 or 24 hours, and this lack of effect occurred whether FK506 was added 24 hours before, or at the same time as ionomycin. At the highest concentration studied, FK506 showed a non-significant trend towards increased death, which could represent a negative effect on neuronal survival that

would be more apparent at higher concentrations.

Staurosporine

Time course

Exposure to staurosporine at a concentration of 300nM caused a less rapid reduction in survival compared with that observed for ionomycin. Again, death was most pronounced in cells grown at low density (50,000 cells per well), where almost half of the observed death occurred within the first 4 hours; at high density, in contrast, half of the observed death occurred within 6 hours (Fig 5.8).

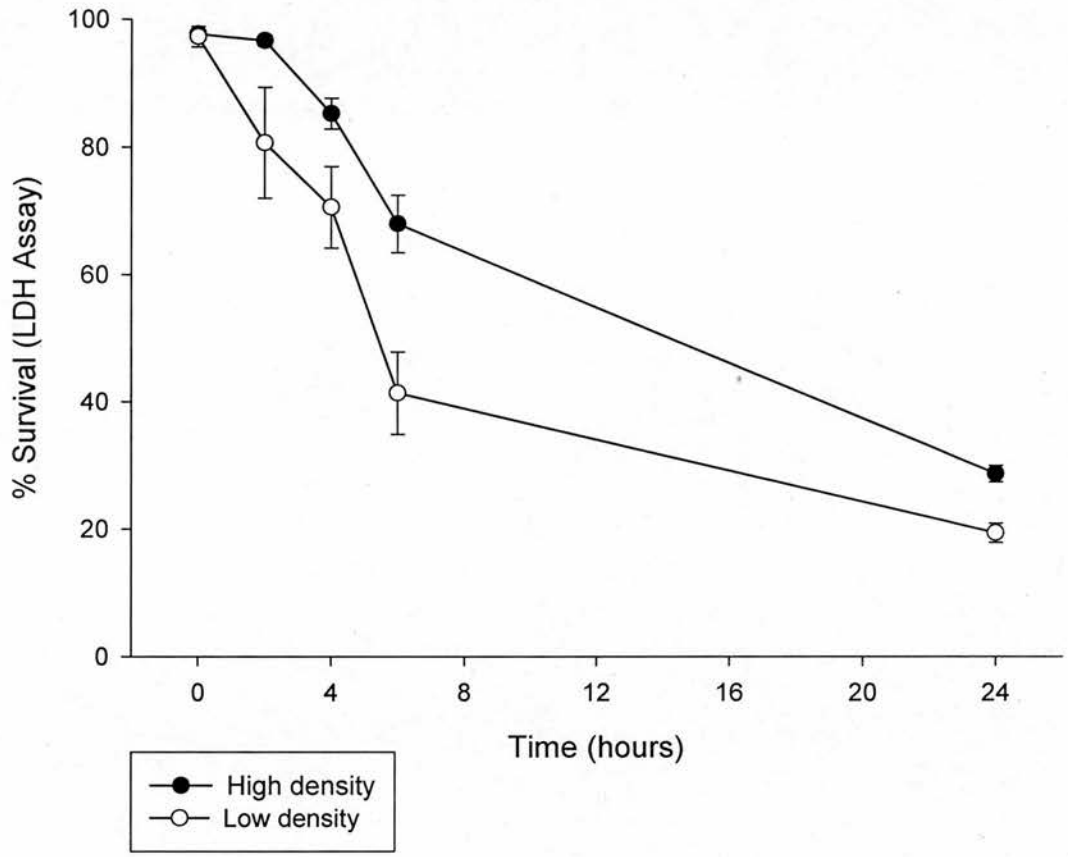
Concentration dependence

Staurosporine induced death was concentration dependent at both low- and high-density. LDH release at 24 hours is shown in Figure 5.9. Under control conditions, the proportionate LDH release at 24 hours was higher in cells grown at low density. Following staurosporine exposure the concentration- response curve for low-density cells was shifted to the left with respect to high-density cells, implying that cells at low density are more susceptible to the toxic effects of staurosporine. Fitting a four component sigmoid curve to the concentration response curve demonstrated an EC_{50} of approximately 30nM at low density ($r^2 = 0.996$), and of 200nM at high density ($r^2 = 0.999$) (Fig 5.10). When the data was normalised to control survival being 100% this difference remained (Fig 5.11), and the curves were significantly different ($F=38.6$, $p<0.001$, two way ANOVA).

Comparison of different endpoints

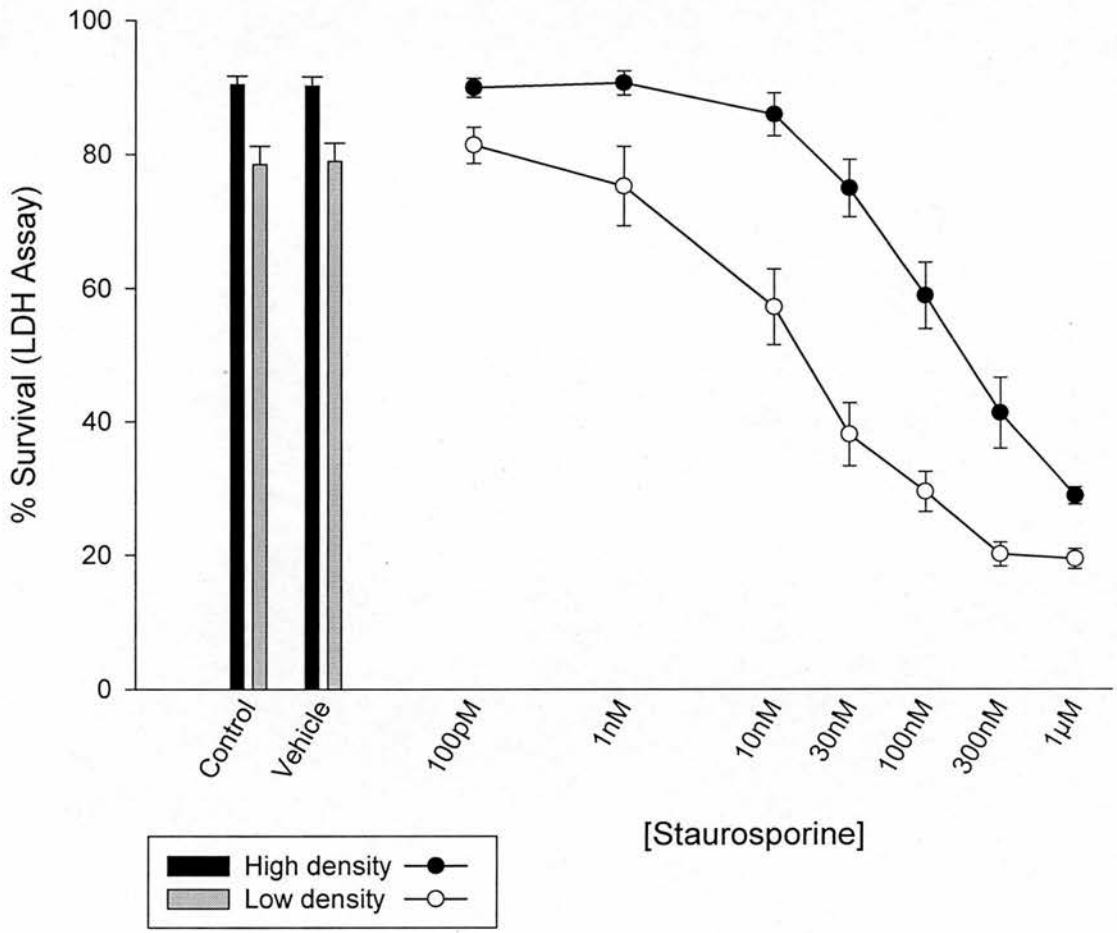
Again, the MTS, LDH and Calcein AM™ assays were compared following exposure to staurosporine for 24 hours. In contrast to the results for ionomycin, the MTS assay is significantly more sensitive to staurosporine-induced death than either the Calcein AM™ or the LDH assays (Figure 5.12) (Two way ANOVA, $F= 11.8$, $p<0.001$). At

Figure 5.8: Timecourse of death in response to staurosporine



Survival of terminally differentiated SHSY-5Y cells at passage 12 to 14 at high (filled circles: 150,000 cells per well) and low (open circles: 50,000 cells per well) density measured using the LDH assay 0, 2 4, 6 and 24 hours following exposure to 1μM staurosporine (mean ± s.e.m.: n=8 per point).

Figure 5.9: Concentration- response curves for staurosporine



Concentration- response curves for LDH release following 24 hours exposure to staurosporine in terminally differentiated SHSY-5Y cells at passage 12 to 14 at high (filled circles: 150,000 cells per well) and low (open circles: 50,000 cells per well) density (mean ± s.e.m.: n=8 per point).

24 hours, staurosporine has a more pronounced effect on mitochondrial respiration than on membrane integrity consistent with it causing apoptotic death.

Characterisation of staurosporine- induced death

Caspase activity

100nM staurosporine caused a significant ($p<0.03$) increase in DEVD cleavage activity (Table 5.2), in contrast to ionomycin, and consistent with the more pronounced effect on mitochondrial respiration described above.

