DEVELOPMENT OF *IN VITRO* MODELS OF NMDA EXCITOTOXICITY AND ASSESSMENT OF EXCITOTOXICITY MODULATION BY NEUROSTEROIDS

by

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Abstract

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Mouse and rat primary hippocampal cultures, along with mouse fibroblast cultures transfected with N-methyl-D-aspartate (NMDA) receptor subunits, were developed as models of NMDA receptor-mediated excitotoxicity. These systems were subsequently used to evaluate the modulation of excitotoxicity by the neurosteroids pregnenolone sulfate and pregnanolone sulfate.

Excitotoxicity was not ellicited reproducibly in the rat hippocampal culture model. Furthermore, immunohistochemical characterization of neurons and glial cells in these cultures was not accomplished. However, measurement of excitotoxicity in the mouse hippocampal culture model was characterized, and thus, pregnenolone sulfate and pregnanolone sulfate modulation of NMDA excitotoxicity was examined. While several concentrations of pregnenolone sulfate appeared to positively modulate NMDA excitotoxicity, vehicle toxicity was marked. Consequently, the modulation of NMDA toxicity by pregnenolone sulfate, at concentrations ranging from 0.001 mM to 0.1 mM, was difficult to establish. In contrast, it was determined with greater confidence that pregnanolone sulfate, at a concentration of 0.05 mM, negatively modulates NMDA toxicity.

A novel model system, consisting of mouse fibroblasts expressing either human recombinant NMDA receptor subunits (NR) 1a/2A or NR1a/2B was successfully developed as a further model of NMDA receptor dependent toxicity. The system was subsequently employed to examine pregnenolone sulfate and pregnanolone sulfate modulation of excitotoxicity. pregnenolone sulfate at a concentration of 0.01mM failed to modulate NMDA receptor mediated toxicity, while 0.10 mM pregnanolone sulfate negatively modulated toxicity in both the NR2A and NR2B expressing cell lines.

In summary, pregnenolone sulfate possibly potentiated NMDA excitotoxicity while pregnanolone sulfate reduced NMDA excitotoxicity in the mouse hippocampal culture model of toxicity. In the mouse fibroblast model of NMDA excitotoxicity, pregnenolone sulfate had no modulatory effect on NMDA receptor mediated excitotoxicity, at a single dose. Pregnanolone sulfate , however, negatively modulated the toxicity, independent of NMDA receptor subunit composition.

Key words: NMDA, excitotoxicity, primary culture, NMDA receptor-transfected fibroblasts, pregnenolone sulfate, pregnanolone sulfate.

CO- AUTHORSHIP

Primary culture and mouse fibroblast experiments described in this thesis were designed by the candidate, Michael Murray Scott in conjunction with his supervisors Dr. K. Jhamandas and Dr. R. Boegman.

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List of Abbreviations and Symbols

α	alpha
γ	gamma
µl	microliter
µM	micromolar
2-AP-7	D-2-amino-7-phosphonoheptanoic acid
L-2-AP4	L-2-amino-4-phosphonobutanoic acid
2R,4R-APDC	2R,4R-4-aminopyrrolidine-2,4-dicarboxylate
3,5-DHPG	(S)-3,5-dihydroxy-phenylglycine
3HPG	(S)-3-hydroxy-phenylglycine
ANOVA	analysis of variance
D,L-AP5	D,L-amino-5-phosphonopentanoic acid
AraC	cytosine arabinoside
ATP	adenosine triphosphate
BAEE	N,α-benzoyl-L-arginine ethyl ester
Bcl-2	anti-apoptotic *caenoraptidis elegans* -like gene product
BSA	bovine serum albumin
$CA_{1,2,3}$	cornu amonis
CaMKII	calcium\calmodulin dependent protein kinase 2
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione

CNS	central nervous system
DAB	diamino benzidine
DCG-IV	(2S, 1'R,2'R,3'R-2)-(2,3-dicarboxycyclopropyl)-glycine
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHPROG	dihydroprogesterone
DIV	days in vitro
DMEM	Dulbecco's modified Eagle's medium
DMEM-base	Dulbecco's modified Eagles medium (base)
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease I
DNQX	6,7-dinitroquinoxaline-2,3-dione
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
EAA	excitatory amino acid
EC	entorhinal cortex
FBS	fetal bovine serum
FCS	fetal calf serum
FUDR	(+)-5-fluorodeoxyuridine
GABA	γ-amino-butyric acid
$GABA_A$	type A GABA receptor
GD	dentate gyrus
GFAP	glial fibrillary acidic protein

G_i	i class G-protein
GluR	ionotropic glutamate receptor
G_o	o class G-protein
G_q	q class G-protein
HBSS	Hank's balanced salt solution
HEK	human embryonic kidney cells
HS	horse serum
IEG	immediate early genes
KA	kainate subfamily of glutamate receptor subunits
KCl	potassium chloride
K_D	equilibrium dissociation constant
KH_2PO_4	potassium dihydrogen phosphate
LDH	L-lactate dehydrogenase
M	molar
MCPG	methylcarboxy-phenylglycine
mg	milligram
Mg^{2+}	magnesium ion
mGluR	metabotropic glutamate receptor
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid

N	number of experiments
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride
NAD	nicotinaminde adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NBQX	2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo-(F)-quinoxaline
NFP	neurofilament protein
nM	nanomolar
NMDA	N-methyl-D-aspartic acid
NO	nitric oxide
NOS	nitric oxide synthase
NR	NMDA receptor
P450	absorbance peak (at 450 nanometers)
PARS	poly-ADP-ribosylsynthase
PBS	phosphate buffered saline
PCP	phencyclidine
PCR	polymerase chain reaction
PKC	protein kinase C
PLC	phospholipase C
PREG	pregnenolone
PREG-S	pregnenolone sulfate
PREGNAN-S	pregnanolone sulfate

PROG	progesterone
PTP	permeability transition pore
RNA	ribonucleic acid
SD	standard deviation
SU	subiculum
TBS	Tris-buffered saline
TBX	tris buffered triton X-100
THPROG	trihydroprogesterone
Tris base	tris[hydroxymethyl]aminomethane
Tris HCl	tris[hydroxymethyl]aminomethane hydrochloride
Ca^{2+}	calcium ion
±	plus/minus

Chapter I

General Introduction

1.1 Historical Overview of Glutamate in the CNS

L-Glutamate, a non essential amino acid, is crucial in mediating excitatory neurotransmission in the central nervous system. This fact was first demonstrated over four decades ago, with the application of glutamate to the cerebral cortex of experimental animals producing both neuronal depolarization and spreading depression (Hayashi 1954, Van Harreveld 1959).

Since that time, numerous other studies have validated the current hypothesis that glutamate excites neurons and acts as a neurotransmitter. For example, glutamate has been shown to be released from brain tissue (Bliss et al 1986), and more specifically from nerve terminals (Nadler et al 1976) upon neuronal stimulation. As well, glutamate uptake processes have been identified both in neurons and in glia (Logan and Snyder 1972, Drejer et al 1982). Additionally, mechanisms which synthesize a releaseable source of L-glutamate have been located in neurons (Patel et al 1982). Lastly, excitatory amino acid (EAA) receptors have been identified on neuronal membranes by using specific antagonists which block neurotransmitter action (Curtis et al 1972). These observations have led to the proposal that glutamate acts as a neurotransmitter in the central nervous system.

In conjunction with these studies, the location of central nervous system neurons which

respond to glutamate was investigated. Localized application of L-glutamate and as well L-aspartate, another dicarboxylic amino acid, to diverse CNS structures was shown to elicit neuronal depolarization, suggesting glutamate may act as a neurotransmitter, in the cerebral cortex, cerebellar cortex, hippocampus, brain stem and the spinal cord. More detailed studies on the release of glutamate and aspartate from axonal terminals, and on the retrograde transport of radiolabelled amino acids, allowed the mapping of specific glutamatergic pathways within these structures of the CNS. For example, cortico-cortical glutamatergic pathways within the cerebral cortex, along with projecting pathways from the cortex to the spinal cord (motor neuronal pathways), substantia nigra, and hippocampus (Fagg et al 1978, Fonnum et al 1979, Kerkerian et al 1983, Cuenod et al 1981) amongst others have been identified. Lastly, the existence of specific glutamatergic neuronal circuits such as the excitatory pathway leading from the entorhinal cortex to the dentate gyrus, CA3, and CA1 areas of the hippocampus (Nadler et al 1976), has been described.

In characterizing glutamatergic neurotransmission, it was also quickly realized that glutamate may act as a toxin in the same pathways in which it functions as an excitatory neurotransmitter. In 1957, Lucas and Newhouse noticed that glutamate administration to piglets caused damage to the developing retina. In the late 1960's, Olney discovered that systemic glutamate administration to infant mice produced neurodegeneration in the retina and other specific areas of the brain (Olney 1969). Subsequently, glutamate-mediated neurodegeneration was observed in other animal species including primates (Olney 1972). Finally, in the ensuing decades, the role of glutamate mediated toxicity as a causative factor in human neurodegenerative diseases such as Huntington's Chorea (Feigin 1998), Parkinson's disease

(Blandini 1996), Alzheimer's disease (Greenamyre 1989) and cerebral ischemia (Rothman 1984) was recognized, providing convincing evidence that glutamate may not only act as a neurotransmitter but as a neurotoxin as well.

1.2 Glutamate Receptors

The most important step in glutamatergic neurotransmission is the interaction of the neurotransmitter with post synaptic receptors. It is via this interaction that complex signals transmitted to the post synaptic neuron are encoded. By creating variations in glutamate concentrations, both temporally and spatially, different glutamate receptors may be activated, to convey different signals. For example, the N-methyl-d-aspartate (NMDA) receptor binds glutamate with a higher affinity than the (AMPA) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor. Thus, NMDA receptors located at the periphery of the synapse, which see reduced concentrations of glutamate owing to its rapid diffusion from the point of release, may be activated by lower glutamate levels (Rusakov and Kullmann 1998). Additionally, different glutamate receptors show differences in voltage-dependant activation and ion conductance (as some glutamate receptors form ion pores)(Choi 1988).These characteristics thus allow the presynaptic nerve depolarization and subsequent release of glutamate to convey highly specific and graded signals to the post synaptic neuron. Remarkably, pertubations in glutamate signaling have been shown to mediate cell death, through the activation of glutamate receptors. Consequently, the structural and functional characterization of different glutamate receptor families has become an important objective.

Currently, there are two main classes of glutamate receptors: Ionotropic receptors, which constitute the ligand gated ionic pores in the cell membrane, and metabotropic receptors, which are coupled to second messenger systems within cells, via Gi/Go or Gq proteins.

1.3 Ionotropic Receptors

The ionotropic receptors have been divided into three subtypes on the basis of activity of three different glutamate analogues: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate.

1.4 NMDA Receptor Pharmacology

The NMDA receptor is an ion pore which allows entry of sodium and calcium across the neuronal membrane and produces a local depolarization in the post synaptic neuron (Watkins 1994). Several agonists of the NMDA receptor have been characterized, specifically, NMDA, ibotenic acid, quinolinic acid (a naturally occurring pyridine dicarboxylate) and glutamate itself. Among the different receptors, the NMDA receptor has the highest affinity for glutamate (Michaelis 1998).

There are several distinctive features of the NMDA receptor that are not shared by the other glutamate receptor subtypes. The NMDA receptor has several modulatory sites (Figure 1). Two main sites are the glycine site, at which this amino acid acts as a co-agonist, and the channel

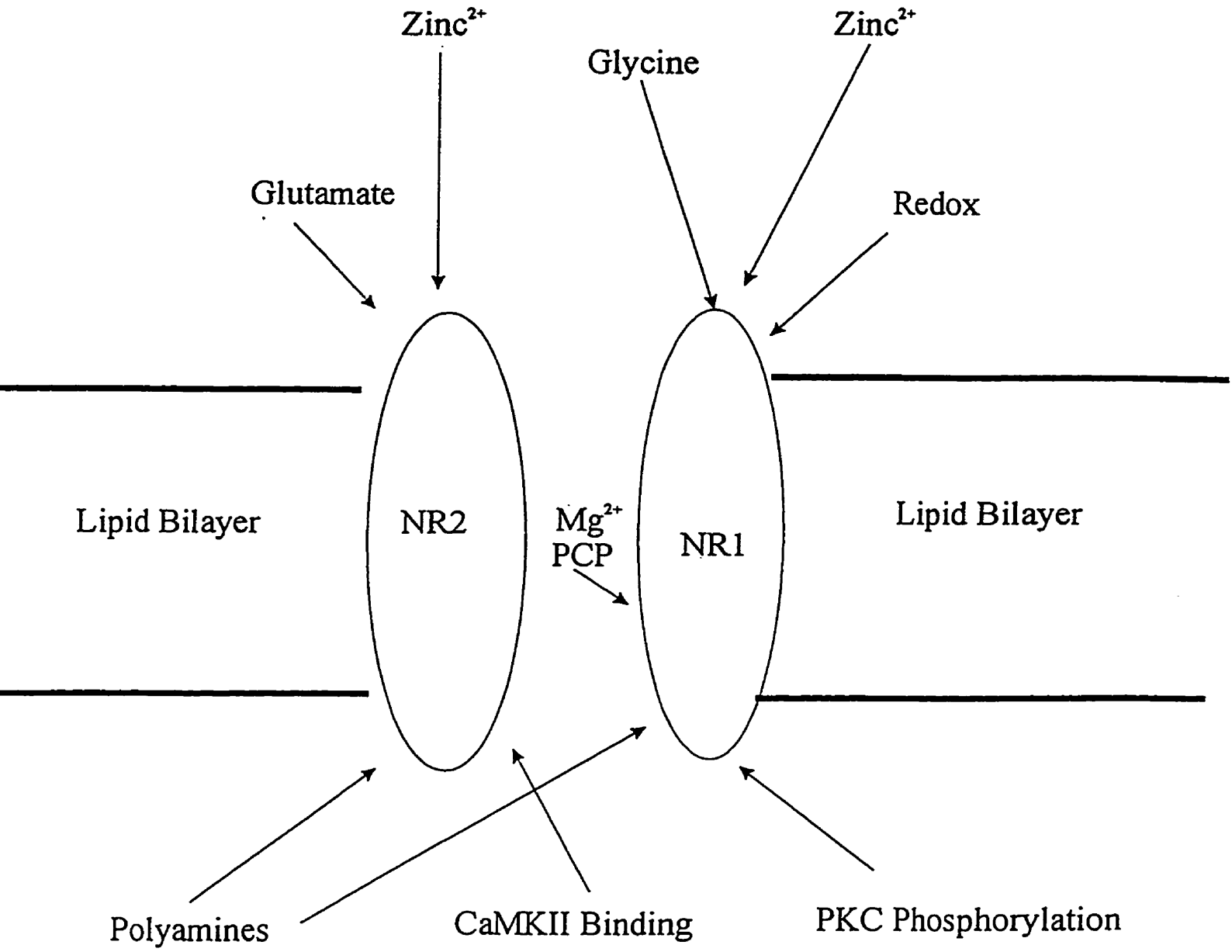


Figure 1. Schematic representation of the NMDA receptor composed of NR1 and NR2 subunits. Location of subunit specific binding or modulatory sites for zinc (Hollmann et al. 1993, Chen et al 1997), glycine (Ivanovoc et al. 1998), glutamate (Ivanovic et al. 1988), polyamines (Kasliwagi et al. 1997), protein kinase C (PKC) (Zheng et al. 1997), calcium/calmodulin dependent protein kinase II (CaMKII) (Stock and Colbran 1988), phencyclidine (PCP) and magnesium (Mg^{2+}) (Sakurada et al. 1993).

site, at which agents such as phencyclidine (PCP) and MK-801 bind to block NMDA receptor function. Additionally there are the zinc, polyamine and redox sites. Binding of glycine at the glycine site is required for glutamate activation of the receptor, while a magnesium ion binds the channel site, inhibiting the receptor, at resting membrane potential. Binding of zinc produces a negative modulation of the receptor, polyamine binding positively modulates receptor activity as does reduction of the redox site(Mayer et al. 1994, Wong and Kemp 1991, Johnson and Ascher 1987). Lastly, unlike the other glutamate receptors, the channel displays relatively long opening and closing times, exhibits little desensitization, and has a large ion conductance (McDermott et al 1986, Mayer et al. 1989).

In recent years, numerous competative and non-competative antagonists for the NMDA receptor have been developed and these are summarized in Table 1. For example, certain antagonists (such as PCP, dizoclipine-HCl, ketamine) bind close to the magnesium binding site and block ion entry (Wong and Kemp 1991). Other antagonists such as D-AP5 act as competitive antagonists and block the agonist binding site on the receptor complex (Davies and Watkins 1982).