Table 5.2: Effect of staurosporine on DEVD cleavage activity

Negative Control	Staurosporine 100nM	Positive Control Dexamethasone treated A1.1 cells
0.056 ± 0.068	0.908* ± 0.215	1.429 ± 0.126

mean ± s.d; relative fluorescent units per mg protein per minute;
*$p<0.03$ (t-test); Data from three independent experiments.

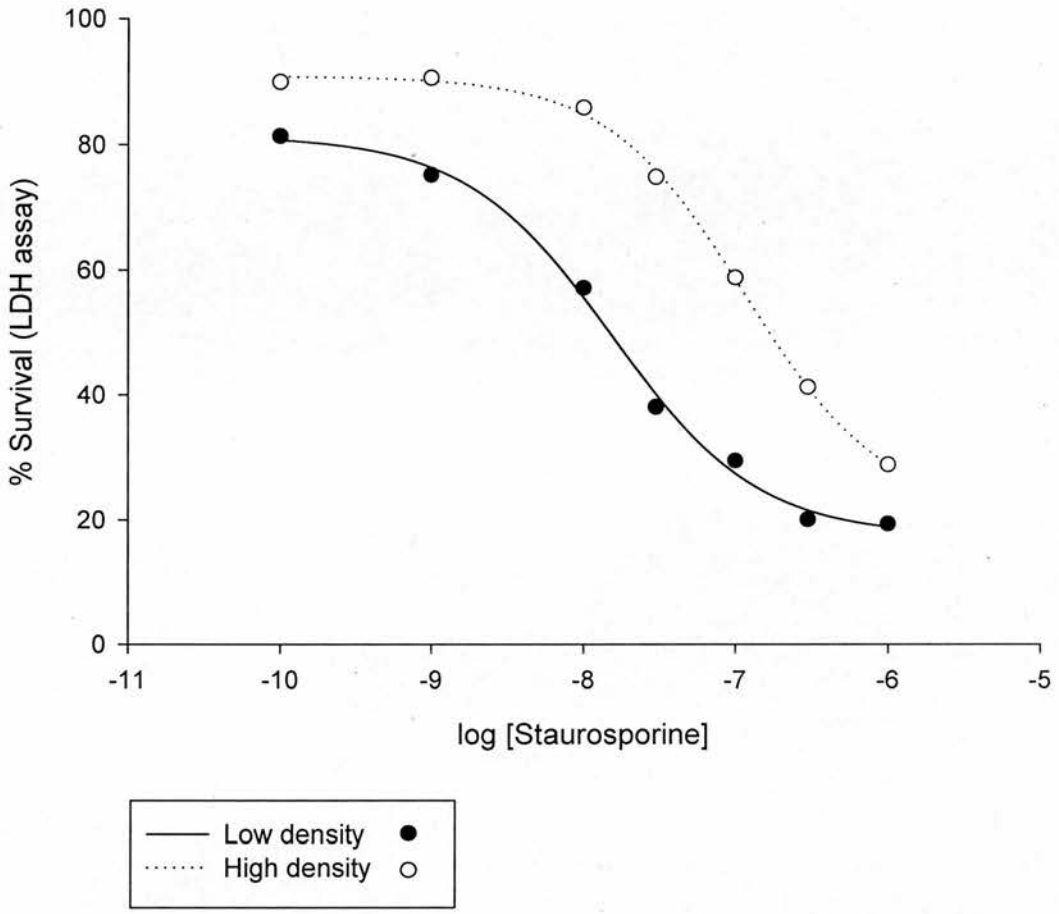
Effect of FK506

No combination of duration of exposure (from 2 to 24 hours), concentration of FK506 (from 1nM to 100nM) or cell density was associated with a protective effect of FK506 in cells exposed to 100nM staurosporine (Figs 5.13 and 5.14). These results were comparable whether FK506 was added 24 hours before or at the same time as staurosporine.

Discussion

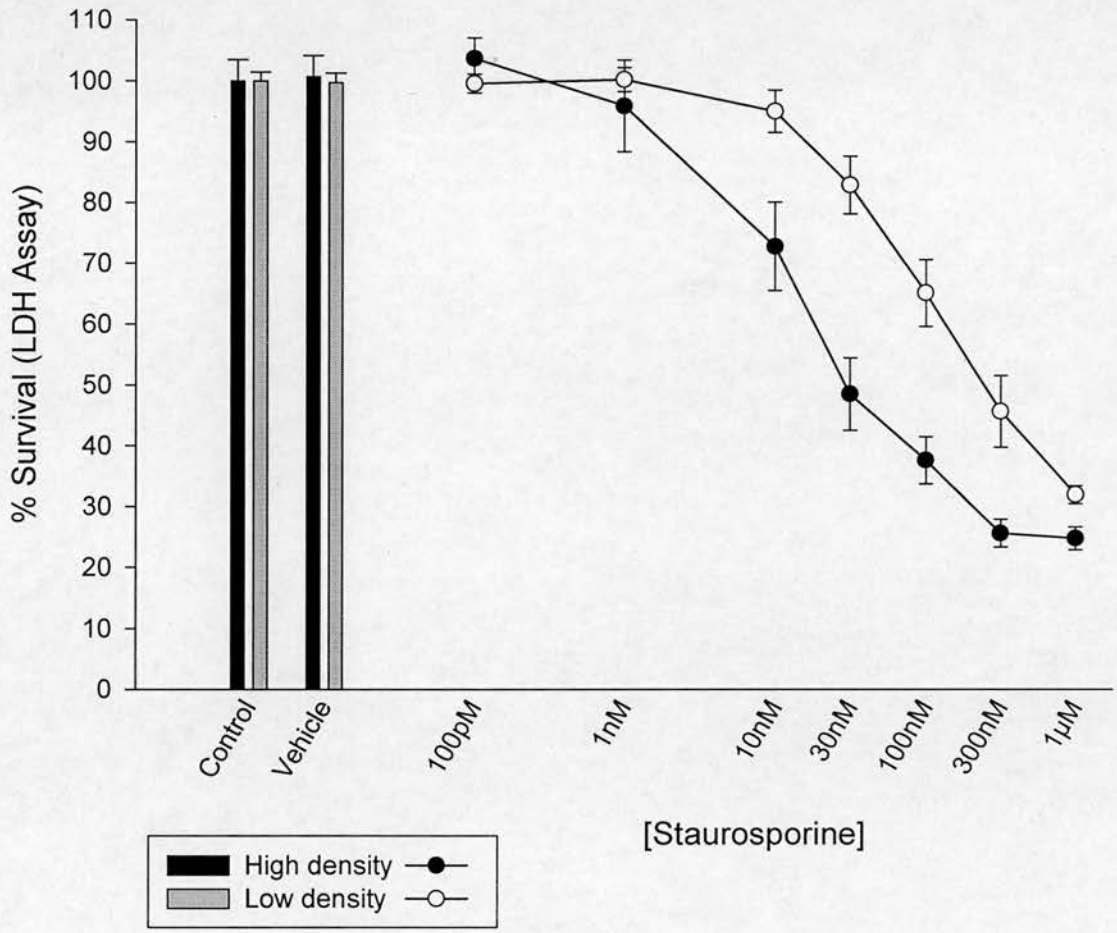
Staurosporine causes a concentration dependent death in SHSY-5Y cells with a timecourse of 4 to 6 hours, consistent with death occuring by apoptosis. The increased estimate of death using the MTS assay of mitochondrial function compared with the LDH assay of membrane integrity implies that membrane integrity is in this case preferentially preserved, as occurs in apoptosis. Furthermore, SHSY-5Y death was associated with caspase activation as manifest by a 16- fold increase in the rate