1.5 Subunit Composition of the NMDA Type Glutamate Receptor

The NMDA receptor, is thought to be composed of 5 subunits (Michaelis 1998), which are drawn from the NR1 or NR2 subfamilies (Monyer 1992). All together, 13 gene products have been identified, by use of a polymerase chain reaction (PCR)- based selection and amplification method. These encode subunits that belong to either the NR1 or NR2 families (Nakanishi 1992).

Subtype	NMDA	AMPA	Kainate	Metabotropic
Subtype Selective Agonists	NMDA	AMPA	Kainate	3,5-DHPG (Group I) 2R,4R-APDC(GroupII) L-2-AP-4 (Group III)
Other Agonists	Ibotenate, Quinolinate	Quisqualate, Kainate, Domoate	Domoate	Quisqualate, Ibotenate, DCG-IV
Allosteric modulators	Glycine, Spermine	Benzothiazides	Concavalin A	
Competitive Antagonists	2-AP5, 2-AP7	CNQX, DNQX, NBQX		Phenylglycine analogs (MCPG, 3HPG)
Antagonists at modulatory sites	5,7-Dichlorokynurenic acid	GYKI 52466		
Ion Channel Inhibitors	PCP, MK-801, Ketamine	Joro Spider Toxin		

Table 1. Antagonists, agonists, and modulators of the glutamate receptor subtypes. Adapted from Michaelis 1998 and Conn and Pin 1997.

The stoichiometry of the NMDA receptor complex is yet to be established, however, it is known that the receptor must include subunits from both the NR1 and NR2 families and that receptors composed solely of NR2 subunits do not form functional channels (Grimwood 1996) . The subunits of the NR1 family all originate from the same gene, yet undergo alternative RNA splicing to produce eight different subunit proteins, designated NR1a-h, while the NR2 family consists of four different proteins encoded by four different genes (Nakanishi 1992). The result of this diversity in subunit combinations is a population of NMDA receptors with a great diversity in channel properties. (Ishii et al. 1993, Hollmann et al. 1993) For example, by altering the splice variants of the NR1 family present in the receptor, it is possible to alter binding of agonists, antagonists and other elements which effect channel function. Furthermore, by altering the type of NR2 subunit incorporated into the receptor, the agonist and antagonist properties can be altered. For example, with the incorporation of the NR2A subunit, agonist binding affinity is maximized and this enhances channel opening and ion conductance, as measured using patch clamped cells (Buller et al. 1994, Grimwood et al. 1996).

The localization of receptors composed of the different combinations of splice variants has yet to be established. However, the localization of NR2 subunits in the mammalian CNS, has been determined using an in situ hybridization technique. The NR2A subunit is the most highly expressed in the cerebral cortex, hippocampus and cerebellar cortex. While the NR2B subunit is expressed in these areas, it is also expressed in the diencephalon and brain stem. Finally, unlike the previously mentioned subunits, the NR2C subunit is expressed predominantly in the cerebellar cortex while the NR2D subunit is found in structures of the diencephalon and brain stem (Ishii et al 1993).

It is now recognized that NMDA receptors comprised of the NR1 subunit in combination with either NR2A, 2B or 2C, have different pharmacological properties. For example, glutamate is most potent at the NR1a/2A receptor, and less potent at the NR1a/2B, NR1a/2C receptor (Grimwood et al. 1996). There is also a difference between the 2A and the 2B receptor subunits when one examines the NMDA redox site. This site exists specifically in the NR1A/2A receptor combination with the two reducible/oxidizable cysteine residues present on the NR1 subunit (Sullivan et al. 1994), their kinetics being modulated by the N-terminus of the NR2A subunit (Kohr et al. 1994). When oxidized, sulfhydryl bridges form in the receptor complex and thus inhibit receptor activity (Reynolds et al. 1990). There are also differences in kinase activity at NMDA receptor subtypes and the channel response to phosphorylation. For example, phosphorylation by PKC in post synaptic densities potentiates the activity of NR1/2A, NR1/2B receptors while it inhibits NR1/2C, NR1/2D subunit containing receptors (Grant et al. 1998). As well, calcium/calmodulin dependant protein kinase (CaMKII) preferentially associates with the NR2B subunit at post synaptic densities, following receptor activation (Stack and Colbran 1998). It is believed that this phosphorylation is important in mediating LTP, as it increases the efficiency of neurotransmission at modified receptors (Fukunaga et al. 1996). Consequently, different phosphorylation signals will differentially modulate LTP, depending on the NMDA receptor type targeted by the kinase.

1.6 NMDA Receptors in Neurotransmission

The NMDA type receptor functions as a "coincidence detector", opening only after stimulation of other glutamate receptors has produced sufficient post synaptic nerve depolarization to lift the magnesium block (Ascher and Nowack 1996). Once activated, the large influx of positively charged ions, especially calcium ions (Macdermott 1986), is capable of activating intracellular signalling systems, involving protein kinase C (PKC), calcium calmodulin dependant protein kinase II (CaM kinase II) (Soderling et al 1994) and nitric oxide synthase (Garthwaite 1988). This in turn alters protein phosphorylation and activates transcription of such immediate early genes as c-fos (Lerea 1997). Consequently, NMDA receptor activation permits long term changes in synaptic function or "plasticity" by effecting the transcription of immediate early genes (IEG's) which regulate target gene transcription (Lerea 1997), promoting (amongst other actions) the physical remodeling of the synapse and altering the number of glutamate receptors present at the synapse.

1.7 AMPA, Kainate and Metabotropic Type Glutamate Receptors

Pharmacological properties of the AMPA, kainate and metabotropic glutamate receptors are summarized in Table 1. Unlike the NMDA receptor, the AMPA receptor is not highly permeable to calcium when it contains the GluR2 subunit. Following activation, in most cases, it permits primarily the passage of sodium ions (Bochet et al. 1994). Interestingly, there are some cells, such as retinal bipolar cells (Gilbertson et al. 1991), non-pyramidal cells of the cerebral

cortex (Jonas et al. 1994) and certain hippocampal neurons (Ozawa 1992), which possess calcium permeable AMPA receptors.

AMPA receptors, like kainate receptors, facilitate rapid excitatory neurotransmission. Consequently, AMPA receptors respond rapidly to agonist binding and desensitize quickly after activation. Like NMDA receptors, several agonists activate the AMPA receptor, including glutamate, AMPA, kainate and quisqualic acid (Keinanen et al 1990).

Several competitive antagonists of the AMPA type glutamate receptor (CNQX, NBQX and DNQX), belonging to the quinoxaline-2,3-dione family, have been identified (Fletcher et al 1988). However, these compounds can also inhibit kainate and in some cases NMDA receptors. The most selective AMPA receptor antagonist is the 2,3 benzodiazepine, GYKI 52466, which acts in a non-competitive fashion (Donevan and Rogowski 1993).

1.8 Subunit Composition of the AMPA Type Glutamate Receptors

The AMPA receptor is composed of five subunits from the AMPA receptor family (Wenthold 1992). This family consists of four different genes producing four types of subunits, named GluR1-4 (Hollman 1989), which, like the NMDA receptor subunit mRNA's, undergo some degree of mRNA processing (Sommer 1990). For example, in one transmembrane domain, the expression of a sequence of amino acid residues is developmentally regulated. The "flip" sequence, as it has been named, appears to be dominant prenatally while the "flop" sequence is seen almost exclusively in the postnatal brain (Monyer et al. 1991). Such changes then influence

channel activity, as the "flop" containing channels have a more rapid desensitization than do the "flip" containing ones (Sommer et al 1990).

AMPA channels are generally composed of GluR2 subunits in combination with subunits of either GluR1,3 and 4 (Michaelis 1998) and appear to be expressed uniformly throughout the brain, with the exception of the GluR4 subunit which is located principally in the cerebellar cortex, dentate gyrus granule cells, and CA1 fields of the hippocampus (Petralia and Wenthold 1992).

1.9 Kainate Type Glutamate Receptor Pharmacology

The most important tool employed to characterize the kainate receptor is probably the AMPA receptor antagonist GYKI 52466. Using patch clamp electrophysiology in cultured hippocampal neurons, this antagonist was found to unmask a short duration, desensitizing current which is mediated by the kainate receptor (Lerma et al. 1997). Without the use of this antagonist, such studies on the nature of the kainate receptor current would not be possible. Interestingly, while AMPA is more selective for the AMPA receptor, kainate is equipotent at both the receptor types (Michaelis 1998).

1.10 Subunit Composition of Kainate-Type Glutamate Receptors

The kainate family of glutamate receptors encompasses 5 different genes and gene products, GluR5-7 and KA1-2 (Pin and Duvoisin 1995). Like AMPA or NMDA receptors, the

inclusion of certain subunits is obligatory in the formation of a functional channel. It appears that only homomeric combinations of the GluR5,6 subunits may form functional channels whereas the GluR7, KA1-2 subunits require the presence of the GluR5,6 subunits in order to generate a working pore (Lomeli et al. 1992).

The RNA editing that occurs in the kainate receptor family resembles that in the AMPA receptor family. At a particular site in the transmembrane domain, alterations in the identity of one particular amino acid residue in the GluR5-6 subunits drastically changes the permeability of the receptor to calcium (Ruano et al. 1995). It is this edited form or calcium impermeable form which predominates throughout the brain, yet, as in the case for the AMPA receptor, there are areas of calcium permeable kainate receptor expression. For example, in certain hippocampal neurons, the only kainate receptor subunits present are the GluR5-6 subunits, which are permeable to calcium (Werner et al. 1991). However, the expression of the other kainate receptors, like that of the AMPA receptors, is fairly uniform throughout the CNS (Michaelis 1998).

1.11 Metabotropic Type Glutamate Receptor Pharmacology

The last category of glutamate receptor is the metabotropic type, which are coupled to intracellular second messenger signaling systems. Not surprisingly, there are similarities between the ionotropic receptor agonists and metabotropic receptor agonists, as each must possess a glutamate binding site. Such agonists include both quisqualic acid (an agonist at the AMPA

receptor class) and ibotenic acid (an agonist at the NMDA receptor class) which also activates metabotropic glutamate receptors (Pin et al 1993). Other agonists and antagonists include certain glycine derivatives. While DHPG and 4CPG are agonists and antagonists of the Class I receptors, respectively, DCG-IV and MCCG are agonists and antagonists respectively of the Class II metabotropic receptors (Pin et al 1993). Further, MAP4 and MPPG act as antagonists of the Class III metabotropic receptors (Pin et al 1993). Table 1 summarizes the pharmacological action of these compounds and others on the metabotropic receptors.

1.12 Subunit Composition and Classification of Metabotropic Glutamate Receptors

There are three subclasses of metabotropic glutamate receptor, one of which (Class I - mGluR1,5) leads to phospholipase C (PLC) activation through communication with Gq like G-proteins (Michaelis 1998), the other two (Class II - mGluR2,3, Class III - mGluR4,6,7,8) inhibit adenylate cyclase through Gi or Go G-proteins (Michaelis 1998). In total, there are 8 different genes which encode metabotropic receptors (mGluR1-8) (Duvoisin et al 1995). Unlike the other glutamate receptors, each gene encodes a protein which functions as an autonomous receptor (Duvoisin et al. 1995). Like other glutamate receptors, there are several splice variants for metabotropic receptors: mGluR1a-c (Pin et al. 1992), 4a-b, 5a-b (Joly et al 1996). While all of the splice variants are functional, they differ in many ways. The mGluR1 splice variants, for example, differ in their potential to activate chloride channels in frog oocyte expression systems, via PLC mediated pathways (Pin et al 1992). With respect to their localization in the CNS, the metabotropic receptors are highly localized, with each gene being expressed in a restricted area

of the brain (Tanabe et al. 1992, Masu et al. 1991, Houamad et al. 1991, Abe et al 1992 Nakajima et al. 1993). For example, mGluR1,2,3 are expressed in the hippocampus and cerebellum.

1.13 Excitotoxicity

"Excitotoxicity", a term coined by Olney, refers to neurotoxicity, which is mediated by over activating glutamate receptors and results from a perturbation in normal glutamatergic neurotransmission (Olney 1967). In the case of cerebral ischemia, extracellular glutamate concentrations rise from below 10μM to 50-60 μM, inducing excitotoxic cell death (Zilkha et al. 1995) Initially, glutamate toxicity was thought to be mediated solely through NMDA receptor activation, leading to sodium and calcium ion influx, osmotic swelling, and lysis of the neuron (Choi et al 1988). However, it was subsequently demonstrated that if the agonist was applied for sufficient time, AMPA and kainate could produce an equivalent level of cell death in susceptible neuronal populations (Koh et al. 1990, Frandsen et al. 1989). In addition, antagonists of type I metabotropic receptors (specifically mGluR5 (Ferraguti et al 1997)) and possibly agonists of type II and III metabotropic receptors, such as DCG-IV, provide neuroprotection in models of excitotoxicity (Bruno et al. 1995), showing how all glutamate receptors not only influence glutamatergic neurotransmission, but also glutamate mediated toxicity.

1.14 Mechanism of Cell Death in Excitotoxicity

In previous studies, cell cultures of glutamatergic neurons were utilized to determine the effects of various EAA receptor specific agonists on the progression of excitotoxicity. As well, primary cultures of CNS neurons have been employed to investigate the mechanism of excitotoxic cell death.

Excitotoxic cell death may manifest itself either as necrosis or apoptosis (Linnik et al. 1993, Loo et al. 1993, Macmanus et al. 1993, Dessi et al. 1993). Apoptotic cell death may be defined by several characteristic observations. For example, cells usually shrink and display cell surface alterations, along with nuclear chromosome condensation and DNA cleavage into small fragments (Kerr et al. 1972, Bursch et al. 1992). Interestingly, these general characteristics may vary between cell type and organism exhibiting programmed cell death (Schwartz et al. 1993). The goal of apoptosis appears to be to minimize the tissue damage by isolating the dying cell from its neighbors. By sequestering toxic constituents of cells in the intracellular space, the apoptotic neuron limits the cell death signal from spilling to adjacent cells (Bonfocco et al. 1995). This is in contrast to necrotic cell death where cell lysis allows large amounts of glutamate and other substances to affect surrounding cells (Leist et al. 1998). Not surprisingly, it is apoptosis which is evident in controlled cell remodeling (Nicotera et al 1997). However, there is a mounting body of evidence that necrosis and apoptosis may not be two distinct pathways, autonomous from each other. Thus, enzyme systems thought to be involved solely in apoptosis, such as Bcl-2 and certain upstream caspases which cleave vital cell proteins, have been observed to play a role in both neuronal necrosis and apoptosis (Leist et al. 1997, Shimizu et al. 1996). The

current understanding is that apoptosis is the default program, while necrosis results when the apoptotic cell death program cannot proceed to completion (Nicotera et al. 1997). Death may be initiated in the apoptotic mode, but if the toxic stimulus is very intense, the cell may undergo necrosis, by circumventing the apoptotic cascade, and lyse.

In primary cultures of CNS neurons, depending on the concentration and length of time of exposure to glutamate, either classical apoptosis, necrosis or combination of the two may be elicited (Bonfoco et al. 1995). Generally short term (minutes) insults at high concentration (1 mM glutamate) produce necrotic death, while long term insults (hours) at reduced concentrations (0.1, 0.01 mM glutamate) produces apoptosis (Bonfoco et al. 1995). It appears then that the execution of a pathway of cell death is related to glutamate channel activity and the degree and type of ion influx. In particular, increases in intracellular calcium ions appear to govern the execution of apoptotic cell death.

As mentioned above, an elevation of free calcium levels is crucial in altering phosphorylation, gene transcription, synaptic plasticity, and it is also important in activating cell death programs. One of the most crucial sites of action of calcium is at the mitochondria (Schinder et al. 1996, Ankarcrona et al. 1995, Bonfoco et al. 1995). Mitochondria are one of the main calcium buffering organelles within the neuron. In times of elevated intracellular calcium, the concentration of calcium inside the mitochondrion increases, from nanomolar to micromolar concentrations (Schinder et al. 1995). If the sustained elevation in calcium persists at a sufficient level, the mitochondrion may lose its membrane potential due to the opening of the permeability transition pore located on the inner membrane. Once this occurs, the inner mitochondrial membrane potential is lost, and along with it, the mitochondrion's ability to carry out oxidative

phosphorylation.(Schinder et al. 1995). If the insult is not substantial enough to produce necrotic cell death, (when the ion influx is sufficient to produce osmotic swelling and lysis), the neuronal membrane potential is lost with the mitochondrion's inability to generate ATP, and an apoptotic death is likely to ensue. In general, the rapid swelling and rapid loss of mitochondrion function usually precipitates a necrotic death, while a loss of function followed by a recovery of mitochondrion membrane potential produces apoptotic death (Nicotera et al. 1997).