Figure 5.10: Curve fitting to staurosporine concentration- response data



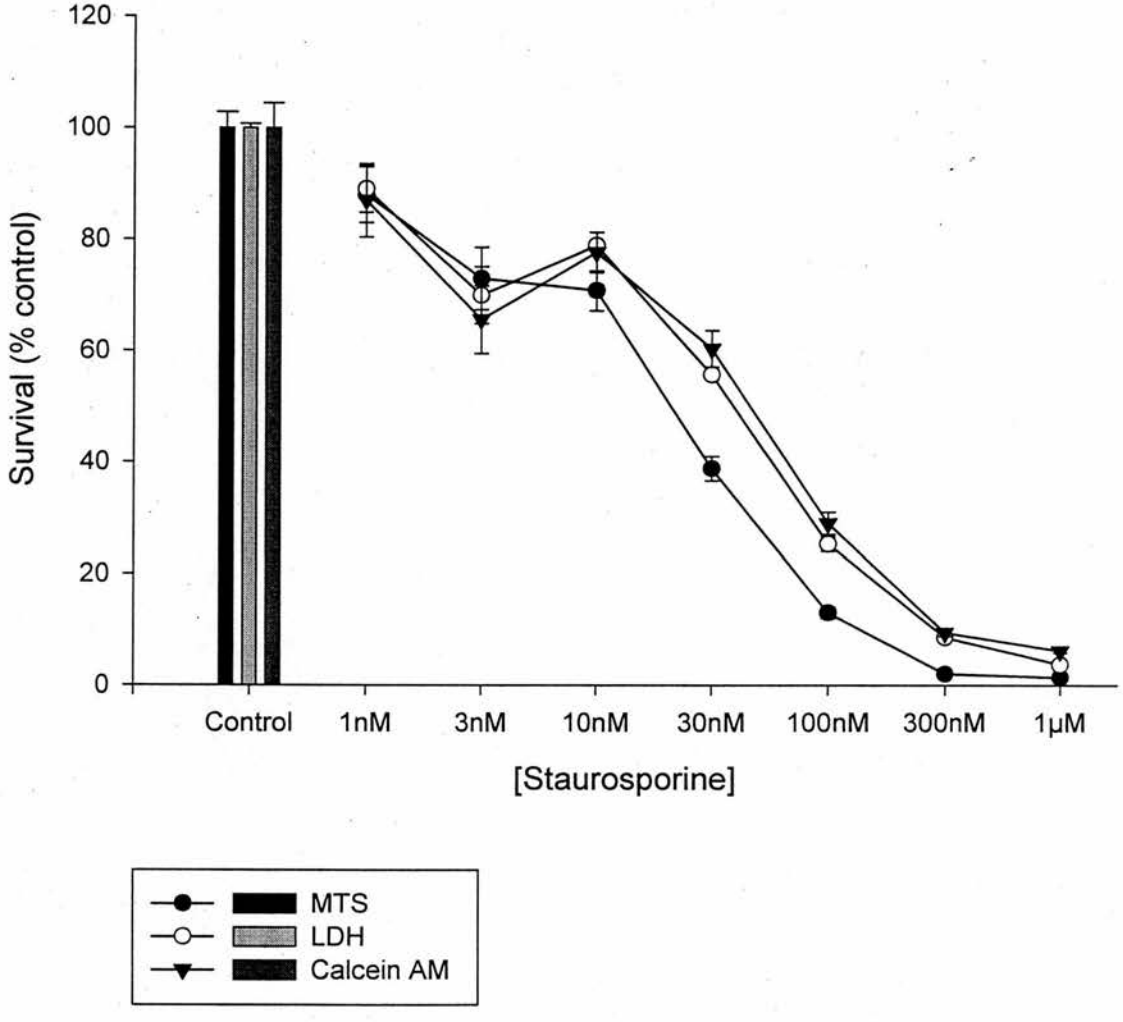
Fitting of staurosporine concentration- response data from Figure 9 to the formula Survival (y) = y_0 + (a/(1+e^((x_0-log[staurosporine])/b) where a, b, x_0 and y_0 are constants. The correlation coefficient r^2 for the line- fit was 0.996 for low density (closed circles) and 0.999 for high density (open circles) cultures.

Figure 5.11: Normalised staurosporine response



Data from Figure 9 normalised to Control survival = 100%. Note that the concentration- response curves remain separated, and the data for low (closed circles) and high (open circles) density cultures are significantly different (mean ± s.e.m.: Two way ANOVA; n=8, $F=38.552$, $p<0.001$).

Figure 5.12: Comparison of different endpoints for staurosporine- induced death



Concentration- response curves for staurosporine- induced death in terminally differentiated SHSY-5Y cells at passage 13 to 18 at high density (150,000 cells per well) measured using the MTS (closed circles), LDH (open circles) and Calcein AM™ (inverted triangles) assay systems. For each assay survival is normalised to Control survival = 100%. Note that while the curves for Calcein AM™ and LDH assays are superimposed one on the other the MTS concentration- response curve is more sensitive to staurosporine (mean ± s.e.m.: n=12 per point).

of cleavage of a synthetic Class II caspase substrate. Taken together, these observations demonstrate that, in common with many neuronal culture systems, SHSY-5Y death following staurosporine exposure manifests several features of apoptosis.

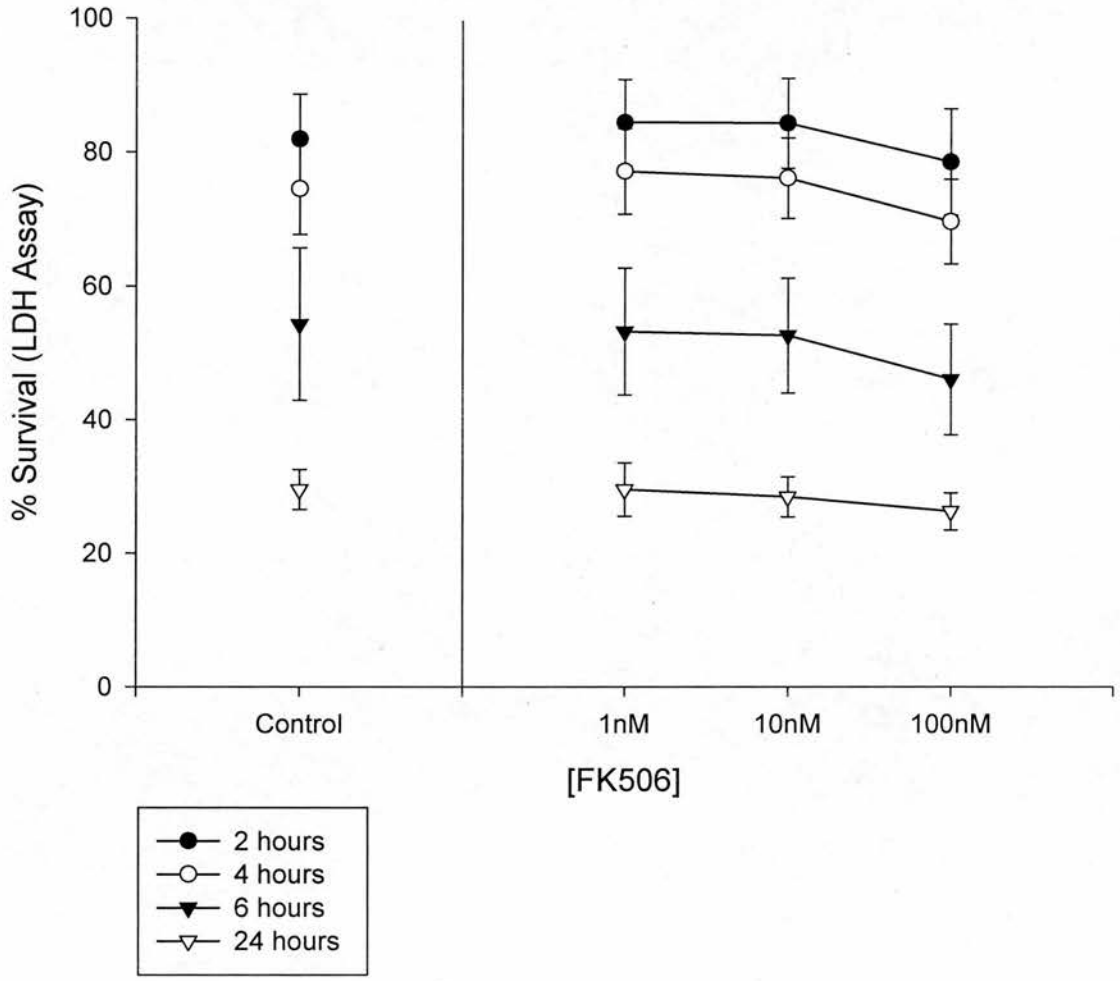
The difference in the toxic effects of staurosporine between high and low density cultures was more pronounced than seen with ionomycin, with a 6 to 7- fold reduction in the EC_{50}. Even when the effect of density on baseline survival is eliminated, there remains a significant effect of density on staurosporine- induced death. Clearly there is some survival influence present in high density cultures, whether this be due to chemical or physical interactions between cells or to the removal of some toxic compound - perhaps even staurosporine itself - from the culture medium. The larger difference in the EC_{50} is consistent with staurosporine causing death more typical of apoptosis and ionomycin causing death more typical of necrosis, as many such survival influences appear able to inhibit apoptosis, while few appear able to inhibit necrotic cell death.

FK506 was without effect in staurosporine treated SHSY-5Y cells. This was the case across a range of concentrations of FK506 against a concentration of staurosporine causing approximately 60% cell death. FK506 was ineffective if given 24 hours before or at the same time as staurosporine, and was also unable to delay death, being without effect at 2, 4, 6 or 24 hours. At the highest concentration, FK506 again showed a non-significant trend towards increased death, as was seen with ionomycin induced death.

Serum withdrawal

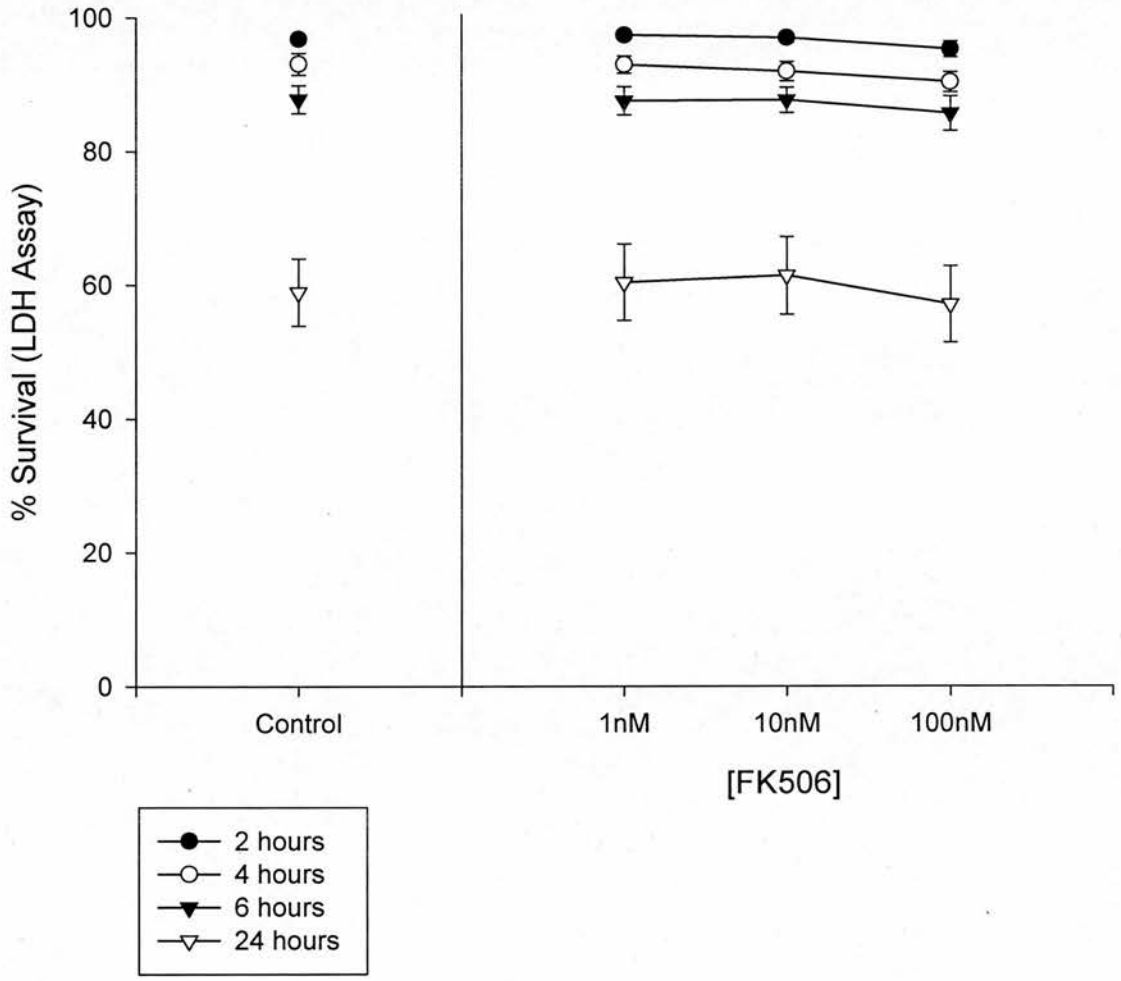
When serum was withdrawn from differentiated SHSY-5Y neurons a proportion of the cells decreased in size, fragmented and eventually detached from the culture dish. At 24 hours of withdrawal, analysis of collected, centrifuged cells using a chromatin stain revealed a proportion of cells with shrunken nuclei containing condensed chromatin (Fig. 5.15). At 48 hours these changes for individual cells appeared more

Figure 5.13: Effect of FK506 on staurosporine- induced death in low density cultures



Survival in low density cultures cells exposed to 100nM staurosporine for 2 (closed circles), 4 (open circles), 6 (closed triangles) or 24 (open triangles) hours in the presence of increasing concentrations of FK506. FK506 was without effect (mean ± s.e.m.: n= 8 per point).

Figure 5.14: Effect of FK506 on staurosporine- induced death in high density cultures



Survival in low density cultures cells exposed to 100nM staurosporine for 2 (closed circles), 4 (open circles), 6 (closed triangles) or 24 (open triangles) hours in the presence of increasing concentrations of FK506. FK506 was without effect (mean ± s.e.m.: n= 8 per point).

pronounced and by 72 hours the chromatin in some cells was crescent shaped and marginalised to the nuclear membrane. At later times however the overall proportion of cells displaying this morphological change was not greatly increased and at 24 and 72 hours many apparently normal cells were observed. It was not possible to quantify the numbers of apoptotic cells due to the limitations of the technique used. Oligonucleosomal DNA fragmentation was not observed.

Apoptosis requires the efficient disassembly of the cell co-ordinated by the activity of one or more members of the caspase family. Using an anti-caspase 3 antibody that detects processed subunits the large p17 subunit of caspase 3 was detected in serum deprived but not in control extracts at 24 hours, indicating processing and therefore activation of the enzyme (Fig. 5.16a). Caspase 3- like activity, measured using cleavage of a fluorogenic peptide substrate (Ac-DEVD-amc; Group II caspase (Thornberry et al., 1997)), was significantly increased following 24 hours of serum deprivation (Table 5.3) ($p<0.05$, t-test). In these and related samples, the proteolysis of a known caspase 3 substrate, PARP, was observed (Fig 5.16b) particularly at 48 hours. Cleaved PARP was undetectable in control extracts.

Table 5.3: Effect of serum withdrawal on DEVD cleavage activity

Negative Control	Serum withdrawal	Positive Control Dexamethasone treated A1.1 cells
0.115 ± 0.025	0.209* ± 0.019	1.885 ± 0.487

mean ± s.d; relative fluorescent units per mg protein per minute;
*$p<0.03$ (t-test); Data from three independent experiments.

FK506 may have neuroprotective properties through an inhibition of neuronal apoptosis. I therefore examined whether FK506 could prevent serum withdrawal induced apoptosis in SHSY 5Y cells. Exposure of serum-deprived cells to FK506 lead to a significant, concentration- dependent increase in survival at 24 hours ($F=8.38$, $p<0.001$, one way ANOVA) as quantitatively assessed using the MTS assay. After 24 hours of serum withdrawal alone cell viability was reduced to 69% of that seen in controls (Fig 5.17); there was no further decline in viability at longer

durations of serum withdrawal. The maximum effect of FK506 was observed at 30nM (viability 12% higher than serum withdrawal alone, $p<0.05$ with Dunnett's method for multiple comparisons). No concentration of FK506 was able to completely prevent the loss of viability that results from serum withdrawal. Indeed, as with the experiments on the effect of FK506 on the toxicity of ionomycin and of staurosporine, there is a suggestion of a toxic effect of FK506 at concentrations greater than 1μM. The survival- promoting effect was not augmented when cells were pre-incubated with FK506 for 24 hours prior to serum withdrawal.

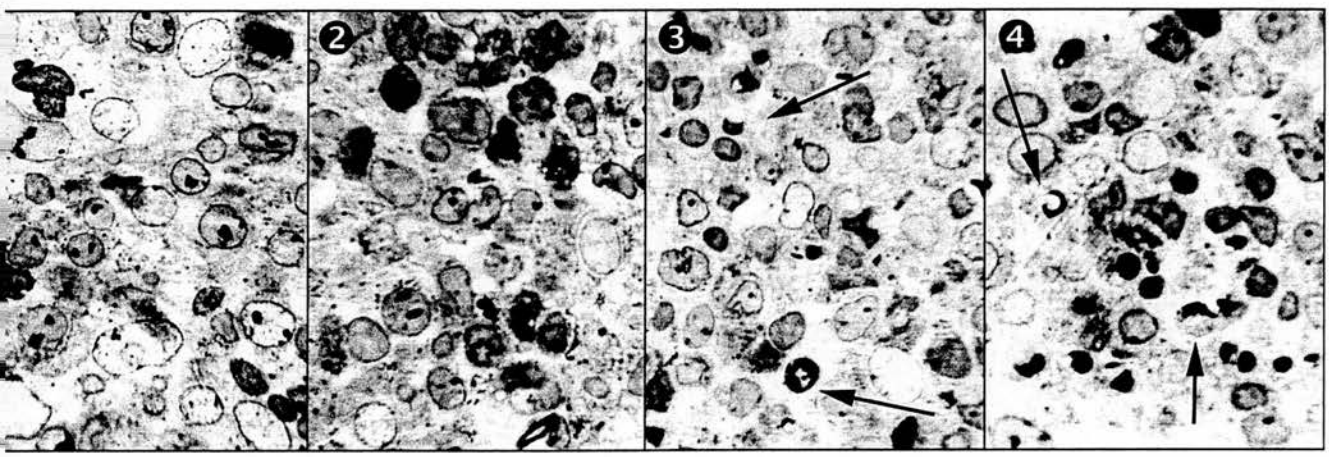
To determine whether the effect of FK506 was due to inhibition of apoptosis I examined its effects on markers of caspase activation. FK506 did not prevent either the processing of caspase 3 to yield the large 17kdal subunit (Fig. 5.16a) nor the proteolytic cleavage of PARP in SHSY-5Y cells deprived of serum for 48 hours (Fig. 5.16b). Furthermore, Ac-DEVD-amc cleavage activity was not reduced by FK506 (Fig. 5.16c). Thus FK506 was able to promote the apparent short term viability of a proportion of SHSY-5Y cells without inhibiting caspase activation or activity.

NGF withdrawal in mouse trigeminal ganglion sensory neurons

To determine whether FK506 had protective effects in other models of survival factor withdrawal induced apoptosis the effect of FK506 on NGF withdrawal-induced apoptosis in cultured embryonic mouse sensory neurons (Allsopp et al., 1993) was examined by Dr. Timothy E Allsopp.