Toxic elevations in intracellular calcium also activate other, parallel cell death cascades. For example, activation of nitric oxide synthase (NOS), due to an increase in intracelluiar calcium (Bredt and Snyder 1989) produces nitric oxide (NO). Subsequently, the NO produced by NOS can combine with superoxide ions to form peroxynitrite, a highly reactive free radical (Stamler et al. 1992). This compound then destroys cells by causing disintegration of the cell membrane through lipid peroxidation (Kooy et al. 1995), damaging cellular proteins and promoting deleterious gene transcription (Remacle et al. 1995). As well, peroxynitrite may activate the enzyme poly ADP ribosyl synthase (PARS) (Szabo 1996). PARS may then poly ADP ribosylate proteins, depleting cellular NAD and consequently ATP, amplifying the ATP deficit caused by mitochondrial failure. Calcium also induces the formation of free radicals from other sources, through the activation of xanthine oxidase (McCord et al. 1985) and phospholipases (Orrenius et al. 1996). Calcium activated proteases may also play a role in activating a class of enzymes important in executing cell death, the caspases. These enzymes are normally inactive at resting calcium concentrations, yet when activated by proteolysis, become capable of cleaving proteins such as α-actin at sites after aspartate residues (Cohen 1997). Recently, enzymes of this family have been shown to play an obligate role in glutamate mediated

neuronal apoptosis in vitro (Du et al. 1997).

1.15 "Fast" and "Slow" Excitotoxicity

As has been mentioned previously, neurons may die by apoptotic or necrotic mechanisms in excitotoxicity. Another dimension in the classification of excitotoxicity is that of "slow" or "fast" excitotoxicity. Figure 2 summarizes and integrates these aspects of glutamate mediated excitotoxicity. In the case of fast glutamate toxicity, excess activation of glutamate receptors provokes an intracellular ion disturbance which leads to cell death. In the case of slow glutamate mediated cell death, it is postulated that normal stimulation of glutamate receptors is sufficient to produce neurodegeneration, (Albin and Greenamyer 1992, Beal and Hyman 1993, Novelli et al. 1988, Henneberry 1989).

The reason for this supersensitivity to glutamate appears to be a deficit in oxidative phosphorylation within the neuron. This results from a perturbation in ion homeostasis leading to mitochondrial dysfunction. Normally, membrane transporters such as the sodium/potassium ATPase, maintain sodium, potassium and calcium gradients across the cell membrane (Albin and Greenamyer 1992, Beal and Hyman 1993, Henneberry et al. 1989). When ATP levels decrease, the ion gradients are also reduced, as sodium, chloride and calcium ions flux into the cell and are not transported back to the extracellular space (Beal and Hyman 1993). Consequently, normal stimulation of glutamate receptors produces calcium influx that summates with the elevated resting calcium levels (Beal and Hyman 1993) to promote apoptotic or necrotic cell death. This process is then dubbed slow or chronic excitotoxicity (Beal and Hyman 1993), as it may develop

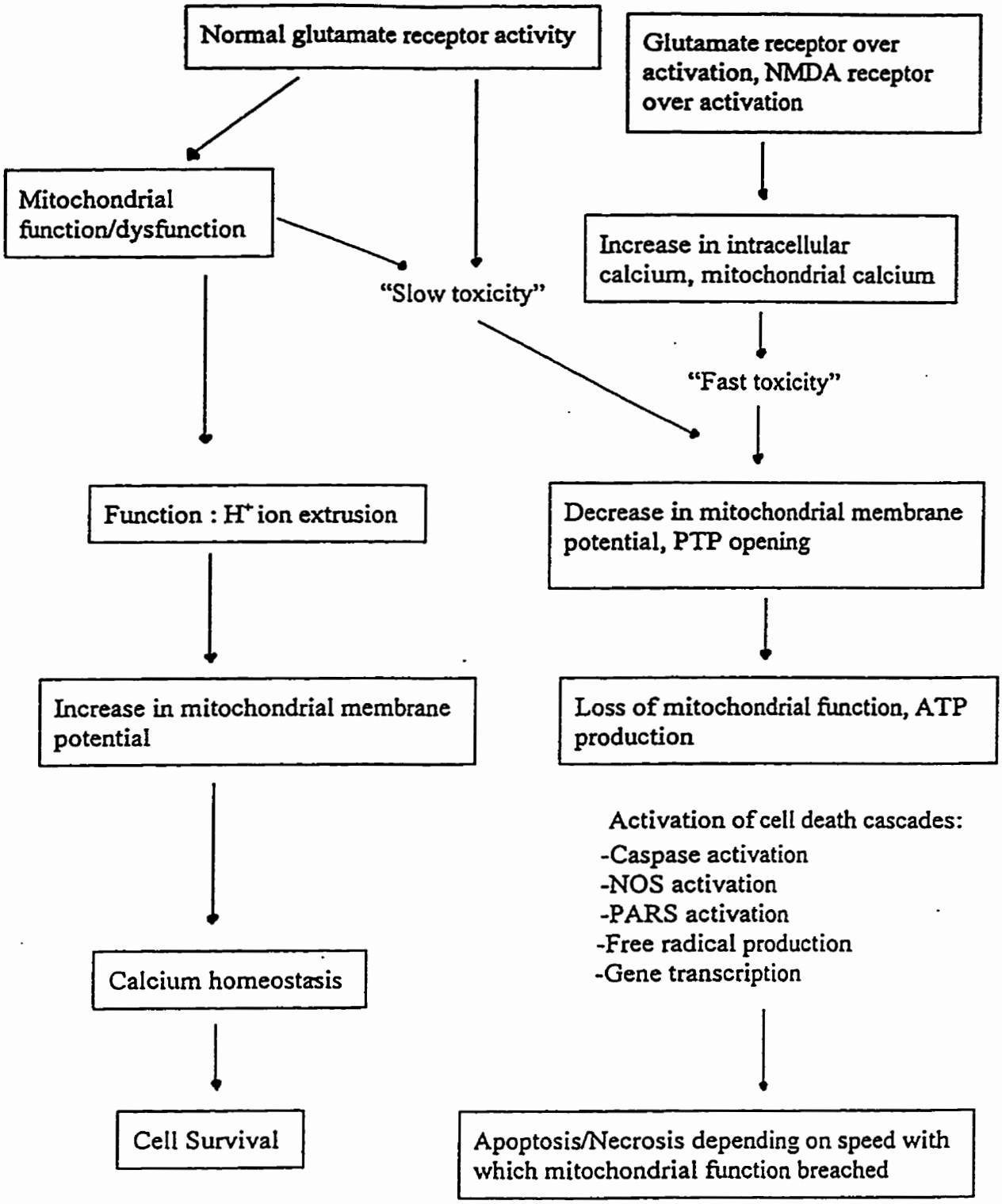


Figure 2. Flow chart integrating concepts of slow and fast toxicity, mitochondrial function, activation of intracellular death promoting cascades, and apoptosis and necrosis.

over days, months or possibly years. In support of this hypothesis, it has been shown that agents such as 3-nitropropionate and malonate which are inhibitors of mitochondrial respiration in vivo (Beal and Hyman 1993) and in vitro (Henneberry et al. 1989), can produce excitotoxic cell death, an effect prevented by NMDA receptor antagonists. This finding is of interest, as normally, NMDA receptor stimulation should not produce cell death.

1.16 The NMDA Receptor and Excitotoxicity

It is now recognized that calcium plays a critical role in excitotoxicity. Thus, targeting the pertubations of calcium homeostasis becomes paramount in the prevention of excitotoxicity. One of the main routes of calcium entry into the neuron is through the NMDA receptor due to its high permeability to calcium and high unitary conductance. Calcium entry is crucial to initiating toxicity, as chelating extracellular calcium prevents excitotoxicity (Tymianski et al. 1994). In addition, calcium dependent cell death is selectively attenuated by NMDA receptor antagonists, as opposed to kainate or AMPA receptor antagonists (Tymianski et al. 1994). It is also apparent that the NMDA receptor has a critical link to neuronal mitochondria, in that uptake of calcium into the mitochondria is faster and "more tightly coupled" during NMDA receptor stimulation, than that seen with kainate/AMPA receptor activation (Peng and Greenamyre 1998).

Further evidence suggesting that the NMDA receptor plays a crucial role in excitotoxic cell death comes from studies on dopamine containing neurons. The NMDA receptor antagonists have been shown to protect dopaminergic neurons against methamphetamine and MPTP toxicity, compounds which are believed to mimick the selective destruction of substantia

nigral neurons in Parkinson's disease. As well, blockade of the NMDA receptor in vitro (Choi et al. 1988, Michaels and Rothman 1990) and in in vivo models of cerebral ischemia (a model of glutamate mediated cell death) (Perez-Pinzon et al. 1995) has been shown to significantly protect neurons from damage. The NMDA receptor thus appears to be an important effector in the calcium-mediated excitotoxic cell death, suggesting that it may be an important target for therapeutic compounds with potential to prevent excitotoxic cell death.

1.17 Disease States Where "Fast" and "Slow" Excitotoxicity May Mediate Neurodegeneration

Far from being an experimental curiosity, it has been hypothesized that fast and slow excitotoxicity may be important in the progression of several neurodegenerative disorders (Beal and Hyman 1993). For example, while cerebral ischemia may be viewed as a combination of fast and slow excitotoxicity (associated with necrosis and apoptosis respectively) (Choi 1996) , the progression of Huntington's, Parkinson's and Alzheimer's disease may be linked to slow excitotoxicity (Beal 1998, Beal and Hyman 1993). In the case of cerebral ischemia, glutamate acts as the causitive factor in neurodegeneration. While necrotic ischemic cell death results from overstimulation of glutamate receptors by high levels of glutamate; apoptotic ischemic cell death occurs in cells which surround this necrotic core of cells. In Huntington's, Parkinson's and Alzheimer's disease, glutamate is not the initiating factor in neurodegeneration. Instead, defects in the nuclear or mitochondrial genome as well as oxidative stress lead to an inhibition of energy production in ageing neurons. This metabolic compromise is exacerbated by glutamate mediated neuronal excitation, resulting in slow excitotoxicity (Beal 1998).

1.18 Modeling Excitotoxicity

Consideration of experimental evidence shows that glutamate mediated excitotoxicity underlies several neurodegenerative conditions and is not merely a phenomenon which is solely observed in experimental models. It is therefore important that neuroprotective strategies be developed to reduce neuron loss. In order to pursue such strategies, appropriate models of excitotoxicity and the disease states exhibiting excitotoxicity must be developed.

1.19 In vitro and in vivo Models of Excitotoxicity

Excitotoxicity has been investigated in intact animals or in the in vitro models involving primary cell culture, culture of cell lines or organotypic tissue slice culture. In vivo models involve the injection of glutamate receptor agonists into glutamatergic pathways in the animal's CNS (Globus et al. 1995). This procedure is capable of mimicking the neuronal loss and behavioral deficits that occur in such disease states as Parkinson's, Huntington's, and Alzheimer's by selectively destroying neurons in the hippocampus, the substantia nigra, striatum and in the forebrain cholinergic system. The advantages of using in vivo models are (i), neuroprotective strategies can be developed in a system where intact , mature glutamatergic neurons exist, expressing glutamate receptor populations and signalling systems which have been well characterized. (ii) The effect of neuroprotective therapies can be measured by a modulation of

the behaviour ellicited upon excitotoxin treatment. There are, however, several problems associated with the use of in vivo models of excitotoxicity: (i) the delivery of excitotoxin could produce significant tissue damage in the targeted area. (ii) A large immune response accompanies the administration of the toxin, and (iii) because of diffusion, the toxin dose delivered to the tissue is uncertain.

The in vitro models of excitotoxicity encompass tissue slice preparations, organotypic cultures, primary cultures of dissociated neurons, and transformed cell lines. The tissue slice preparation involves slicing adult animal brain tissue, isolating and then culturing a particular structure in the CNS . A major advantage of this technique is that the connectivity of the neurons and glia within the structure is preserved to some extent and the nature of this preparation makes it an attractive model for use in excitotoxicity studies. A major disadvantage of using tissue slices is that the thickness of the slice (500 micrometers) may prevent even diffusion of chemicals throughout the preparation. This problem can be resolved by using a modification of this technique, where by thinly sliced tissue of 50 - 100 μm are cultured (Gahwiler et al. 1997).

Another in vitro preparation which is useful in the investigation of excitotoxicity is the primary culture of dissociated neurons (Koh et al. 1990). The advantage of this model is that the administration of toxin and toxin antagonists can be accomplished easily, environmental conditions may easily be controlled and variability amongst animals is reduced as multiple treatments may be performed on a single culture. The problem of employing a tissue culture approach, however, is that the dissociation of the embryonic tissue disrupts the neuronal and glial connections. Neuronal interactions in culture can be different from those seen in vivo, as are other physiological parameters, such as extracellular volume.

One other in vitro model of interest is that of transformed cell lines. This system utilizes transformed cells, such as mouse fibroblasts or human embryonic kidney (HEK) cells expressing specific receptor proteins (Grimwood et al. 1996, Raymond et al. 1995). Specifically, glutamate receptor subtypes can be introduced into the cell line, which normally does not express such receptors. Studies on receptor function can be conducted in isolation, without the participation of other receptor subtypes. In this way, the effectiveness of therapies targeted to a particular glutamate receptor or receptor subunit can be evaluated. The main disadvantage of this system stems from its simplicity. Although one can ask very specific questions when employing this system, the extrapolation of results to in vivo excitotoxicity must be made carefully, since the cells are not of neuronal origin and thus they may not undergo excitotoxic cell death as do neurons.

In conclusion, there are several methods one may employ in the investigation of excitotoxicity. However, for the purposes of investigating the actions of particular compounds at specific glutamate receptors, in vitro studies , using dissociated tissue cultures and transformed cell lines seems highly appropriate.

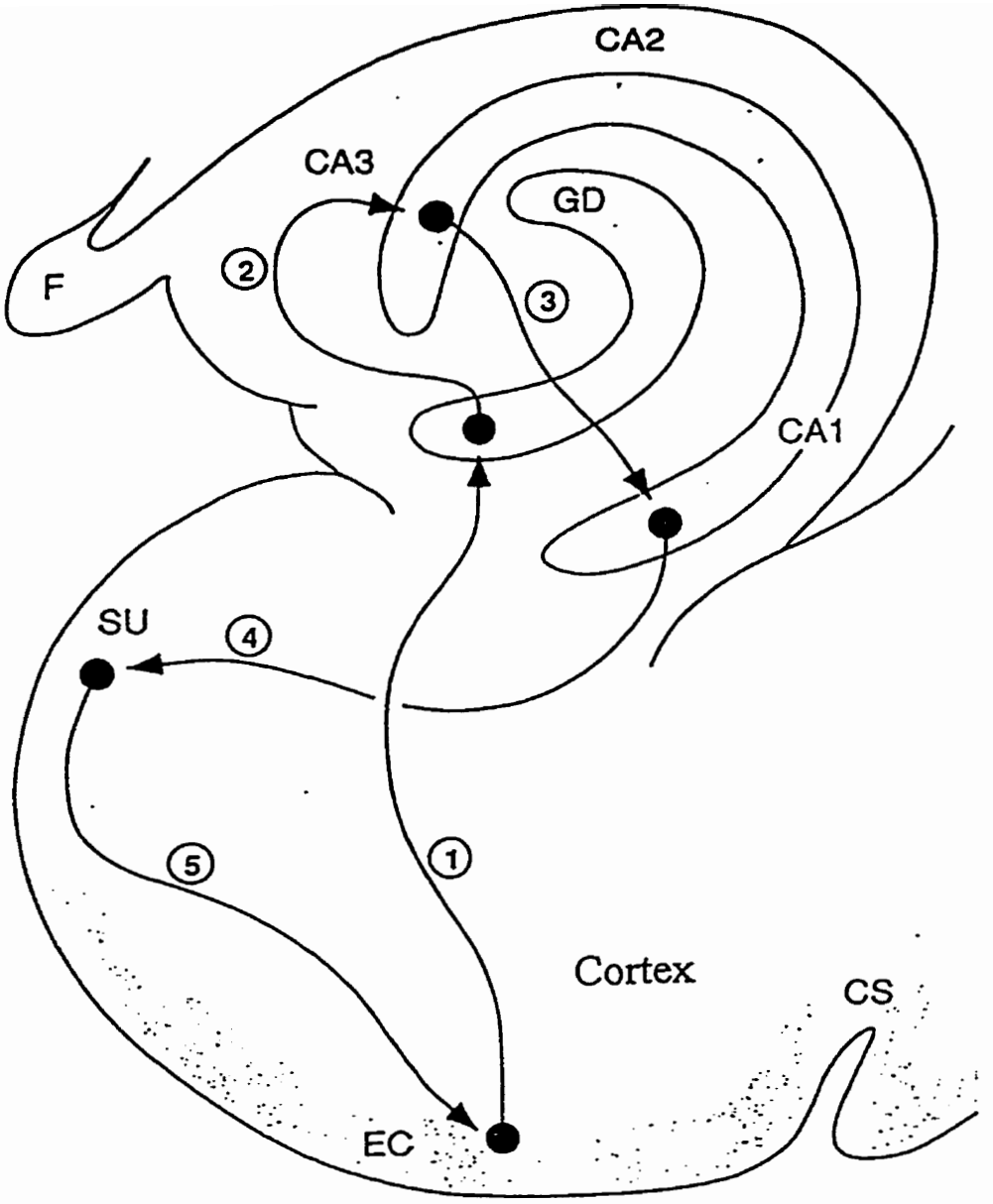
1.20 Dissociated Primary Cultures as In Vitro Models of Excitotoxicity

An in vitro model which may be employed in the investigation of excitotoxicity is that of primary cultures of CNS neurons. The main requirement for selecting specific CNS neurons is that they release glutamate and bear glutamate receptors. The hippocampus, a structure in the CNS rich in glutamatergic neurons (Brown and Zandor 1990), lends itself well as a model of

excitotoxicity in vitro.