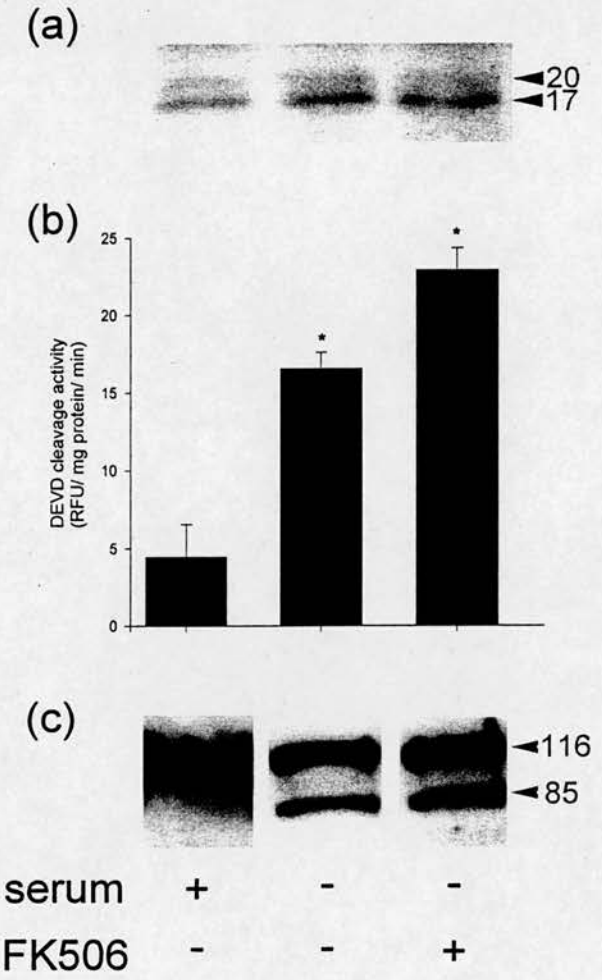
More than 90% of these cells (determined by direct counting of neurons) die within 48 hours of NGF withdrawal (Fig. 5.18). FK506 was only able to suppress a small proportion of this death ($F=15.48$, $p<0.001$) with maximum effect seen at a concentration of 100 nM (survival 18% of NGF-treated controls, compared with survival of 4% in the absence of FK506; $p<0.05$ with Dunnett's method for multiple comparisons). At higher concentrations FK506 did not promote survival and at the optimum concentration (100 nM) FK506 was unable to promote the long-term

Figure 5.15: Effect of serum withdrawal on nuclear morphology



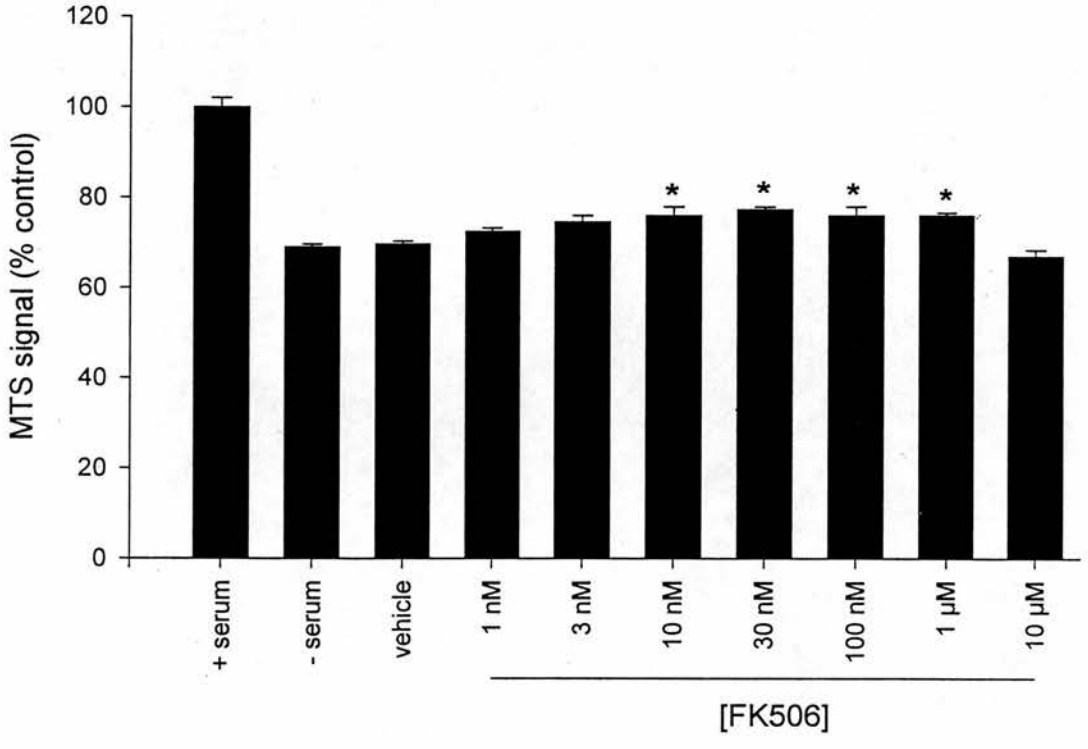
Terminally differentiated SHSY-5Y cells were either maintained in serum-containing medium (1) or deprived of serum and retinoic acid for 24 (2), 48 (3) or 72 (4) hours. Cells were then collected, rinsed in ice cold PBS and pelleted in preparation for fixation and embedding. Thick sections were cut and stained with toluidine blue. At 24 hours some increased chromatin condensation is apparent and by 48 hours there is crescent-shaped chromatin condensation (arrows). At 72 hours smaller chromatin fragments consistent with nuclear break up are observed against a background of cells which appear normal.

Figure 5.16: Serum withdrawal results in caspase 3 processing, poly (ADP-ribose) polymerase (PARP) cleavage and DEVD-amc cleavage activity



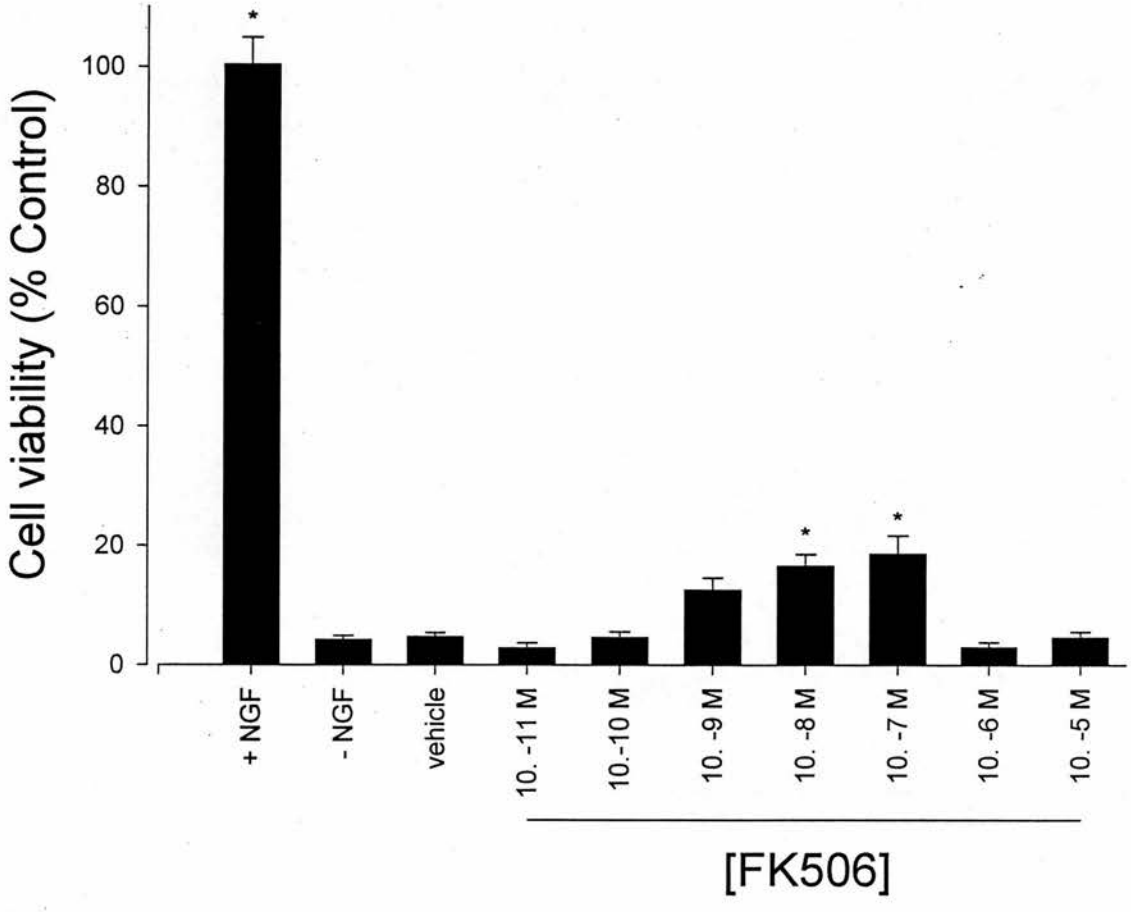
Neurons were maintained under control conditions (lane 1) or subjected to serum deprivation for 48 hours in the absence (lane 2) or presence (lane 3) of 100nM FK506. Extracts were prepared for SDS-PAGE analysis with immunoblotting for (a) caspase 3 or (c) PARP processing. Serum withdrawal results in accumulation of caspase 3 p17 subunit and PARP proteolysis. FK506 does not prevent either of these effects. (b) Neurons were serum-deprived for 24 hours then collected, extracted and caspase 3-like Ac-DEVD-amc cleavage activity analysed (Life Sciences Resources; Fluoroskan Ascent) using appropriate excitation (340 nm) and emission (460 nm) filters. Serum withdrawal leads to 3 fold increase in DEVD-amc cleavage activity ($p<0.05$, One way ANOVA with Dunnett's method for multiple comparisons) and FK506 has no effect on this activity.