The hippocampus may be broken down into several anatomically distinct regions: the dentate gyrus, and regions inside the hippocampal formation, the CA1, CA2 and CA3 regions (Parent 1996, Nadler et al. 1976). The most important cell types in the various regions are the dentate granular cells in the dentate gyrus, and the pyramidal cells in the hippocampal formation, as they constitute the main glutamatergic pathway in the hippocampus (Amaral and Insausti 1990). There are also numerous other types of neurons such as the basket cells, which are located within the hippocampus and modulate the glutamatergic pathways (Amaral and Insausti 1990). One of the main circuits in this structure is a closed glutamatergic loop, running from the cortex into the hippocampus, and connecting back to the cortex (Amaral and Insausti 1990) (Figure 3.). Thus, the entorhinal cortex sends glutamatergic projection neurons to the dentate gyral granule cells, which in turn send "mossy fibre" projections to the CA3 region of the hippocampal formation. The CA3 neurons then send Schaffer collaterals to the CA1 region, which projects back to the entorhinal cortex. The signaling within this loop is quite complicated, as afferent inputs to the hippocampus synapse not only on dentate gyral cells, but on all pyramidal cells, bypassing steps in the previously mentioned pathways (Nadler et al. 1976,Amaral and Insausti 1990).

When generating primary hippocampal cultures, all of the dentate gyral and hippocampal neurons are utilized, with glutamatergic synapses reforming in culture. Once in culture, these embryonic neurons develop into mature cultures, showing glutamate receptor expression which is similar to that seen in the adult animal (Williams et al. 1993). Indeed, this system has been used frequently to investigate excitotoxicity and neuroprotection. (Prehn et al. 1993, Prehn et al.



Hippocampal Formation

Figure 3. Schematic of the main afferent glutamatergic input to the hippocampal formation from the cortex. Adapted from Parent 1996.

1. Input from entorhinal cortex (EC) to granule cell layer of dentate gyrus (GD)
2. Mossy fibre connection to the CA3 region of the hippocampus (CA3).
3.Schaffer collaterals connect the CA3 and CA1 regions of the hippocampus (CA1).
4.CA1 region sends projection to the subiculum (SU).
5. Subiculum connects back to the entorhinal cortex (EC).

1996). Previous studies have used cortical cultures to explore excitotoxicity mediated by NMDA, AMPA and kainate (Fradsen et al. 1989, Fradsen and Schousboe 1990, Koh et al. 1990). Thus, the use of CNS glutamatergic neurons in culture as a model of excitotoxicity is well established, and is a valuable tool in the investigation of new agents with potential to modulate this phenomenon.

1.21 Transformed Cells as a Model of NMDA Receptor Dependant Toxicity

Primary cultures are an important model for use in the investigation of excitotoxicity. However, in examining novel neuroprotective compounds, it is difficult to investigate receptor selective effects and almost impossible to examine receptor subtype selective effects of these compounds. Recently, novel expression systems have been developed, by transforming mouse fibroblast or HEK 293 cells with cDNA's encoding particular glutamate receptor subunits from the NR1 and NR2 families (Raymond et al. 1996, Priestly et al. 1995, Varney et al. 1996). The receptors expressed in these cells exhibit characteristics of NMDA receptors expressed in vivo (Priestly et al 1994, Kendrick et al. 1998). For example, strong, concentration-dependant depolarizations are observed in patch clamped cells which have been exposed to glutamate (Priestly et al. 1995). Importantly, these currents were similar in amplitude to those seen in primary cultures of hippocampal neurons (Priestly et al 1994). In addition, glycine has been shown to be an important co-agonist at these recombinant receptors, substantially increasing the current amplitude evoked by glutamate. This action is similar to the modulatory action of glycine seen in NMDA receptors expressed in vivo (Priestly et al. 1994).

NMDA receptor-dependent cell death or excitotoxicity is also evident in this expression system, as induction of receptor expression is toxic to the host cells, unless NMDA receptor antagonists are included in the culture media (Raymond et al. 1996, Priestly et al. 1995, Varney et al. 1996). As channel characteristics of the transfected cell resemble those of native receptors in vivo, cells expressing NMDA receptor subunits are attractive models for studying the protective effects of compounds in excitotoxicity. Furthermore, such an expression system may allow the investigation of subunit specific effects of potential neuroprotectants.

1.21 Neurosteroids as Potential Neuroprotectants

Neuroactive steroids or neurosteroids, are steroid hormones synthesized within the central nervous system from the precursor cholesterol (Rupprecht et al. 1996). Cholesterol is converted to pregnenalone (PREG) by a cytochrome P450 isozyme in myelinating glial cells. PREG may be sulfated, generating pregnenolone sulfate (PREG-S) or pregnanolone sulfate (PREGNAN-S) or may be subsequently be metabolized to numerous other steroidal compounds, within the CNS (Figure 4)(Le Goascogne et al. 1987, Baulieu 1997). For example, PREG may be reduced to progesterone (PROG) and then to tertrahydroprogesterone (THPROG). Alternatively, PREG may be converted to dehydroepiandrosterone or dehydroepiandrosterone sulfate (DHEA, DHEA-S) (Rupprecht et al. 1996). Interestingly, it was this compound which was first detected in the CNS at concentrations which were greater than those observed in the plasma, suggesting the steroid was in fact synthesized and acted in the CNS, promoting the concept of the neuroactive steroid (Corpechot et al. 1981, Baulieu 1981). Neuroactive steroids have

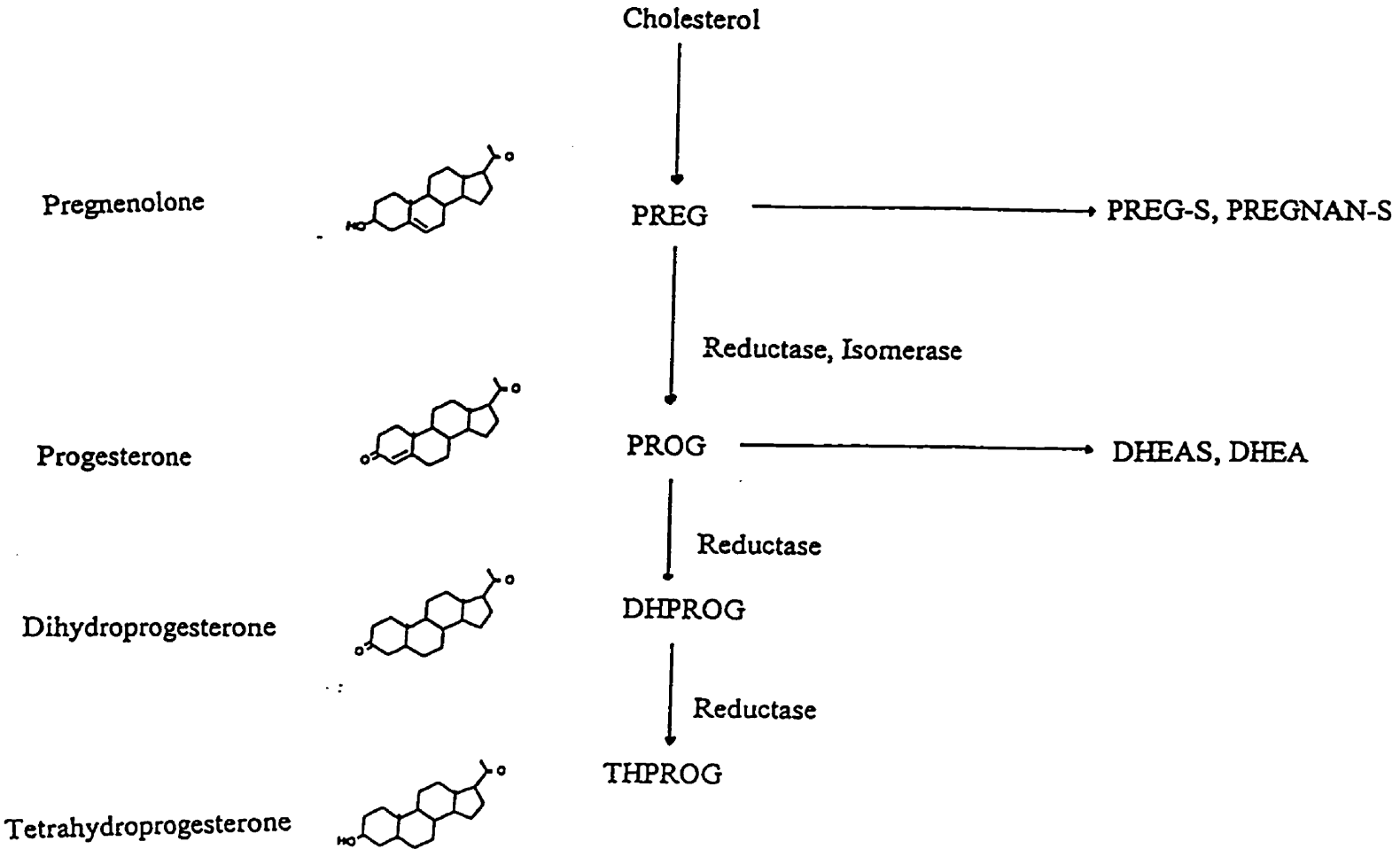


Figure 4. Diagram showing the synthesis of neuroactive steroids derived from cholesterol. Adapted from Rupprecht et al. (1996)

subsequently been shown to be capable of altering behaviour. For example, DHEA and PREG-S have been implicated in the modification of aggressive behavior in male rats. Aggression has been shown to decrease when DHEA is administered, by selectively decreasing PREG-S concentrations (Young et al. 1994). As well, PREG and PREG-S concentrations in the CNS, especially in the hippocampus, appear to influence cognitive performance in rodents (Baulieu 1997).

Further, PROG administration can increase levels of THPROG, a steroid which acts at the inhibitory $GABA_A$ receptor. It is hypothesized that the changes in THPROG levels may modulate behavior in both pregnancy and stress (Purdy et al. 1991, Majewska 1992).

There are numerous postulated sites of action for the neurosteroids in the CNS. The classical view of steroid action is that these compounds exert their effects by binding to intracellular receptors, which in turn may act as trans-activating factors (Whelling 1997). In the CNS, the progesterone receptor is capable of binding PROG, along with PREG and its derivatives, after conversion back to PROG (Rupprecht et al. 1996). The effects of binding to the progesterone receptor has numerous consequences, including inhibition of cell differentiation and growth in glial cells (Jung-Testas 1991, 1992). Neuroactive steroids also influence the activity of neurotransmitter receptors, located on the cell membrane (summarized in table 2). Pregnenolone sulfate (PREG-S) along with DHEA-S both appear to negatively modulate the $GABA_A$ receptor, while THPROG and its sulfate THPROG-S positively modulates this receptor (MacDonald and Olsen 1994). This site of action of the neurosteroids appears to be autonomous from other modulatory sites such as the benzodiazepine binding site. Further, the actions at this site appear to be dependent on $GABA_A$ receptor subunit combination. The mechanism of steroid action at

the receptor is thought to involve modulation of channel open time (Peters et al. 1995). In addition, neurosteroids are capable of either increasing or decreasing the frequency of single channel openings, depending on the neurosteroid. A further action of neurosteroids at the $GABA_A$ receptor complex is that of $GABA_A$ receptor activation. Unlike other $GABA_A$ receptor modulators, neurosteroids are capable of directly activating the $GABA_A$ receptor. Thus, the neurosteroids are capable of producing hypnotic, anxyolytic and analgesic effects (Lambert et al. 1996, Backstrom 1995). The neurosteroids may also act at the NMDA receptor, either directly or indirectly. The indirect actions are believed to be achieved through activation of the sigma receptor, a G-protein linked receptor, which has been shown to have numerous modulatory effects on cell physiology and behavior. For example, sigma receptors have been shown to mediate actions of certain anti psychotic agents, such as haloperidol (Su 1993). As well, sigma receptors are capable of modulating glutamate release presynaptically and offer protection against hypoxia in vitro (Nakazawa et al. 1998). Interestingly, sigma receptors are also capable of positively modulating NMDA receptor function. Using NMDA receptor-mediated release of norepinephrine as a measurement of sigma receptor modulation of the NMDA receptor in the hippocampus, it was observed that while DHEA-S acts as a sigma receptor agonist, PREG-S acts as an inverse agonist and PROG, an antagonist (Monet et al. 1995).

There are steroids such as PREG-S and PREGNAN-S which can directly modulate NMDA receptor activity. The sites of action of these two steroids, unlike the common steroid binding site seen in the $GABA_A$ receptor, are different. While PREGNAN-S antagonizes channel conductance in a non-competitive manner, its actions are unaffected by the presence of PREG-S, which acts to increase channel conductance upon glutamate binding (Park-Chung et al.

1997). The sites of steroid action are also unrelated to other modulatory sites, such as the glycine binding site, polyamine binding site, redox site and MK-801 binding site (Park-Chung et al. 1997). In addition, it is not known whether modulation of the NMDA receptor is subunit dependent (Park-Chung et al. 1997). Interestingly, the reduction and elimination of stereospecificity of PREG-S changes the properties of these two steroids at the NMDA receptor (Park-Chung et al. 1994). While PREG-S positively modulates the NMDA receptor, PREGNAN-S negatively modulates the NMDA receptor, along with AMPA and kainate receptors, when both are applied at μM concentrations (Park-Chung et al. 1994). Neurosteroids have also been shown to be neuroprotective in primary culture models of NMDA induced excitotoxicity.A dose of 0.10 mM DHEA and 0.010 mM DHEA-S significantly reduced the toxicity of 1mM NMDA in rat cortical cultures (Kimonides et al. 1998).

In conclusion, the discovery that neurosteroids may act rapidly as non toxic modulators at membrane bound receptors, specifically effecting NMDA receptor activity, suggests that they may be candidates as neuroprotectants, preventing excitotoxic cell death. Of particular interest is PREGNAN-S which negatively modulates the NMDA receptor, and may prove to be neuroprotective in the previously mentioned models of excitotoxicity.

Steroid	Modulation of Activity at NMDA Receptor	Modulation of Activity at $GABA_A$ Receptor	Modulation of Activity at Sigma receptor	Effect on Behavior
DHEA-S	Positive	Negative	Positive	Calming
PROG	-	-	Negative	-
PREG-S	Positive	Negative	Negative	Increase in Aggressiveness, Increase in Cognitive Abilities
PREG	Positive	-	-	-
PREGNAN-S	Negative	Positive	-	-
THPROG	-	Positive	-	Calming
THPROG-S	-	Positive	-	Calming

Table 2. Summary table of select actions of certain neuroactive steroids.

1.22 Rationale and Specific Objectives

Excitotoxicity has been implicated in such disease states as Alzheimer's, Parkinson's, Huntington's and cerebral ischemia. It has been postulated that the NMDA receptor plays an important role in mediating calcium entry into the cell and calcium dependent over load which results in excitotoxic neuronal cell death. Thus, by decreasing the activity of the NMDA receptor, excitotoxic cell death is prevented. The work conducted during this thesis research examines the effects of neurosteroids, which act directly at the NMDA receptor, to modulate NMDA receptor dependent excitotoxic cell death. Specifically, primary hippocampal cell culture models of excitotoxicity were established in order to examine the ability of pregnanolone sulfate and pregnenolone sulfate to modulate NMDA receptor dependant cell death. Further, transformed cell lines expressing specific NMDA receptor subunits were used to investigate the subunit dependant effects of these neurosteroids on NMDA receptor mediated excitotoxicity.

Specific Objectives:

Objective 1. To establish and characterize a primary hippocampal cell culture model of NMDA mediated excitotoxicity.

Objective 2. To establish mouse fibroblast cultures expressing either NR1a/2A or NR1a2B subunits as a model of NMDA mediated excitotoxicity.

Objective 3. To investigate whether PREG-S and PREGNAN-S are capable of modulating NMDA receptor mediated toxicity in primary hippocampal cell cultures and transformed mouse fibroblasts expressing either NR1a/2A or NR1a/2B receptors.

Chapter 2

Materials and Methods

2.1 Chemicals and Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, Canada (Oakville, ON) and were of tissue culture grade. Adult male Sprague-Dawley and adult female untimed pregnant rats were purchased from Charles River (Montreal, P.Q.). Horse serum was obtained from ICN pharmaceuticals (Montreal, PQ). Minimum essential medium (MEM), GlutaMAX and L-glutamine were procured from Life Technologies (Burlington, ON). Tissue culture 24 and 96 well plates were purchased from Fisher Scientific Canada (Mississauga, ON). Tissue culture media filters and sterile conical tubes were obtained from Starstedt (Montreal, PQ). All non-sterile solutions, other than tissue culture media, were filtered using 0.2μm syringe filters from Fisher Scientific, Canada. All solutions were prepared using deionized water obtained through a Nanopure water purification system (Barnstead Sybron, Boston, MA, USA). Pregnenolone sulfate and pregnanolone sulfate were both purchased from Steraloids Inc., (Wilton, NH) Carbon dioxide was purchased from BOC gases (Kingston, ON). The Spectramax multiwell plate scanner was purchased from Fisher Scientific (Nepean ON). The Cary 100 Bio spectrophotometer was purchased from Varian Canada Inc. (Mississauga, ON) Mouse Ltk^- cells expressing either NR1a/2A or NR1a/2B NMDA receptors were a generous gift from Dr. Paul Whiting at Merck Sharpe and Dohme (U.K.). Primary anti-neurofilament antibody was

purchased from Dako Corp. (Carpinteria, CA), while primary anti-glia fibliary acidic protein antibody was obtained from Chemicon (Temecula , CA). Secondary antibodies (horse anti-mouse goat anti-rabbit) and the Vectastain antibody detection kit were obtained from Vector laboratories Inc. (Burlingame, CA).

2.2 Composition of Solutions

Calcium, magnesium free Hank's balanced salt solution

KH_2PO_4	0.44 mM
KCl	5.4 mM
NaCl	136 mM
$NaHCO_3$	4.2 mM
Na_2HPO_4	0.34 mM
D-Glucose	5.6 mM

Dulbecco's modified Eagle's medium (DMEM)
As per Sigma-Aldrich cat# 5280 with added

D-Glucose	32.7 mM
Insulin (as zinc suspension)	100 IU/L

Dulbecco's modified Eagle's medium (Base) (DMEM base)
As per Life Technologies cat# 23800-022 with added

D-Glucose	32.7 mM
L-glutamine	2 mM

N2 supplement

D-Glucose	32.7 mM
Transferrin (Human-iron saturated)	5 mg/L
Progesterone	0.02 mM
Selenium oxide	0.046 mM
Insulin (as zinc suspension)	100 IU/L

Fetal calf serum and fetal bovine serum preparation
serum compliment was heat inactivated by incubating at 56°C for 20 minutes

0.1M TBS

Tris HCl	167.8 mM
Tris base	320.4 mM
NaCl	308.0 mM

DAB solution
0.1M TBS 40 ml
nickle chloride 200ul
30% hydrogen peroxide 20ul
Diaminobenzidine (DAB) 0.093 mM

Prehn solution (Hepes-buffered saline)

NaCl	146 mM
Hepes	10 mM
$CaCl_2$	2 mM
KCl	5 mM
Glucose	10 mM
Glycine	100 μM

GlutaMAX

dipeptide of L-glutamine and L-alanine

2.3 Preparation of Primary Cultures of Hippocampal Neurons from Rat Embryos

Cultures were prepared according to the conditions specified by Xiong et al. (1997). Untimed pregnant Sprague-Dawley female rats or mice (17-19 days pregnant) were anaesthetized and decapitated. Ten to fourteen rat embryos were obtained from an individual pregnant rat, removed aseptically, and placed in ice cold (HBSS). Embryos were immediately decapitated and the heads placed in ice cold HBSS. The brains were extracted from the skulls, the meninges pulled away, and the hippocampal formations removed under a dissecting microscope providing a magnification of 10X. The hippocampal formations of ten to fourteen embryos were suspended in HBSS containing trypsin (25 000 BAEE units/ml) and DNase (25 Kunitz units/ml), and inverted several times. The tissue was incubated at 37^0C for 25 minutes, after which time, the HBSS was removed and replaced with serum supplemented DMEM or MEM and the

mixture titurated 20 times using a pasteure pipette. The suspension was centrifuged at 1,000 rpm for 10 minutes to pellet the tissue and free cells. The supernatant was removed, and fresh serum supplemented DMEM or MEM was added. The mixture was further titurated 20 times and centrifuged for 10 seconds at 1,000 rpm, pelleting intact tissue and leaving dissociated cells in the supernatant. The supernatant was collected and fresh serum supplemented media added. This last step was then repeated several times, until no further tissue was seen as a pellet after centrifugation. The resulting cell suspension was sampled and the cell density and viability estimated using a haemocytometer. With respect to viability, cell suspensions exhibiting greater than 90% viability were used to generate cultures, a condition met by all cell suspensions prepared during the course of these experiments. Using the cell density data, cells were plated at a calculated density of 300,000 cells per well on 24 well tissue culture plates which had been pre treated, for two days with a solution of 0.1mg/ml poly-D-lysine.

2.4 Culturing of Primary Hippocampal Cultures

The method of primary culture preparation remained constant throughout the period of experimentation. The culturing of the primary cultures, however, varied substantially as culture conditions were optimized for the long term culture (greater than seven days) of hippocampal neurons. Table 3 includes a list of culturing conditions employed , in chronological order, starting from the initial conditions (1) and terminating with most recent (5) used by Xiong et al. (1997)

Table 3. Conditions employed for culturing rat hippocampal cultures (Mitotic inhibitors: Cytosine arabinoside (AraC), 5-Fluorodeoxyuridine (FUDR)).

	1	2	3	4	5
Day 0	Plated using 1%FCS in DMEM + N2	Plated using 1%FCS in DMEM	Plated using 10%FCS in DMEM with 2mM glutaMAX	Plated using 10%FCS in DMEM with 2mM glutaMAX	Plated using 10%FCS + 10%HS + DMEM with 2mM glutaMAX
Day 1					
Day 2	DMEM + N2 + 10uM AraC	DMEM + 1%FCS+ 10uM AraC	DMEM + 10%FCS + 10uM AraC	DMEM + 10%FCS + 10uM FUDR + 5uM uridine	DMEM +10%FCS + 10%HS + 10uM FUDR + 5uM uridine
Day 3					
Day 4	DMEM + N2	DMEM + 1%FCS	DMEM + 10%FCS	DMEM + 10%FCS	DMEM +10%FCS + 10%HS
Day 5					
Day 6					
Day 7	DMEM + N2	DMEM+ 1%FCS	DMEM + 10%FCS	DMEM + 10%FCS	DMEM + 10% FCS
Day 8					
Day 9					
Day 10		DMEM + 1% FCS	DMEM + 10%FCS	DMEM + 10%FCS	
Day 11					
Day 12					
Day 13					
Day 14					

2.5 LDH Assay conditions

The LDH assay, used to assess cell health, was conducted according to the method of Koh et al., (1988). A 400 µL volume of sample was added to 2.2 mL of 0.1M sodium phosphate buffer (pH 7.5). 200 µL of a 1.4 mM nicotinamide adenine dinucleotide (reduced form) (NADH) solution was added, and vortexed. The reaction mixture was incubated at 25°C for 5 minutes, to eliminate non substrate dependent oxidation of NADH. This condition was verified at the start of each assay, by monitoring the change in absorbance of the reaction mixture over time without substrate. A 200 µL aliquot of a 11.5 mM solution of sodium pyruvate was added to the reaction mixture, and the absorbance change resulting from the oxidation of the enzyme co-factor NADH to NAD was monitored at 340 nM at 25°C for 1 minute using a Cary 100 Bio spectrophotometer. Units of enzyme activity were then calculated by determining the rate of change of the reaction mixture's absorbance over time, where 1 arbitrary unit represented the absorbance change of 0.001 absorbance units over 1 minute.

2.6 Excitotoxicity Assay

Mature 12 days *in vitro* (DIV) cultures were washed twice with a salt exposure solution as per Prehn et al. (1994). Cell cultures were then exposed to NMDA or drug treatments in Prehn's solution for 10 minutes at room temperature. After this, cultures were washed once with DMEM base, then incubated with DMEM base for 24 hours. 400 µl of the 500 µl of supernatant was then sampled, and used in the LDH assay. This fraction contained the released LDH activity, which resulted from cell death and lysis. The remaining DMEM base was aspirated off, and 500 µl of fresh DMEM base added. The cultures were frozen and thawed several times, and 400 µl of the media was used in the LDH assay. The frozen and thawed fraction represented latent LDH enzyme activity, which is contained in viable intact cells, and is released upon freeze thawing. Expressing the released LDH activity as a percentage of total LDH activity gives the fraction of dead cells present in the culture.

2.7 Preparation of Rat Hippocampal Homogenate for use in LDH Assay Characterization

Brains from 3 adult Sprague-Dawley rats were sliced along the saggital fissue, and the hippocampi removed. Tissue was homogenized in a solution of 0.1% tritonX-100 and 0.1M phosphate buffer, and refrigerated.

2.8 Determination of Protein

Protein content of hippocampal homogenate was determined using the method of Lowry et al. (1951). Sample protein, along with bovine serum albumin (BSA) standards was digested overnight in 1 ml of 1M NaOH. 100ul of the digested tissue was added to 750 ul of distilled water. 2.25 ml of a 2%Na_2CO_3 : 1% $CuSO_4$: 2% NaK tartarate solution was added to the tissue-distilled water mixture, and incubated for 10 minutes at room temperature. 250 µl of Folin-Ciocalteau's Phenol reagent (1M) was added to the mixture. After a 30 minute incubation, the absorbance of the mixture was recorded at 750 nm using the Cary bio 100 spectophotometer. Sample absorbance was related to a standard curve generated from absorbances of the BSA standards, and the protein content of the sample was determined.

2.9 Trypan Blue Dye Exclusion Assay

Cells were incubated with a 0.4% solution of trypan blue in 0.9% NaCl for 10 minutes. The trypan blue solution was then removed and cultures fixed in a 4% paraformaldehyde solution for 10 minutes. Cultures were rinsed with a solution of 0.9% NaCl . Viable cells which excluded the dye and dead cells which included the dye were counted in four different fields of view of each culture well. The four fields of view were averaged and the percent cell death was expressed as (the number of cells including dye)/(total number of cells)

2.10 Immunohistochemistry

Rat hippocampal cultures were grown on 10mm round coverslips coated with poly-D-lysine, at a density of 300 000 cells per well and were cultured using the same conditions as outlined above. After 12 DIV, cells were washed once with 0.1M phosphate buffered saline (PBS), then were fixed using 4% paraformaldehyde in PBS for 10 minutes. Cells were then dehydrated with sequential washings with 50% then 80% then 50% acetone.

Immunohistochemical detection of glial fibliary acidic protein (GFAP) and neurofilament protein (NFP) was performed according to the method of Walsh et al.(1998). All manipulations were conducted in the tissue culture plate wells, without removing the fixed coverslips. The tissue was rinsed with 0.1M tris buffered saline (TBS) and was subsequently incubated with a 0.3% hydrogen peroxide in TBS solution for 30 minutes in order to eliminate endogenous peroxidase activity. Tissue was rinsed again with TBS and incubated with a 10% bovine serum albumin (BSA) plus 0.25% triton-X100 in TBS (TBX) for 30 minutes. This was folowed by a rinse with 0.1M TBS and incubation with the primary antibody for 2 days, either with GFAP or NFP in 3% BSA in TBX. Controls were prepared in the absence of primary antibody. Neurofilament monoclonal mouse anti-human primary antibody was used at a dilution of 1:500 from stock, while GFAP monoclonal rabbit anti-human primary antibody was used at a dilution of 1:1000 from stock. The primary antibody was rinsed away with TBS and the tissue incubated with secondary antibody for 2 hours, either with horse anti-mouse at 1:200 for the NFP tissue or with goat anti-rabbit at 1:200 for the GFAP tissue. The tissue was subsequently rinsed, and incubated for 30 minutes with an avidin/biotin mixture from the Vectastain avidin /biotin

secondary antibody detection system kit (Vector Laboratories Inc. CA). The mixture was removed with a TBS rinse and the tissue exposed to a diamino benzidine (DAB) solution for 1 minute. The DAB solution was removed and the tissue washed with TBS and dehydrated by adding, sequentially, 50%, 75%, 85%, 95% and 100% ethanol. Fixed and stained tissue was exposed to Permount, and the coverslips removed from the tissue culture plate wells, and mounted on glass microscope slides. This procedure was also performed using adult rat sagittal sections embedded in paraffin. Sections were treated exactly as the fixed coverslips.

2.11 Cell Culture of Transformed Cell Lines

Frozen cell stocks, of Ltk$^-$ mouse fibroblasts containing cDNA encoding human NR1a and NR2B or NR1a and NR2A NMDA receptor subunits were obtained from Dr. Paul Whiting at Merck Sharpe and Dohme (UK). Stocks were thawed and grown in 20% FBS/DMEM in 100mm tissue culture dishes for 2 weeks. Cells containing the transgene were selected for using the antibiotic G148 at 5mg/mL for seven days. After which time cultures were split, and further frozen stocks prepared by freezing cells in 7% DMSO plus 20% FBS/DMEM in liquid nitrogen. The remaining cells were grown in 100mm culture dishes until a full monolayer developed. The cultures were then dissociated using trypsin at 0.5mg/ml in HBSS. 40000 cells were plated per well in 10% FBS/DMEM on a 96 well plate, and utilized in toxicity assays. The remaining cells were plated on new 100mm tissue culture plates and grown in 10% FBS/DMEM . This cycle was repeated every 4-5 days, when cells in the 100mm culture plates grew to form a confluent monolayer.

2.12 Toxicity Assay in NR1a/2A and NR1a/2B Containing Fibroblasts

Expression of the NMDA receptor subunits in these cells is under the control of a dexamethasone response element. In order to investigate toxicity mediated by the NMDA receptor, expression of the receptor subunits is obviously necessary. After 1 DIV, 1μM dexamethasone in 10% FBS/DMEM was added to the culture plate wells for 6 hours to induce NMDA receptor expression. During the induction phase, 0.50 mM ketamine was also present in the medium combination, as it was previously determined that this non-competitive NMDA receptor antagonist was required to prevent toxicity from endogenous excitatory amino acids, released by the fibroblasts. After the 6 hour exposure period, wells were washed twice with DMEM base twice and then were incubated with either neurosteroid, the competitive NMDA receptor antagonist AP5, AP5 and neurosteroid or the vehicle (1%DMSO) in DMEM.. After 24 hours, cell health was assessed using the acid phosphatase assay.

2.13 Acid Phosphatase Assay

The acid phosphatase assay was conducted according to the method of Connolly et al. (1986). Cells are first washed with 0.1M phosphate buffered saline (PBS) and incubated at 37°C for 1 hour in 100 μl of a 0.1M sodium acetate buffered solution containing 10 mM Sigma 104 phosphatase substrate and 15 μl of triton-X100. The reaction was stopped by adding 10 μl of a 1M sodium hydroxide solution. The absorbance resulting from the formation of reaction product was measured at 405 nm using a spectramax multiwell plate scanner.

Chapter 3

Results

3.1 Establishment of the LDH Assay

The LDH assay was developed to quantitate excitotoxicity in primary cultures of hippocampal neurons. It has been previously shown by Koh et al. (1988) that by expressing the LDH activity released by dying cells into the culture media as a percentage of the total LDH activity, the percentage of dead cells in the culture can be estimated. Using rat hippocampal homogenate to standardize the assay, the LDH assay showed a linear increase in LDH enzyme activity relative to the amount of protein present (Figure 5).