Figure 5.17: Effect of serum withdrawal on the viability of terminally differentiated SHSY-5Y cells



Neurons were serum deprived for 24 hours in the absence of FK506 (vehicle) or the presence of various FK506 concentrations. Viability was subsequently assessed using the MTS assay (see Materials & Methods) and data is expressed as viability as a percentage of the maximum MTS signal in control cultures. 24 hours of serum deprivation causes a decline in viability to 69% of non-deprived controls. FK506 treatment results in a promotion of viability ($F=8.38$, $p<0.001$, $n=12$) which is concentration- dependent and which reaches a maximum of 77% of control at 10 nM (*$p<0.05$, Dunnett's post hoc test).

Figure 5.18: Effect of NGF withdrawal on the viability of mouse trigeminal ganglion sensory neurons



Embryonic sensory neurons were initially grown in the presence of NGF (4 ng/ml) and then deprived of NGF by extensive washing. The number of viable neurons possessing a phase bright soma and intact neurites longer than 3 times the cell soma diameter were initially counted in a defined area. 48 hours later the number of remaining viable neurons in each experimental condition was assessed and the data expressed as a percentage of the maximum cell survival seen in control (+NGF) cultures. NGF deprivation, shown here on a logarithmic scale, causes a decline in viability to 4% of control. FK506 treatment resulted in a concentration-dependent promotion of viability ($F= 15.48$, $p<0.001$, $n=16$) to a maximum of 18% of control at 100 nM (*$p<0.05$, Dunnett's post hoc test).

viability of NGF-deprived neurons. Nearly all neurons maintained by FK506 treatment at 48 hours were dead by 72 hours of NGF deprivation (data not shown). Thus FK506 was able to delay the death of a proportion of these neurons following NGF deprivation.

Discussion

SHSY-5Y cells apoptose in response to serum deprivation

Terminally differentiated SHSY-5Y neurons undergo apoptosis in response to serum withdrawal, as shown by changes in nuclear morphology, caspase processing and activation and endogenous caspase substrate proteolysis. 24 hours serum withdrawal leads to a 31% reduction in survival measured using the MTS assay. This is the first demonstration of apoptosis occuring in response to serum withdrawal in differentiated SHSY-5Y cells.

There was no further decline in viability from 24 to 72 hours, so the maximum reduction in survival was 31%, indicating that many terminally differentiated SHSY-5Y cells are resistant to serum withdrawal induced apoptosis. Survival beyond 72 hours was not measured because it would not be possible to distinguish between the effects of withdrawing serum and of falling retinoic acid concentrations due to its instability in solution; in SHSY-5Y cells withdrawal of retinoic acid has been reported to cause apoptosis (Lovat et al., 1997). In Chapter 4 I have demonstrated substantial heterogeneity of SHSY-5Y cells following differentiation, with many cells retaining non-neuronal morphologies and remaining negative for the differentiation markers MAP-2 and $p21^{waf-1}$. Furthermore, being of malignant origin these cells may have blunted responses to apoptotic stimuli. There may be differences between cells in the integrity of death pathways and this may give rise to substantial heterogeneity in the response to injury.

Oligonucleosomal DNA fragmentation was not observed in SHSY-5Y cells following serum withdrawal using standard techniques that were able to detect such DNA laddering in dexamethasone treated A1.1 T cell hybridoma cells. While DNA

laddering is considered to be one of the cardinal features of apoptosis, there are reports of apoptotic cell death occuring without oligonucleosomal DNA fragmentation in hepatocytes exposed to transforming growth factor ß (Oberhammer et al., 1993) MOLT-4 cells exposed to staurosporine (Falcieri et al., 1993) and PC-12 cells subjected to serum withdrawal (Mesner et al., 1992). Alternatively, it may be that because only one third of cells die their fragmented DNA contributes a smaller proportion of total DNA which is therefore more difficult to detect on agarose gels. It would be possible to address this question directly using alternative means of demonstrating DNA fragmentation, for instance by growing cells in the presence of bromodeoxyuridine (BrdUr) and staining electrophoresed DNA with an anti-BrdUr antibody, or using a ligation mediated PCR based technique to amplify the DNA fragments prior to conventional ethidium bromide/ agarose gel electrophoresis.

FK506 has no effect on markers of apoptosis following serum withdrawal

FK506 partially protects SHSY-5Y neurons from the decline in viability seen following serum withdrawal. While this death response manifests several hallmarks of apoptosis, the promotion of survival occurs without a detectable effect on caspase 3 processing or cleavage of Ac-DEVD-amc or PARP. Given the limited effect on survival, it may be that western blotting is not sufficiently sensitive to detect changes in caspase 3 processing or PARP cleavage. However, the lack of effect on Ac-DEVD-amc cleavage activity at 24 hours suggests that FK506 does not inhibit caspase activity following serum withdrawal. Alternatively, FK506 may inhibit caspase activity earlier in the course of the response to serum withdrawal, reflected in increased survival at 24 hours, with this inhibitory effect being lost by 24 hours. This could be due to a reduction in the concentration of FK506 at 24 hours, as described in Chapter 2, or to FK506 being able to delay but not prevent apoptosis following serum withdrawal.

The MTS assay was chosen as the primary endpoint because it appeared to be more sensitive to the development of apoptotic death following staurosporine exposure than either the LDH or the Calcein AM assays. As the MTS assay measures mitochondrial respiratory activity, it is conceivable that FK506 could be promoting

an increase in mitochondrial activity independent of an effect on cell survival. However, there was no such promotion of MTS signal in the continuing presence of serum, and a similar effect of FK506 was observed in preliminary experiments using the LDH assay. While Gold and colleagues have shown a trophic effect of FK506 on SHSY-5Y cells (Gold et al, 1999), this occurs at lower concentrations (10 pM to 10 nM) than the survival promoting effect observed here. Furthermore, since FK506 had no effect on the MTS signal under control conditions a direct trophic effect is unlikely. Therefore, the most reasonable explanation for the observed effect of FK506 following serum withdrawal in SHSY-5Y cells is an effect on cell survival.

Effect of FK506 on mouse trigeminal ganglion cells

Embryonic mouse sensory neurons undergo apoptosis when deprived of NGF and the reduction in viability is partially inhibited by FK506. The effect of FK506 was quantified by counting the number of remaining cells and is therefore not reliant on the MTS assay. As with SHSY-5Y cells FK506 had no effect on viability under control conditions, providing further evidence against a neurotrophic mechanism for the observed effects.

As with SHSY-5Y cells, the effect of FK506 is small, with a maximum of less than 20% of neurons remaining viable following 48 hours of NGF deprivation. This compares with rescue rates of over 90% in cells treated with the caspase inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde (Allsopp et al., 1998).

Mechanism(s) of survival promoting effect of FK506

The mechanism(s) through which FK506 protects cells from the consequences of survival factor withdrawal is not clear. In both SHSY-5Y cells and mouse trigeminal sensory neurons less than one third of dying cells can be rescued, and in SHSY-5Y cells there is no inhibition of caspase activity. Together with the near total inhibition of NGF withdrawal induced apoptosis seen in trigeminal ganglion sensory cells treated with caspase inhibitors this suggests that the protective effect of FK506 may not be mediated through an inhibition of apoptosis.

FK506, cyclosporin and their analogues have been shown to augment the neurite outgrowth-promoting effect of NGF on dorsal root ganglion explants and PC12 cells (Steiner et al., 1997), and Gold et al have recently shown that a similar effect of picomolar concentrations of FK506 on SHSY-5Y cells is due to inhibition of the activity of FKBP 52 (Gold et al., 1999b). However, the lack of effect of FK506 under control conditions on the end points used in both SHSY-5Y cells and trigeminal ganglion sensory neurons in the present study argues against a neurotrophic mechanism for the observed promotion of viability.

The immunosuppressant cyclosporin inhibits glutamate induced apoptosis in cerebellar granule cells, and it is thought that this may occur through inhibition of mitochondrial processes (Ankarcrona et al., 1996). However, this is not likely with FK506, as in contrast to cyclosporine, FK506 has no effect on the mitochondrial permeability transition (Friberg et al., 1998).

It may be that FK506 is able to inhibit some but not all components of the apoptotic machinery, or that it is able to delay but not prevent caspase activation. FK506 is known to inhibit the calcium dependent phosphatase calcineurin (protein phosphatase 2B), from which a number of consequences for neuronal survival might be predicted.