3.2 Establishment of Primary Hippocampal Cultures as a Model of Excitotoxicity

Cultures obtained using conditions 1 through 4 as outlined in table 3 did not survive until day 12 in vitro (12 DIV), and thus could not be used in the assessment of excitotoxicity. However, cultures prepared using culture condition 5 , as outlined in table 3, did survive. These conditions were the same as those of Xiong and Macdonald (1997). The most significant changes from condition 4 to condition 5 were (i) changing from using fetal calf serum (FCS) supplemented DMEM to FCS + horse serum (HS) supplemented DMEM for the initial 2 days in vitro, (ii) stopping culture medium replacement after day 7 in vitro and (iii) adding insulin as a

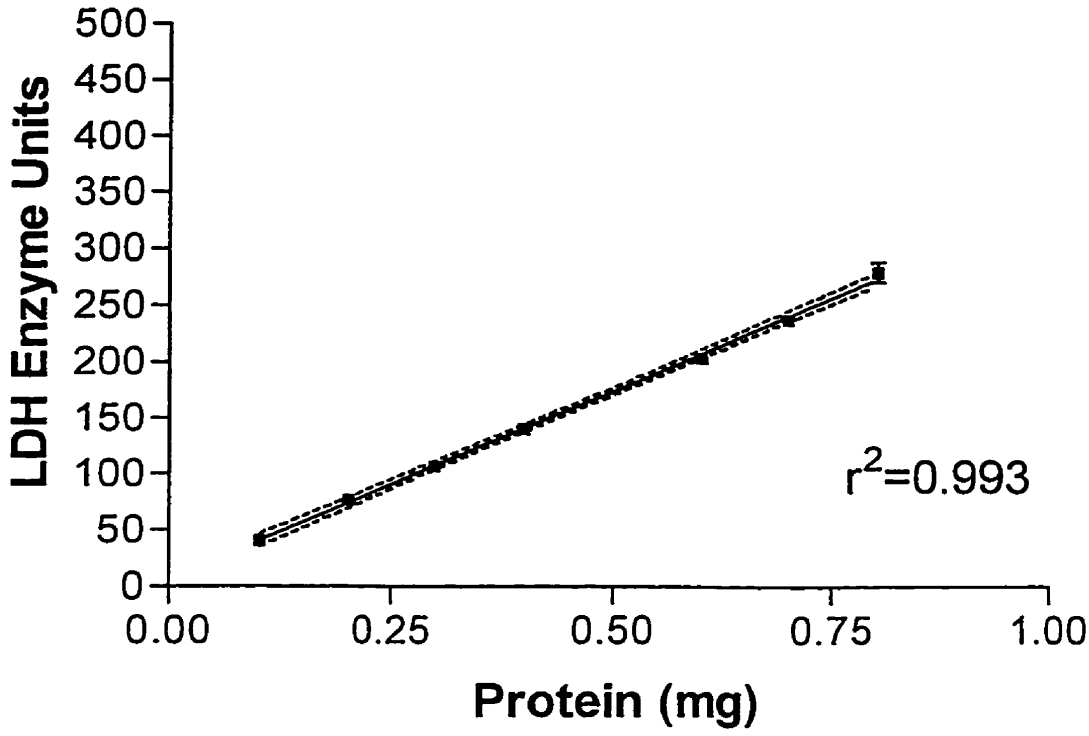


Figure 5. Change in LDH enzyme activity (LDH Enzyme Units) versus protein (mg). Each data point represents the mean ± SD of N = 1 experiments in triplicate. Dashed lines represent 95% confidence interval.

suspension, instead of as a solution in 0.1M hydrochloric acid. Neurons were viable (Figure 6.) and exhibited NMDA receptor mediated cell death (Figure 7). Figure 8 shows the concentration-dependant toxicity of NMDA in these cultures. Maximal NMDA induced toxicity was obtained using 100 μM NMDA, which caused a 33 ± 1 % release of total LDH activity from the culture, compared to 11.6 ± 3 % release of total LDH activity from the control washed cultures. Since these cultured neurons could not be prepared reproducibly, an alternate primary culture model of excitotoxicity was used. The murine hippocampal cultures were generated in Dr. John Macdonald's laboratory at the University of Toronto, and were used in subsequent studies (section 3.4).

3.3 Immunohistochemical Characterization of Hippocampal Cultures

Rat coronal sections were immunostained for both NFP and GFAP according to the method of Walsh et al. (1998). Figures 9 and 10 show the selective staining for GFAP and NFP, with no cross reactivity. Neurons were identified by nuclear staining as well as by axonal staining (Figure 9). The polarity of the neurons, extending deep into the cortex, is readily visible. Glial staining in these tissue slices is characterized by cell body staining and with the staining of radial processes (Figure 10).

Stained 12 DIV rat hippocampal cultures look remarkably different. Neuronal cell bodies stain lightly (Figure 11), however, there is similar staining of neurons in control cultures which were not exposed to the primary NFP antibody (Figure 12). Glial cells cover the entire surface of the tissue culture well. All areas of the cell stain for GFAP. Structures which resemble neurons

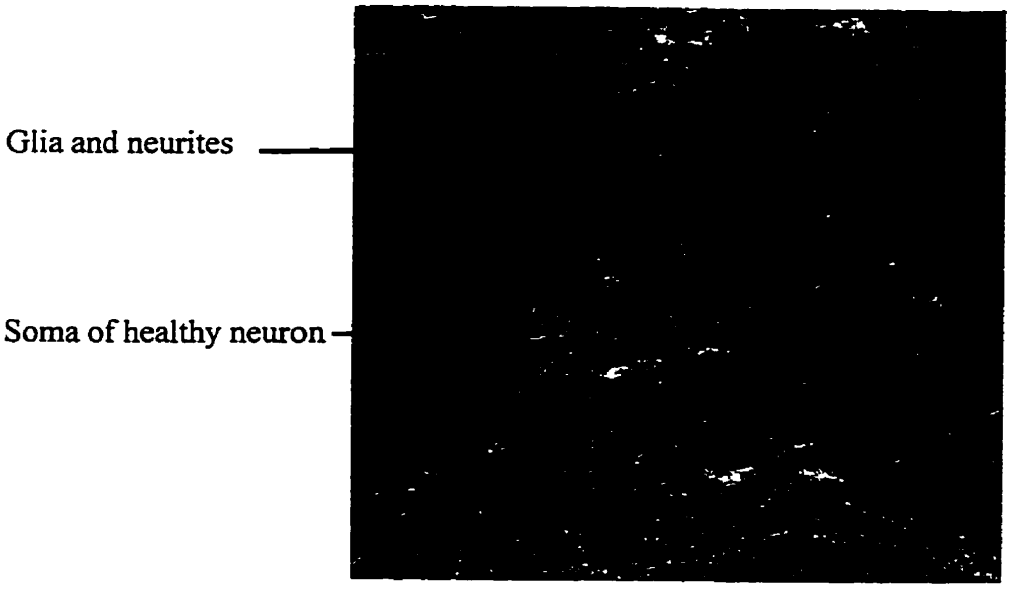


Figure 6. Viable 12 DIV rat hippocampal primary cultures (200x).

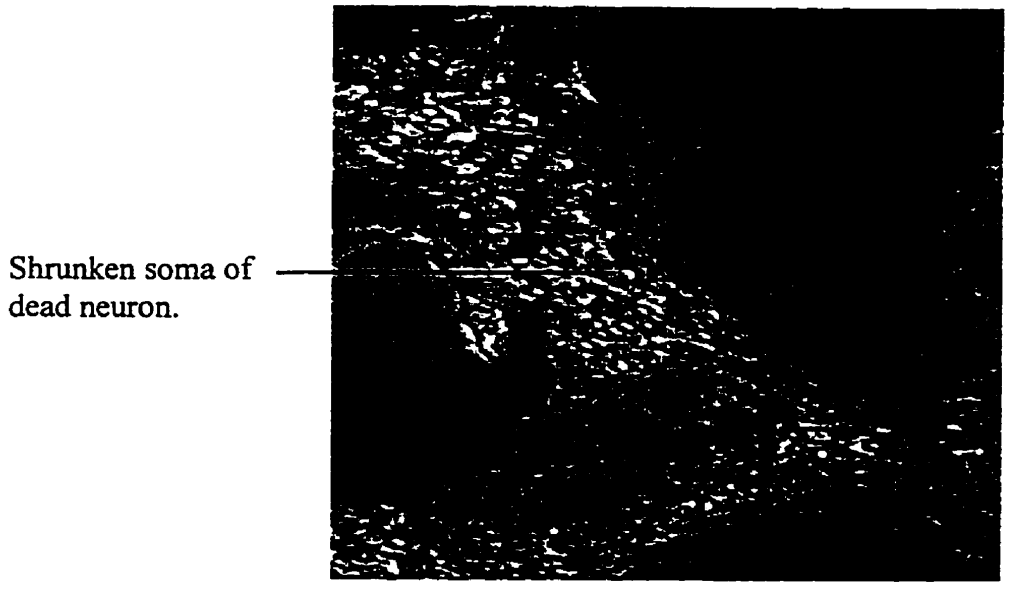


Figure 7. 12 DIV cultures exposed to 0.1 mM NMDA for 10 minutes (200x).

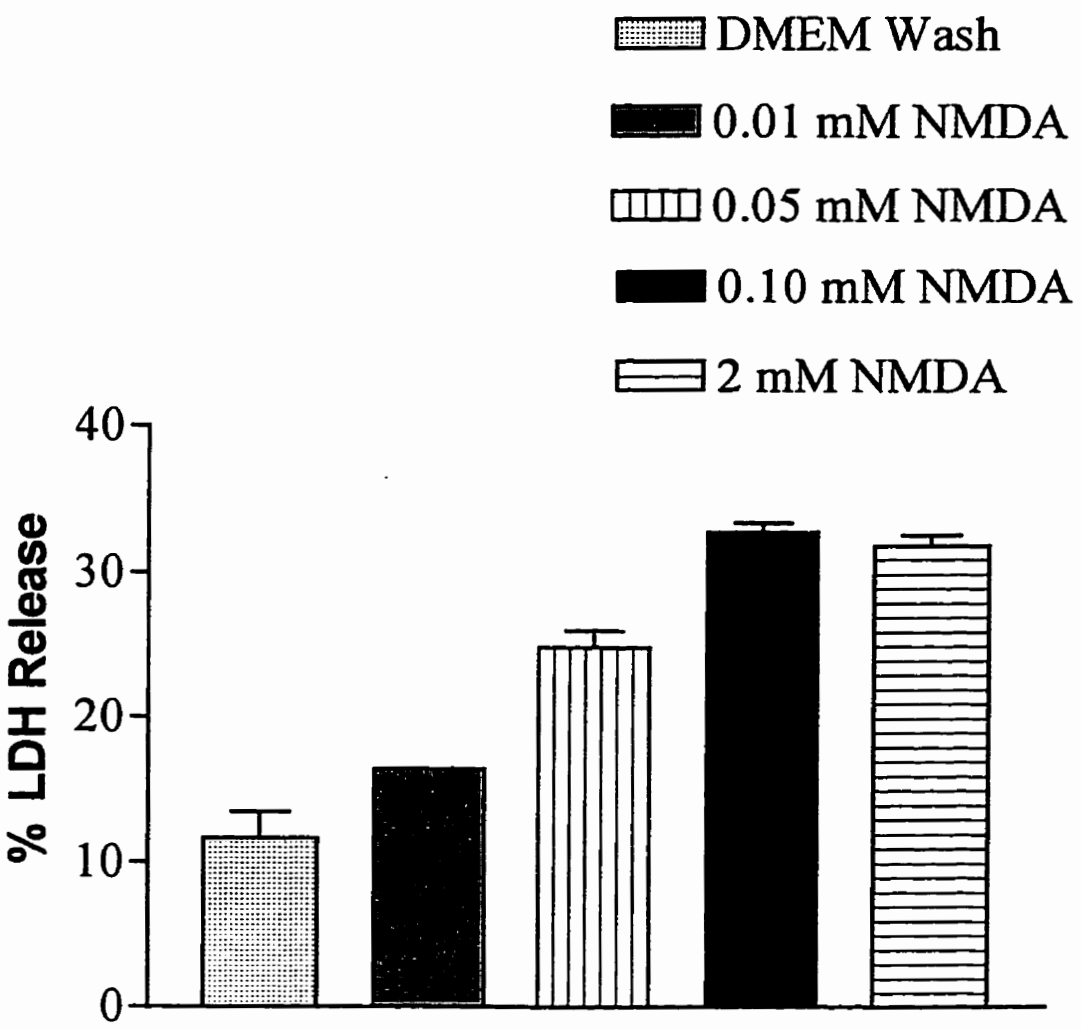


Figure 8. Concentration dependence for the effect of NMDA on LDH release in 12 days in vitro (DIV) rat hippocampal cultures. Data presented as mean ± SD for N=1 experiment.

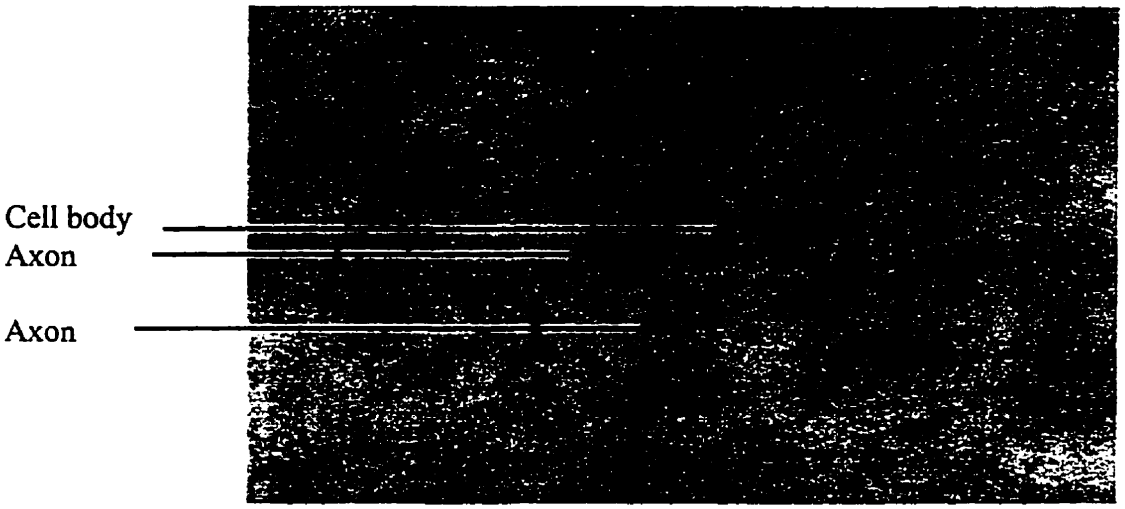


Figure 9. Anti-NFP stained coronal section of rat cortex (100x). Neuronal cell bodies stain dark, as do axons. Axons run parallel to one another.

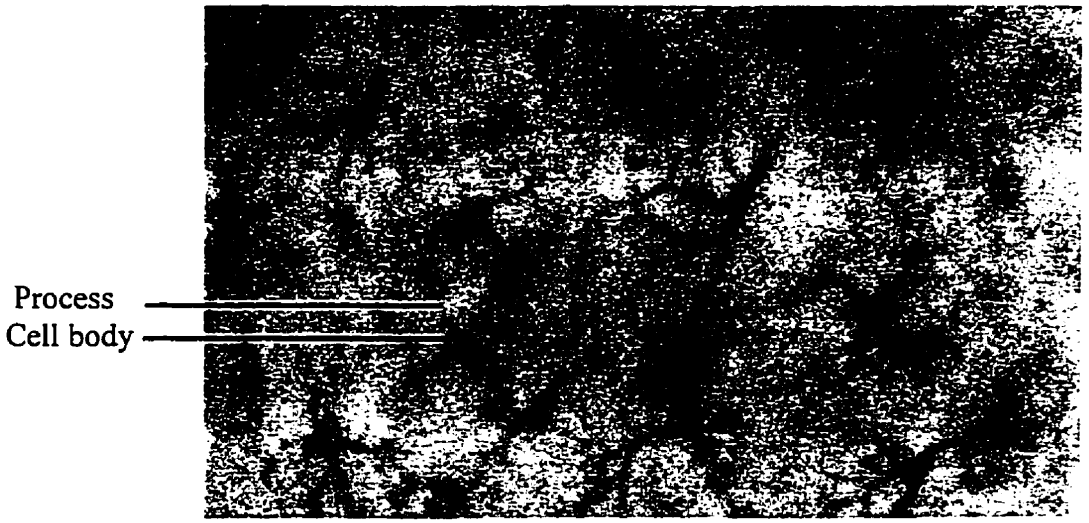


Figure 10. Anti-GFAP stained coronal section of rat cortex (100x). Cell bodies stain dark, as do processes.

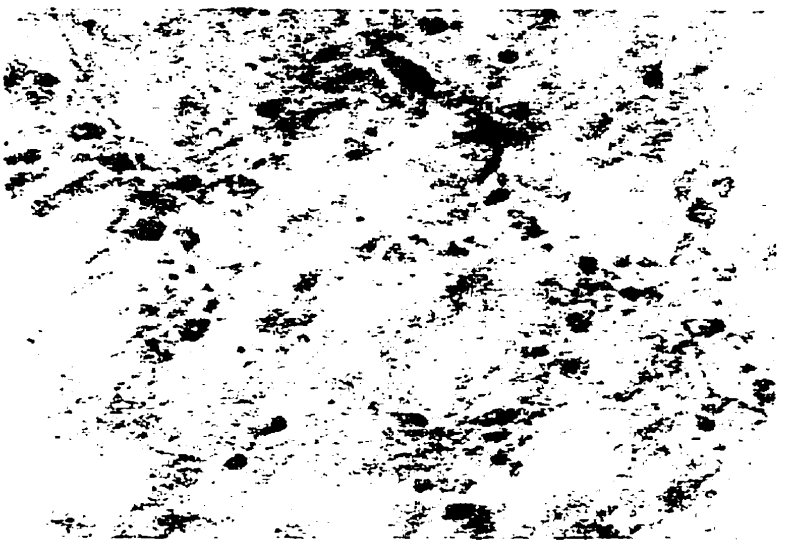


Figure 11. NFP staining in 12 DIV rat hippocampal cultures (200x). Neuronal cell bodies stain lightly, with little evidence of axon staining.

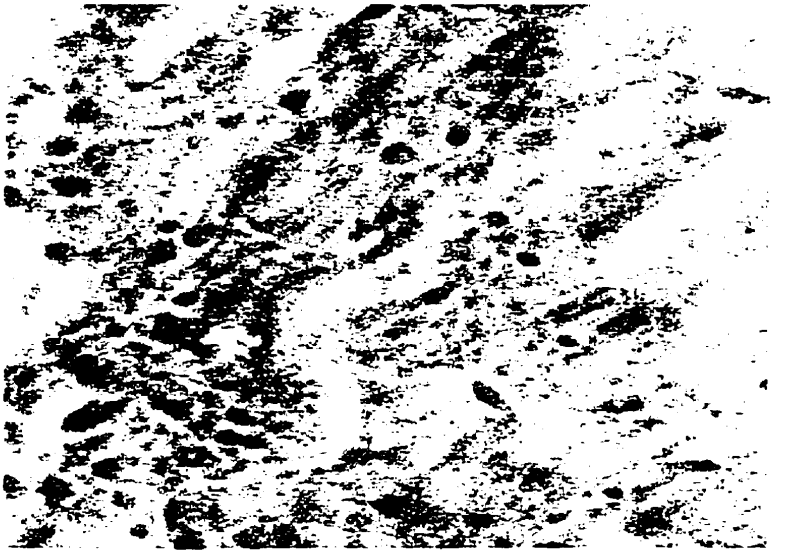


Figure 12. Control NFP staining in 12 DIV rat hippocampal cultures (200x). Staining identical to Figure 11.

also stain for GFAP (Figure 13). There is an absence of GFAP staining in the control cultures, which were not exposed to primary GFAP antibody (Figure 14). Thus, even though neuronal cells and glial cells could be readily identified in culture by assessing cell morphology, the immunostaining could not selectively differentiate between the two cell types. Alternate immunostaining conditions, from those quoted in chapter 3, were tried without success. For example, in an attempt to reduce neuronal staining with GFAP due to possible high levels of endogenous neuronal peroxidase activity, extended peroxidase treatment was tried, ranging from 1 to 2 hours, in place of the 30 minute treatment. Conditions were also altered in the NFP staining protocol. Increased dilutions of primary NFP antibody were utilized, from 1:1000 to 1:2000 from the stock, to prevent a possible crowding and inhibition of antibody binding at the NFP epitope. Neither of the condition changes improved the NFP staining or reduced the neuronal staining on glial stained coverslips.

3.4 Characterization of Excitotoxicity in Murine Hippocampal Cultures

As mentioned in section 3.2, hippocampal cultures could not be prepared reproducibly, and thus could not be used to assess NMDA mediated excitotocity. Consequently, glutamate mediated excitotoxicity was examined in an alternate primary culture model, that of murine hippocampal cultures (see Xiong and Macdonald 1997). In switching to the murine model, it was expected that problems with reproducibility and with high levels of non-specific toxicity in the rat model would be avoided.

It was observed that these neurons were sensitive to glutamate-mediated toxicity (Figure

Figure 13. GFAP staining in 12 DIV rat hippocampal cultures (200x). Both neurons and glia stain for GFAP.

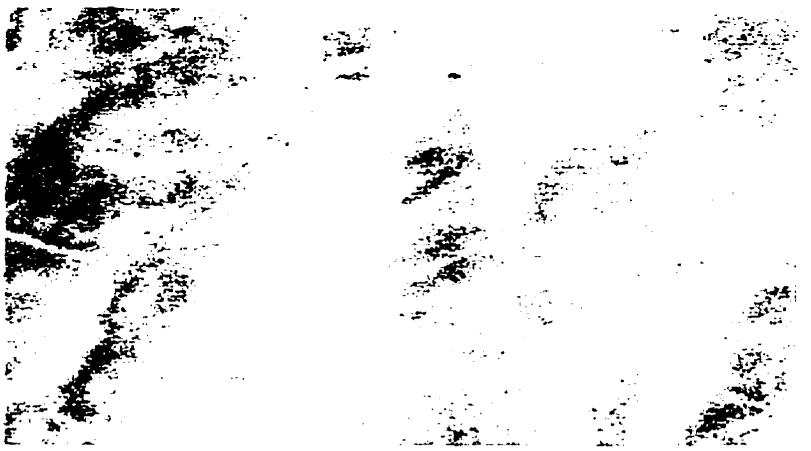


Figure 14. Control GFAP staining in 12 DIV rat hippocampal cultures (200x). Absence of glial and neuronal staining.

15). A glutamate concentration of 0.50 mM produced 69.1 ± 5.3 % release of total LDH enzyme activity, compared to a value of 22.7 ± 10.7 % in the sham washed cultures (Figure 15). Using this model, the effects of PREG-S and PREGNAN-S acting on the NMDA receptor were examined.

3.5 NMDA Receptor Dependent Toxicity in Murine Hippocampal Cultures

Figure 16 shows NMDA receptor dependent toxicity in the murine hippocampal cultures. The cells cultured for the experiments examining neurosteroid modulation of the NMDA-mediated excitotoxicity were prepared in a manner different from those in section 3.4. Specifically, fewer cells were plated on each tissue culture plate. For this reason, lower LDH release in the NMDA excitotoxicity assay was observed, compared to the cultures used to characterize glutamate mediated toxicity in section 3.4. For example, since neurons are the only cells which exhibit excitotoxicity mediated by glutamate or NMDA, the released LDH activity in these cultures would be lower than seen in the previous culture, as fewer neurons were present. Furthermore, in both cultures, it was estimated through visual inspection that glial cell numbers remained constant. Thus the percent LDH release would decrease, as there would be little change in latent LDH activity, which glial cells contribute to principally, while released activity contributed by neurons would decrease. Consequently, 0.50 mM NMDA ellicited a 32.2 ± 11.8 % release of total LDH activity compared to 20.6 ± 5.1 % release of total LDH activity from control sham washed cultures (Figure 16). This NMDA mediated toxicity was blocked by co-treatment with MK-801. Exposure of cultures to 20 μM MK-801 combined with 50 μM NMDA

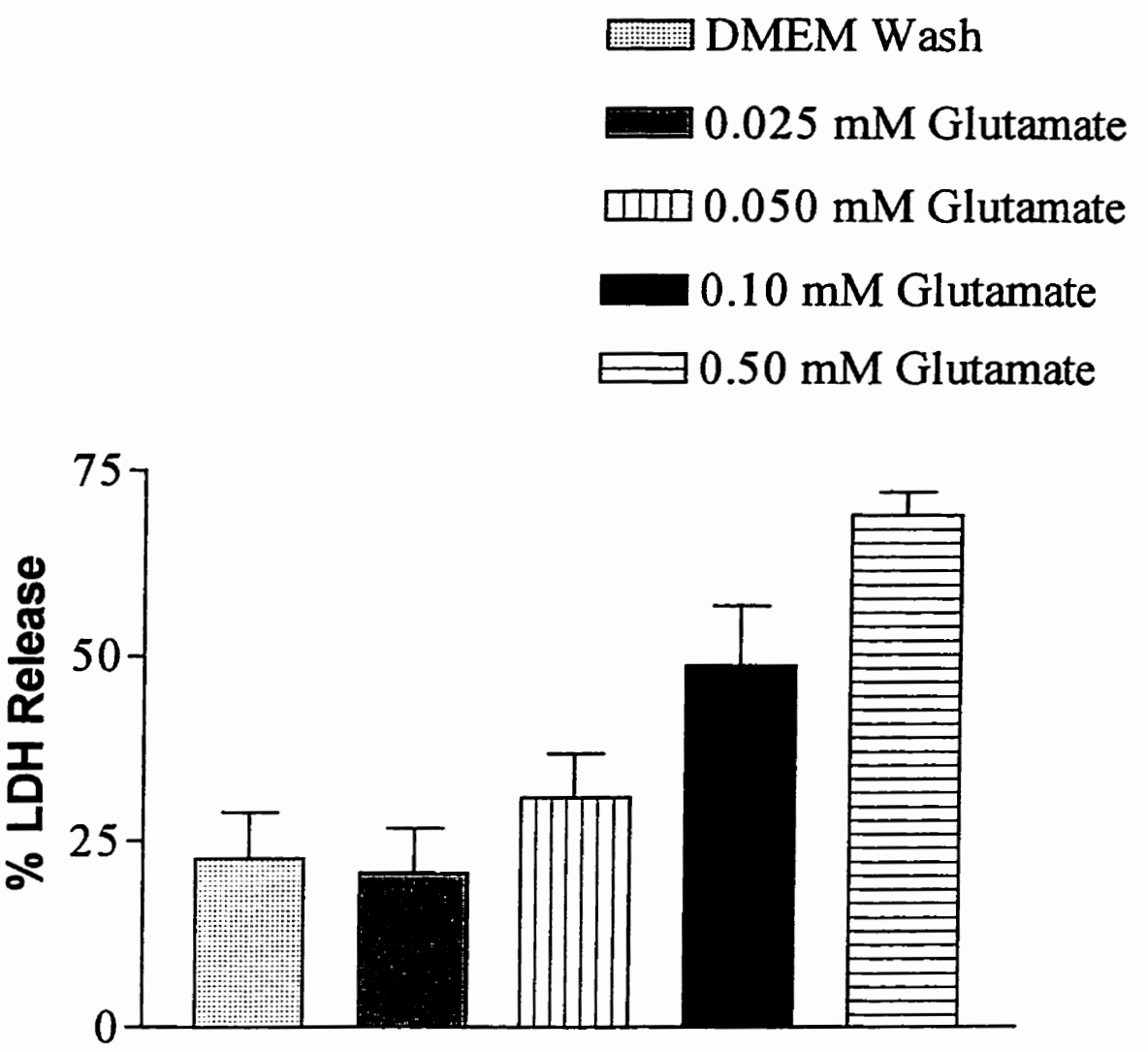


Figure 15. Concentration dependence for the action of glutamate in 12 days in vitro (DIV) mouse hippocampal cultures. Data presented as mean ± SD for N = 1 experiments carried out in 3 sister cultures.

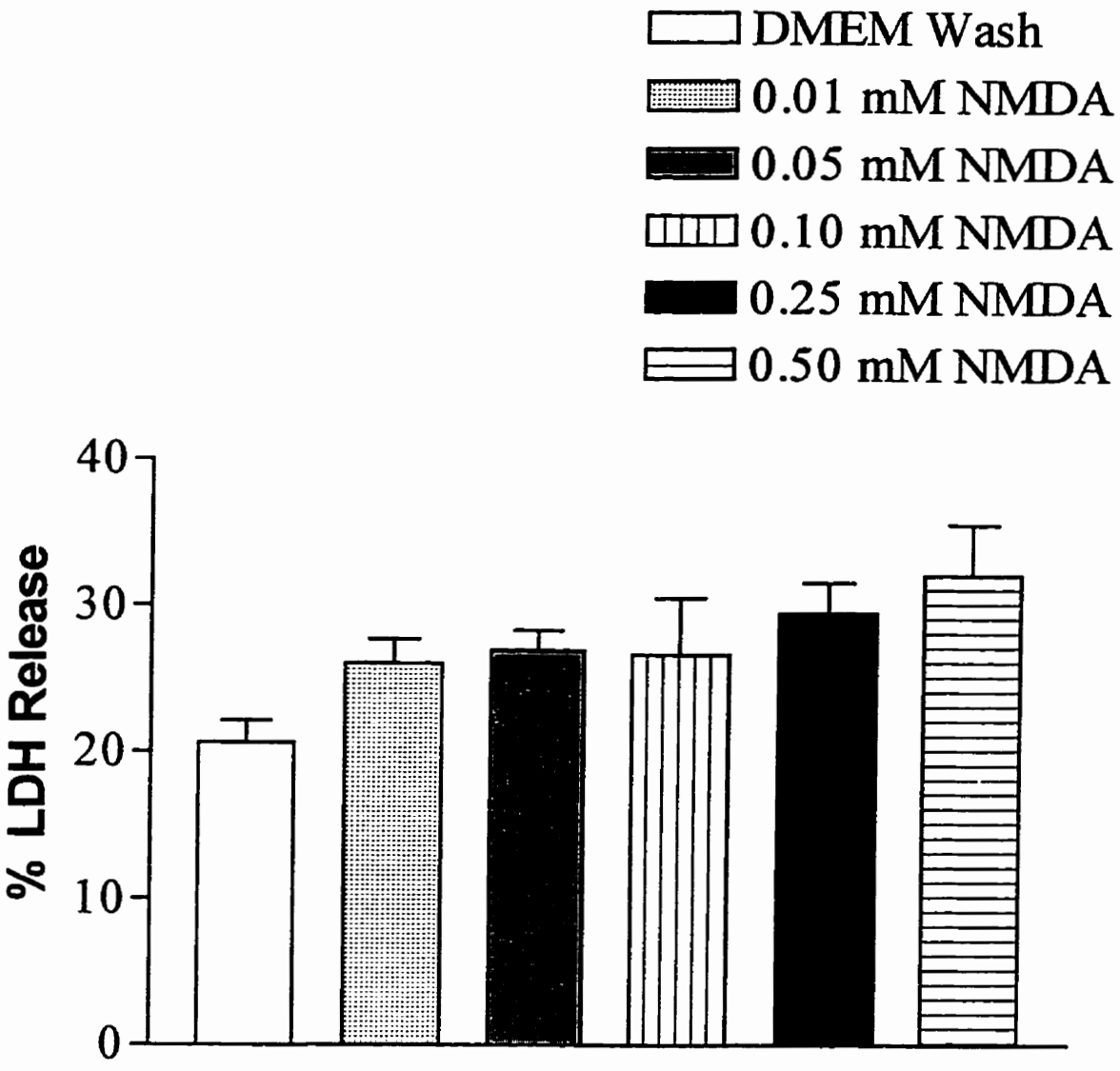


Figure 16. Concentration dependence for the action of NMDA in 12 days in vitro (DIV) mouse hippocampal cultures. Data presented as mean ± SD for N = 6 experiments carried out in 3 sister cultures.

resulted in a 20.4 ± 4.5% release of total LDH activity compared to 50 μM NMDA alone, which caused a 37.4 ± 15% release of total LDH enzyme activity (Figure 17).

3.6 Characterizing the Effects of Pregnanolone Sulfate and Pregnenolone Sulfate on NMDA Receptor-Dependant Toxicity

The mouse hippocampal culture model was used to investigate the ability of PREG-S and PREGNAN-S to modulate NMDA mediated excitotoxicity. Figure 18 shows that PREG-S treatment combined with NMDA treatment produced a significant increase in NMDA induced excitotoxicity, at concentrations ranging from 0.001 mM to 0.1 mM steroid compared to NMDA treatment alone. While NMDA mediated toxicity resulted in a 25% release of total LDH activity, PREG-S treatment with NMDA treatment resulted in a release of 47% of the total LDH enzyme acitvity from the cultures. However, this apparent modulation of NMDA toxicity may result from vehicle toxicity, as 20% cylodextran, which was used to solvate the 0.1 mM PREG-S, produces a release of 32% of the total LDH activity. Furthermore, the toxicity resulting from 0.01 and 0.001 mM PREG-S treatment is equally uncertain, since control treatments using 10% cyclodextran, which was used to solvate the 0.01 and 0.001 mM concentrations of PREG-S, were not performed.