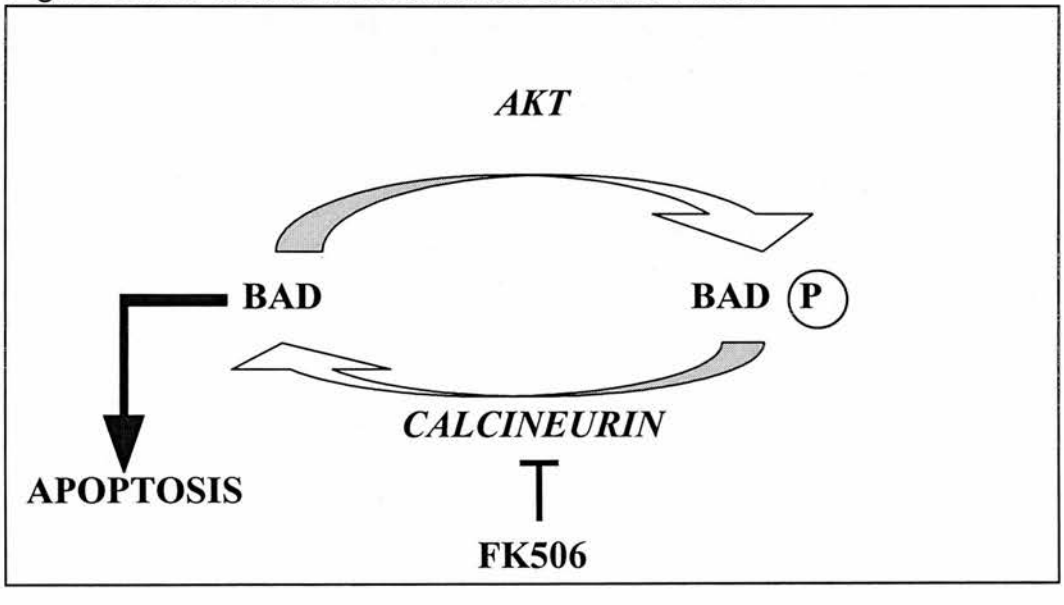
Survival signals such as insulin- like growth factor maintain neuronal survival by stimulating phosphatidyl inositol 3' kinase (PI3K), and inhibition of PI3K by compounds such as wortmannin results in cell death with many features of survival factor withdrawal induced apoptosis (D'Mello et al., 1997). PI3K activation leads to activation of protein kinase B (Akt) (Dudek et al., 1997). Akt activation leads to reduced caspase 9 activity (Cardone et al., 1998); to activation of the transcription factor NF kappaß (Ozes et al., 1999; Romashkova and Makarov, 1999), which can in turn induce TRAF1, TRAF2, c-IAP1 and c-IAP2 leading to inhibition of apoptosis (Wang et al., 1998); to retention of the Forkhead transcription factors FKHRL1 and AFX in the cytoplasm, inhibiting their proapoptotic activity (Brunet et al., 1999: Takaishi et al., 1999); and to phosphorylation of the proapoptotic Bcl2 family member Bad.

Overexpression of calcineurin kills cells by apoptosis (Shibasaki and McKeon 1995) and phosphorylation of Bad inhibits apoptosis (Zha et al 1996, Datta et al 1997); preventing Bad phosphorylation at serine136 by expressing mutant Bad where this residue is replaced by alanine abrogates the survival promoting effect of IGF-1 in cerebellar granule cells (Datta et al., 1997b). Recently Wang et al have shown that calcineurin can dephosphorylate Bad at multiple sites; that following L-glutamate treatment of primary rat hippocampal neurons Bad is dephosphorylated and translocated to mitochondria; and that these changes are blocked by FK506 and by overexpression of mutated calcineurin with dominant-negative activity (Wang et al., 1999).

Following survival factor withdrawal, decreased PI3K activity may lead to reduced Akt activity; under the influence of calcineurin, Bad phosphorylation may diminish with a consequent increase in its apoptosis- inducing activity. Therefore by reducing calcineurin activity FK506 may delay the apoptosis-promoting activity of Bad (Figure 5.19).

A number of experimental approaches could be adopted to further explore the mechanisms of FK506's neuroprotective actions. Determining whether FK506

Figure 5.19: Possible mechanism of action of FK506



analogues that are unable to inhibit calcineurin have survival promoting effects, or whether FK506 protects cells when calcineurin expression is reduced by antisense techniques, would confirm that FK506 mediates its effect through an effect on calcineurin. Testing the effect on SHSY-5Y cell survival of PI3K inhibitors such as wortmannin or LY 294002 would demonstrate whether or not survival influences were mediated through tonic activation of a survival factor–PI3K– Akt pathway in these cells. Finally, the effects of serum withdrawal and FK506 on serine136 phosphorylation of BAD could be examined using the specific antibody generated by Datta et al (Datta et al., 1997b).

GENERAL DISCUSSION AND CONCLUSIONS

Using three different insults I have demonstrated 3 different patterns of death in terminally differentiated SHSY-5Y cells. The calcium ionophore ionomycin caused rapid loss of mitochondrial function and membrane integrity, and FK506 was not able to inhibit that death. The protein kinase inhibitor staurosporine caused a slower loss of first mitochondrial function and then membrane integrity, and while this death had some features of apoptosis FK506 was again without effect.

Withdrawal of serum resulted in SHSY-5Y apoptosis as manifest by nuclear changes and caspase activation, and this reduction in survival was partially inhibited by FK506. As has been found in some other systems, SHSY-5Y apoptosis was not accompanied by oligonucleosomal DNA fragmentation; however, it may be that the use of more sensitive techniques would be able to detect its presence. FK506 was without effect on caspase activation at 24 hours, but any inhibition might be manifest only at earlier time points.

How FK506 protects these cells is not clear, and further experiments are needed to define its mechanism of action in this system. However, given that only one third of cells die following serum withdrawal and that FK506 is only able to rescue around one third of these, it may be difficult to design experiments where small changes in the efficacy of FK506 would be apparent. Furthermore, since two-thirds of cells do not appear to be affected by serum withdrawal, and two thirds of the remainder do

not appear to be affected by FK506, changes in protein expression or enzyme activity in the remaining ninth may be difficult to detect. In spite of these reservations, I believe further study of serum withdrawal induced apoptosis in terminally differentiated SHSY-5Y cells offers the potential to increase our understanding of the mechanism of action of FK506.

CHAPTER 6

DISCUSSION

FK506 is one of a number of chemicals, along with staurosporine, rapamycin and wortmannin, that occur naturally in fungi and that have profound effects on signal transduction in mammalian cells. This conservation of effect across species barriers suggests that these compounds may be involved with modulating fundamental processes common to much of the living world. With FK506 in particular, the involvement of its binding proteins in diverse signaling pathways including calcium release, the transforming growth factor ß receptor and steroid receptor complexes along with its ability, in complex with FKBP12, to inhibit the protein phosphatase calcineurin gives it many possible mechanisms of action. Where biological effects are of potential therapeutic importance, as with the immunosuppressive and neuroprotective effects of FK506, understanding the mechanism(s) that subserve these effects may help in the search for and the design of other drugs with greater efficacy and lower toxicity.

FK506 has potent neuroprotective properties in focal cerebral (Sharkey and Butcher, 1994) and transient global (Tokime et al., 1996) ischaemia and improves motor performance following middle cerebral artery occlusion (Sharkey et al., 1996). In primary neuronal cortical culture, FK506 inhibits glutamate- induced neuronal death (Dawson et al., 1993). In this thesis I have described the approach I have taken to identify those actions of FK506 responsible for its neuroprotective properties.

Previously, these neuroprotective properties had been attributed to inhibition of calcineurin-mediated dephosphorylation of nitric oxide synthase leading to reduced nitric oxide production and reduced free radical mediated damage. Evidence in support of this hypothesis came from primary cortical culture, where the neuroprotective effect of FK506 was associated with increased phosphorylation and reduced activity of nitric oxide synthase (Dawson et al., 1993). However, these authors did not demonstrate that inhibition of NOS was required for the

neuroprotective effect of FK506. Nonetheless, considerable evidence from both pharmacological nNOS inhibition with 7-nitroindazole (Yoshida et al., 1994) and from experiments in transgenic animals lacking nNOS (Hara et al., 1996; Huang et al., 1994; Panahian et al., 1996) suggests that nNOS makes an important contribution to neuronal death in stroke and that its inhibition has neuroprotective effects.

In Chapter 3 I describe experiments testing the hypothesis that FK506 is able to inhibit NMDA induced NOS activity in cerebellar prisms *ex-vivo*. In prisms from neonatal rats NMDA induces a rapid rise in NOS activity that in turn stimulates the enzyme guanylate cyclase and leads to the accumulation of cyclic GMP. Using a radioimmunoassay for cGMP I have demonstrated that cGMP accumulation begins within a minute of NMDA stimulation and is inhibited by the NMDA receptor antagonist MK-801 and the broad spectrum NOS inhibitor L-NAME. The NO donor SNP stimulated cGMP production, and this was not inhibited by either MK-801 or L-NAME. These results are consistent with NMDA inducing accumulation of cGMP through stimulation of NOS. I have gone on to demonstrate that in cerebellar prisms FK506 augments protein phosphorylation following NMDA stimulation, although I have not examined directly the effect of FK506 on NOS phosphorylation. In this system FK506 was entirely without effect on NMDA stimulated cGMP production.

The effect on protein phosphorylation demonstrates that FK506 is able to inhibit calcineurin in this preparation. It may be that calcineurin and NOS exist in separate cellular compartments, or that while inhibiting NO mediated cGMP accumulation FK506 simultaneously promotes cGMP accumulation through an alternative pathway; unfortunately it was not possible to measure NO production directly. However, the most credible explanation for these data is that in neonatal cerebellar prisms FK506 does not inhibit NOS activity.

The extent to which it is possible to generalise from this finding to the effect of FK506 on nNOS activity in adult cortex following middle cerebral artery occlusion is not clear. Attempts to measure cGMP production in prisms from adult rat hippocampus and cortex were unsuccessful because levels of cGMP produced were

below the limits of detection of the radioimmunoassay used.

The absence of an effect on cGMP production found here is in contrast to the findings in primary cortical culture of Dawson et al (Dawson et al., 1993) that FK506 inhibits NMDA mediated cGMP production. This may reflect a regional difference in the regulation of NOS activity. Alternatively, it may be that regulation of nNOS activity in dissociated culture does not reflect the situation in *ex vivo* preparations and *in vivo* following stroke, where FK506 may not inhibit nNOS activity.

Recent evidence supports the view that the neuroprotective effect of FK506 is not dependent on inhibition of nNOS activity. Cortical cultures derived from mice lacking nNOS are partially protected from NMDA- induced death, but not to the same degree as wild type cultures protected with FK506. Furthermore, in cultures lacking nNOS FK506 was still neuroprotective, reducing death by almost 30% (Dawson et al., 1996), demonstrating that nNOS is not required for the neuroprotective effect of FK506. More recently, the same group has reported that nNOS activity is not reduced by neuroprotective treatment with FK506 following middle cerebral artery occlusion in rats (Toung et al., 1999). That is to say, the effect of FK506 on nNOS activity in animal models of focal cerebral ischaemia mirrors the effect shown in Chapter 3 in cerebellar tissue prisms ex vivo, and not the effects reported earlier from primary cortical culture.

If FK506 is exerting its neuroprotective effects through other mechanisms, what might these be? FK506 is known to inhibit activation- induced apoptosis in T lymphocytes (Bierer et al., 1990), a process which involves marked increases in intracellular calcium similar to those seen in neurons exposed to hypoxia or excitotoxins. Apoptosis is now recognised as an important mode of neuronal death in cerebral ischaemia (Choi, 1996; Linnik, 1996), and FK506 might be reducing infarct size in focal cerebral ischaemia by inhibiting the apoptotic contribution to neuronal death.

To examine the role of FK506 in neuronal apoptosis I have examined apoptotic cell

death in a SHSY-5Y neuroblastoma cell line. This cell line was chosen because as a human cell line its characteristics are potentially more generalisable to human neurons than cell lines derived from other animals. Of the human neuronal cell lines available, SHSY-5Y cells were easier to grow and differentiate than for instance NTera II cells (Pleasure et al., 1992; Pleasure and Lee, 1993).

Initial experiments used differentiated SHSY-5Y cells at high passage. Neither their morphology nor their response to various toxins was constant. I therefore identified a source of low passage SHSY-5Y cells and compared their behaviour with high passage cells under differentiating conditions. A retinoic acid / serum reduction differentiation regime was chosen because other regimes such as those using phorbol esters or cAMP analogues might have a greater confounding impact on neuronal second messenger systems involved in signaling death pathways than retinoic acid. This work is described in Chapter 4, where I demonstrate that treatment of low passage cells with retinoic acid and serum reduction for 7 days results in the development of neuron- like morphology, with stabilisation of cell number and the development of immunopositivity for the neuronal marker MAP2 and the cell cycle G_0 marker $p21^{waf}$.

In Chapter 5 I describe the response of terminally differentiated SHSY-5Y cells to three different death inducing stimuli. The calcium ionophore ionomycin caused rapid and simultaneous loss of mitochondrial respiratory activity and membrane integrity (determined using the MTS and LDH assays respectively) and had no effect on endogenous (PARP) or synthetic (Ac-DEVD-amc) Caspase 3 substrate cleavage. This demonstrates that in SHSY-5Y cells at the concentrations studied ionomycin does not cause apoptotic death. This contrasts with findings from primary neuronal culture, where at similar concentrations it has been reported to induce apoptosis (Hatanaka et al., 1996; Takei and Endo, 1994). While FK506 has been reported to inhibit ionomycin- induced responses in T- lymphocytes (Mattila et al., 1990) it was without effect on ionomycin- induced cell death in SHSY-5Y cells.

The broad spectrum protein kinase C inhibitor staurosporine also caused death in

SHSY-5Y cells in a concentration dependent fashion, but the onset of the death was slightly slower, and there was a significant difference between the MTS and LDH assays. The decline in viability determined using the MTS assay, an index of mitochondrial function, was more pronounced than that seen using the LDH assay, a measure of membrane integrity. Because loss of membrane integrity is a held to be a late event in apoptosis, this finding suggests that staurosporine may be causing apoptosis in SHSY-5Y cells. This is supported by the increased cleavage of the synthetic caspase 3 substrate Ac-DEVD-amc seen following staurosporine treatment.

Staurosporine was chosen as a prototypical inducer of neuronal apoptosis. The mechanism of this action is not clear, as in addition to its effect on protein kinase C staurosporine activates a p57 Jun Kinase (Yao et al., 1997). It has also been reported to have NGF- like effects on PC12 cells (Yao et al., 1997), G protein mediated effects on phospholipase D activity in peritoneal neutrophils (Kanaho et al., 1992), and it may induce ceramide pathways in primary culture of embryonic chick cortical neurons (Wiesner and Dawson, 1996). Irrespective of the mechanism through which staurosporine induces apoptosis in SHSY-5Y cells FK506 was again without effect.

Serum withdrawal caused the apoptosis of a proportion of SHSY-5Y cells, as evidenced by characteristic nuclear changes, caspase activation and cleavage of caspase substrates. FK506 partially reduced the decline in viability seen following serum withdrawal, but was without discernable effect on caspase activation. However, given the magnitude of the FK506 effect changes in caspase activity below the limits of detection of the methods used, or indeed caspase inhibition occuring at an earlier timepoint, cannot be excluded. While FK506 has been reported to have neurotrophic effects in cell culture, these generally occur at lower concentrations to those at which it has its protective effect here. No effect of FK506 on MTS signal was observed under control conditions, making this an unlikely explanation for the observed effects.

SHSY-5Y apoptosis following serum withdrawal results from the withdrawal of some factor or factors present in serum whose continuing presence is required for

cell survival. Neuronal apoptosis induced by withdrawal of such factor is though to be mediated through a reduction in the tonic activity of a PI3K pathway that normally has a number of apoptosis- inhibiting consequences. One such consequence is increased Bad phosphorylation, and by reducing calcineurin activity FK506 may delay Bad dephosphorylation following serum withdrawal.

It is not yet clear to what extent deactivation of this PI3K pathway is responsible for neuronal apoptosis following stroke. Experiments targeting components of other pathways, such as those with transgenic animals lacking the DNA damage sensing protein p53 (Crumrine et al., 1994), or with pharmacological inhibition of NMDA receptors (Didier et al., 1996; Ikeda et al., 1996), suggest that these pathways are important routes to apoptosis following stroke. Involvement of the PI3K signaling in neuronal injury following stroke has yet to be demonstrated. However, Bad phosphorylation is not solely a consequence of PI3K pathway activation, as it is also a target for the MAP kinase survival promoting pathway (Bonni et al., 1999). Furthermore, expression of a dominant negative form of calcineurin inhibits L-glutamate mediated excitotoxicity in hippocampal cells (Wang et al., 1999). Therefore, the effect of FK506 may be mediated through such an interaction even if a PI3K pathway is not itself activated in focal cerebral ischaemia.

Abrupt changes in mitochondrial permeability (the mitochondrial permeability transtion) are thought to act as a signal for cell death, and cyclosporine blocks the mitochondrial permeability transition. However, this cannot explain the neuroprotective properties of FK506 as FK506 has no effect on the mitochondrial permeability transition (Friberg et al., 1998).

FK506 has been shown to have neurotrophic properties in cell culture (Gold et al., 1999b) and in neurite regrowth following injury (Steiner et al., 1997). While the effect on SHSY-5Y cells following serum withdrawal is unlikely to be due to such an effect, for the reasons given above, it may be that the neuroprotective effects of FK506 following focal cerebral ischaemia are due to such neurotrophic properties rather than to an effect on neuronal apoptosis.

In conclusion, I have demonstrated that FK506 can be without effect on NOS activity in brain tissue. Secondly, I describe the terminal differentiation of SHSY-5Y cells. In these cells both serum withdrawal and staurosporine cause a decline in viability that has some of the features of apoptosis; the effect of serum withdrawal, but not staurosporine, is partially reversed by FK506.

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