PREGNAN-S had an inverse effect on NMDA mediated toxicity. Treatment of mouse hippocampal cultures with PREGNAN-S combined with 0.050 mM NMDA produced a significant reduction in toxicity relative to 0.050 mM NMDA treatment alone (Figure 19). Treatment of cultures with 0.050 mM PREGNAN-S combined with 0.050 mM NMDA resulted

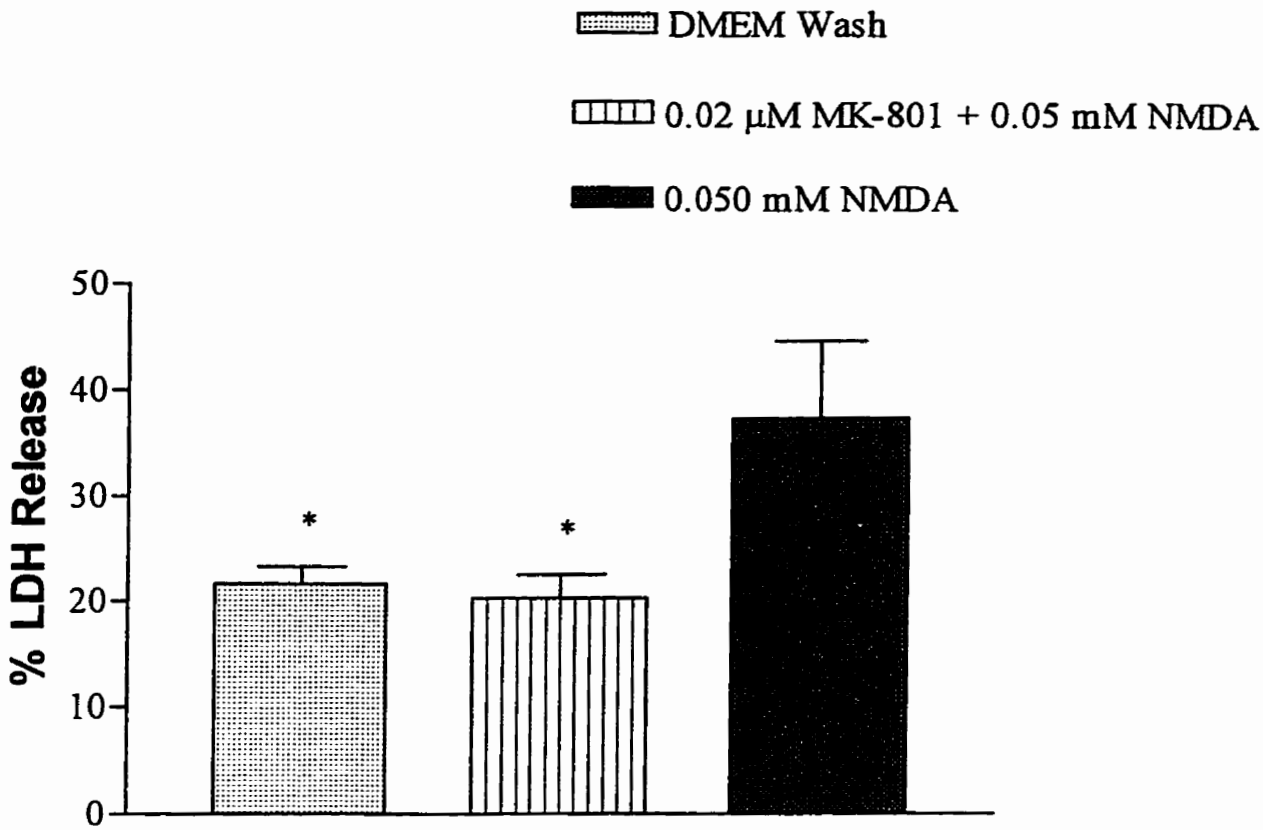


Figure 17. MK-801 antagonism of NMDA toxicity in 12 days in vitro hippocampal cultures. Data presented as mean ± SD for N = 2 experiments caried out in 3 sister cultures. * = significantly different from 0.050 mM NMDA using 1-way ANOVA, $p<0.05$, with Newman-Keuls post-hoc test.

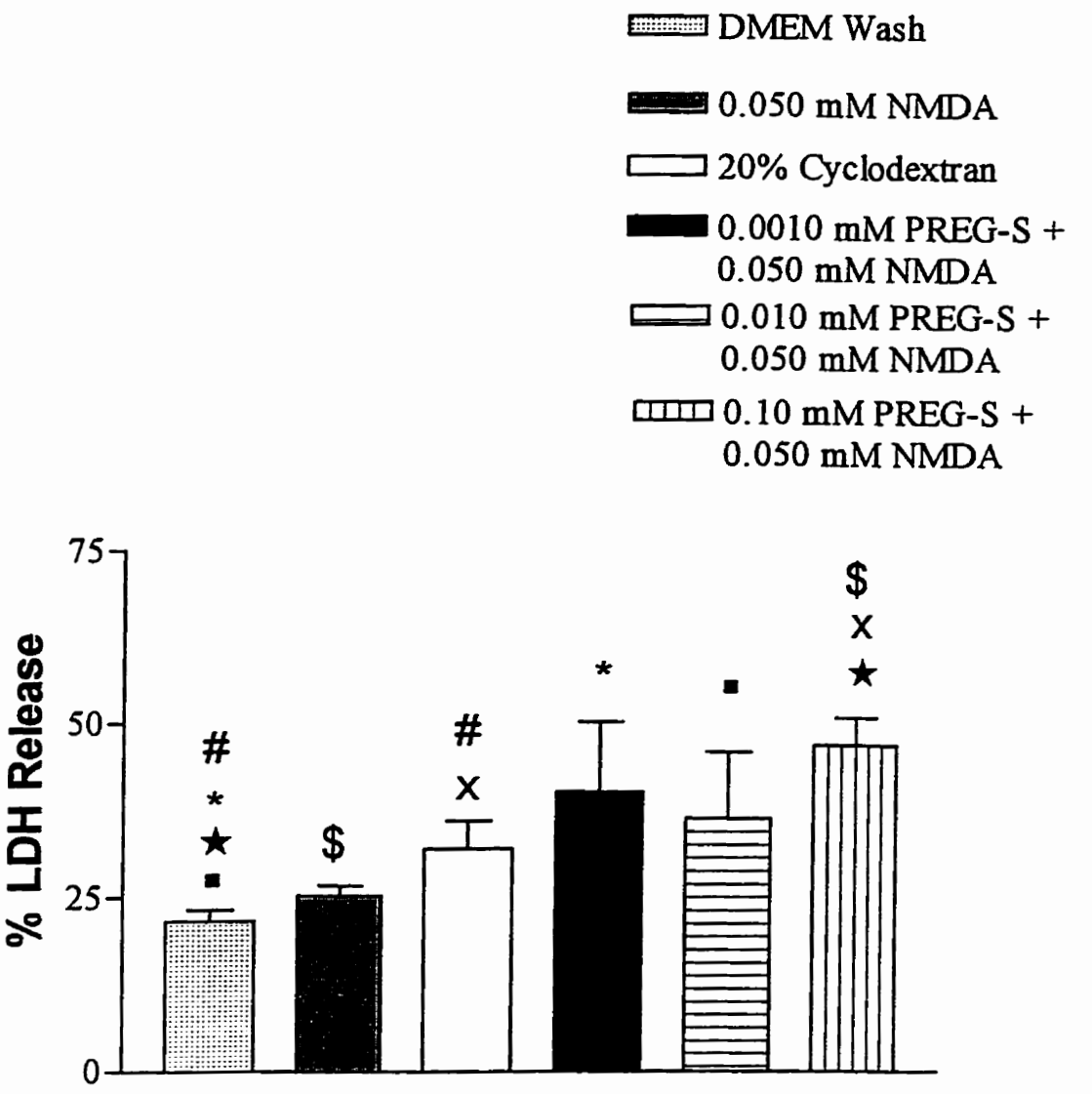


Figure 18. Effect of Pregnenolone Sulfate (PREG-S) on NMDA excitotoxicity in 12 days in vitro (DIV) mouse hippocampal cultures. Data presented as mean ± SD for N = 6 experiments carried out in 3 sister cultures. ▪,★$,*,#,X = significantly different from each other using 1-tailed t-tests. $p<0.05$.

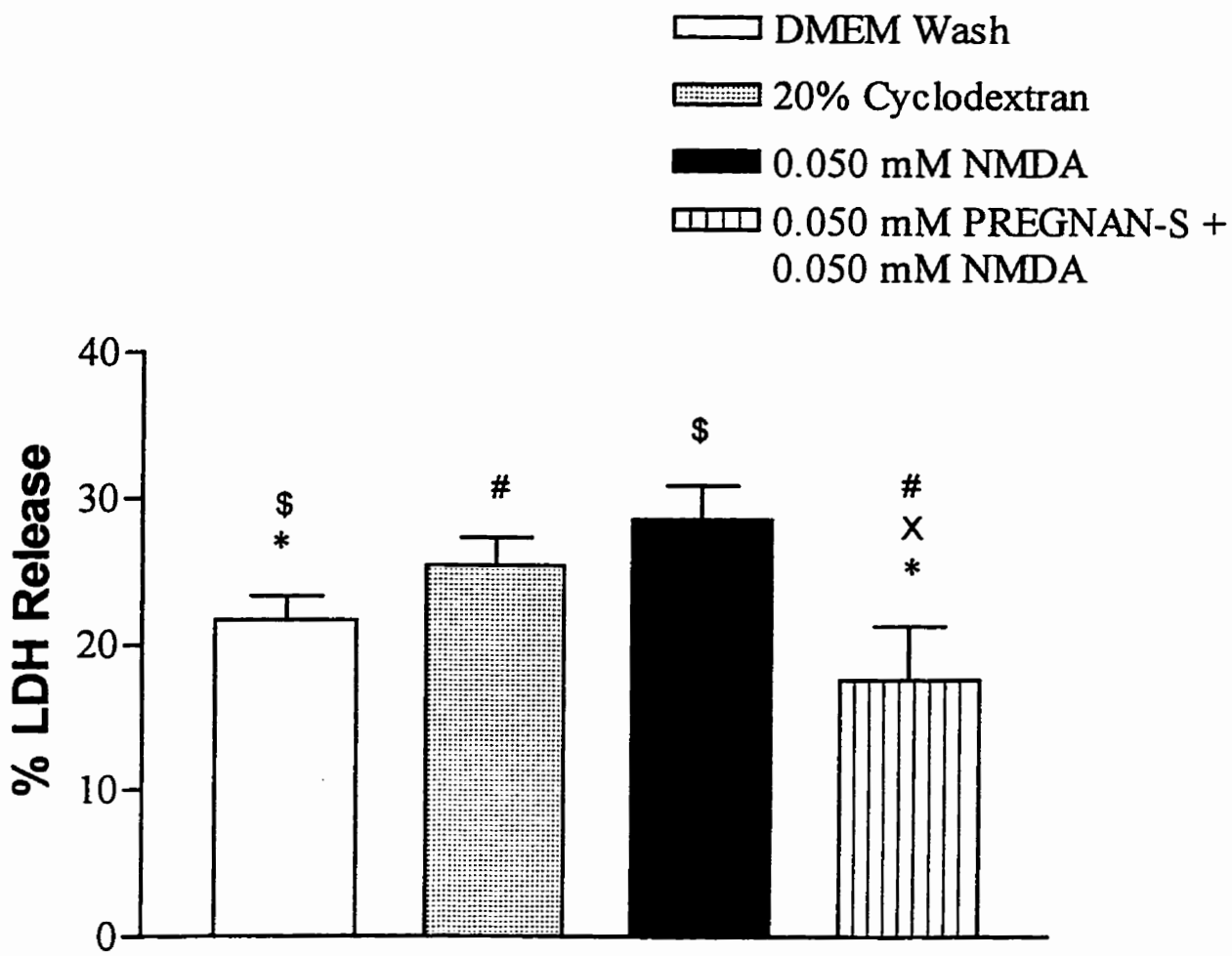


Figure 19. Effect of pregnanolone sulfate on NMDA excitotoxicity in 2 DIV mouse hippocampal cultures. Data presented as mean ± SD for N =6 experiments carried out in triplicate. *,X,$,# = significantly different from each another using 1-tailed tests. $p<0.05$

in a 17.7 ± 9 % release of total LDH activity compared to a 28.6 ● 5.7 % release of total LDH activity in 0.050 mM NMDA treated cultures (Figure 19). Again, 20% cyclodextran treatment appeared greater than control washed cultures, but the difference was not statistically significant. Regardless of possible vehicle toxicity, pregnanolone sulfate was still capable of negatively modulating NMDA mediated toxicity.

3.7 Murine Fibroblasts as a Model of NMDA Receptor-Dependant Toxicity

Based on the murine hippocampal culture data, it appears that pregnanolone sulfate protects against NMDA recptor induced cell death, while pregnenolone sulfate either potentiates or has no effect on NMDA receptor mediated toxicity. Unfortunately, additional experiments could not be carried out using this model system which could clarify further the modulatory effects of neurosteroids at the NMDA receptor. Thus, the modulation of NMDA receptor dependant excitotoxicity by pregnanolone sulfate and pregnenolone sulfate was investigated in fibroblasts expressing NMDA receptors. Induction of NMDA receptor expression in mouse fibroblasts using 1 μM dexamethasone resulted in 100% cell death, which was induced by washing and incubating the cells for 24 hours in DMEM. This toxicity results from the stimulation of NMDA receptors by endogenous excitatory amino acids released by the fibroblasts into the culture medium (Grimwod et al. 1996). This toxicity was significantly reduced by including the competitive NMDA receptor antagonist D,L-AP-5 (0.5 or 1mM) during the 24 hour DMEM incubation period (Figure 20). Toxicity was assessed by using a trypan blue dye exclusion method, as described in chapter 2. These initial studies thus confirmed the

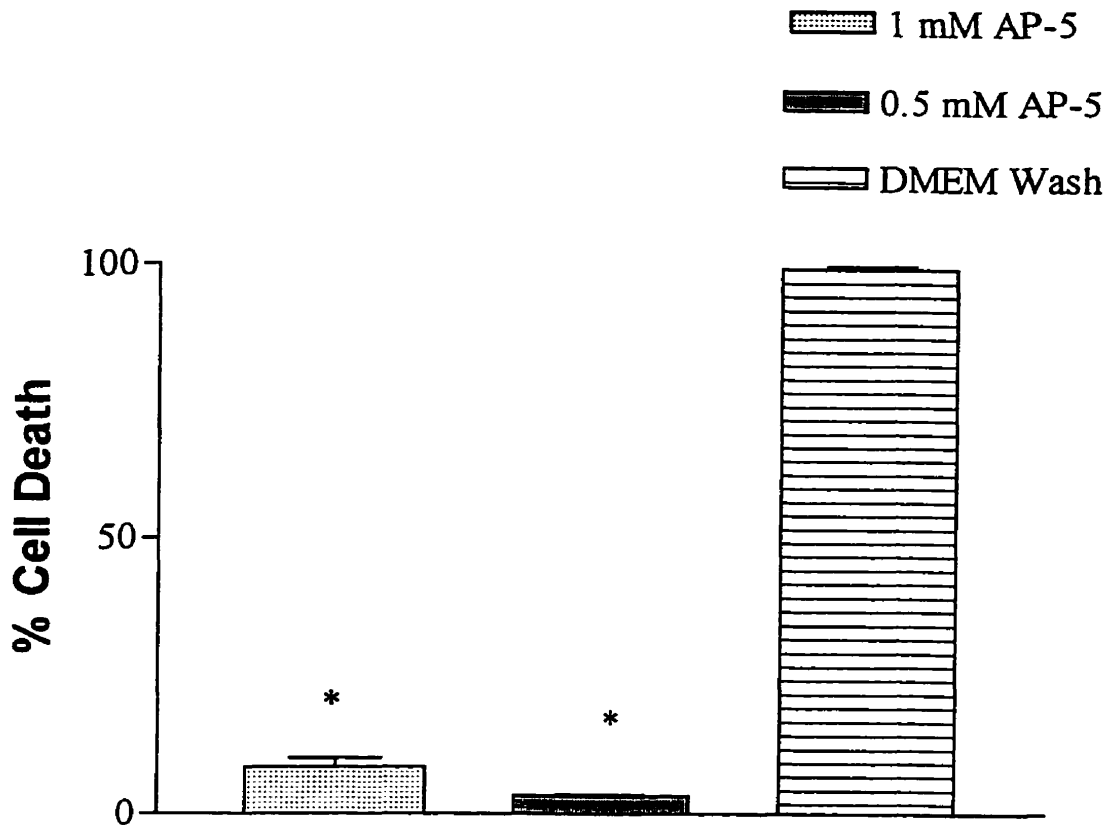


Figure 20. Effect of D,L-AP-5 on cell viability. Data presented as mean ± SD for N = 3 cultures carried out in 3 sister cultures. * = significantly different from DMEM wash using 1-way ANOVA $p<0.05$ with Newman-keuls post-hoc test.

observations made by Grimwood et al. (1996), that in the absence of NMDA receptor antagonists the fibroblast cultures degenerate spontaneously, after induction of NMDA receptor expression in these cells.

3.8 Development of the Acid Phosphatase Assay

The acid phosphatase assay was developed to assess excitotoxicity in the mouse fibroblast model, as the LDH assay proved unreliable in this system. The acid phosphatase assay was also preferrable to using the trypan blue dye exclusion method, as individual fibroblasts are difficult to identify and count when they form a monolayer in culture. This assay is based on the conversion of the acid phosphatase substrate Sigma-104 to a yellow product by cell membrane associated acid phosphatase enzyme. An increase in phosphatase activity indicates an increase in cell number and therefore cell viability. The acid phosphatase assay was shown to produce a linear increase in phosphatase product with an increase in cell number (Figure 21). Subsequently, this assay was used to measure NMDA receptor-induced toxicity in the transfected mouse fibroblast cells, replacing the trypan blue dye exclusion method.

3.9 Effect of D,L-AP-5 on NMDA Mediated Toxicity in Mouse Fibroblasts

NMDA receptor mediated toxicity in the mouse fibroblast model was characterized using the NMDA receptor competitive antagonist D,L-AP-5. It was subsequently determined that this antagonist prevented toxicity in fibroblasts expressing NMDA receptors in a concentration

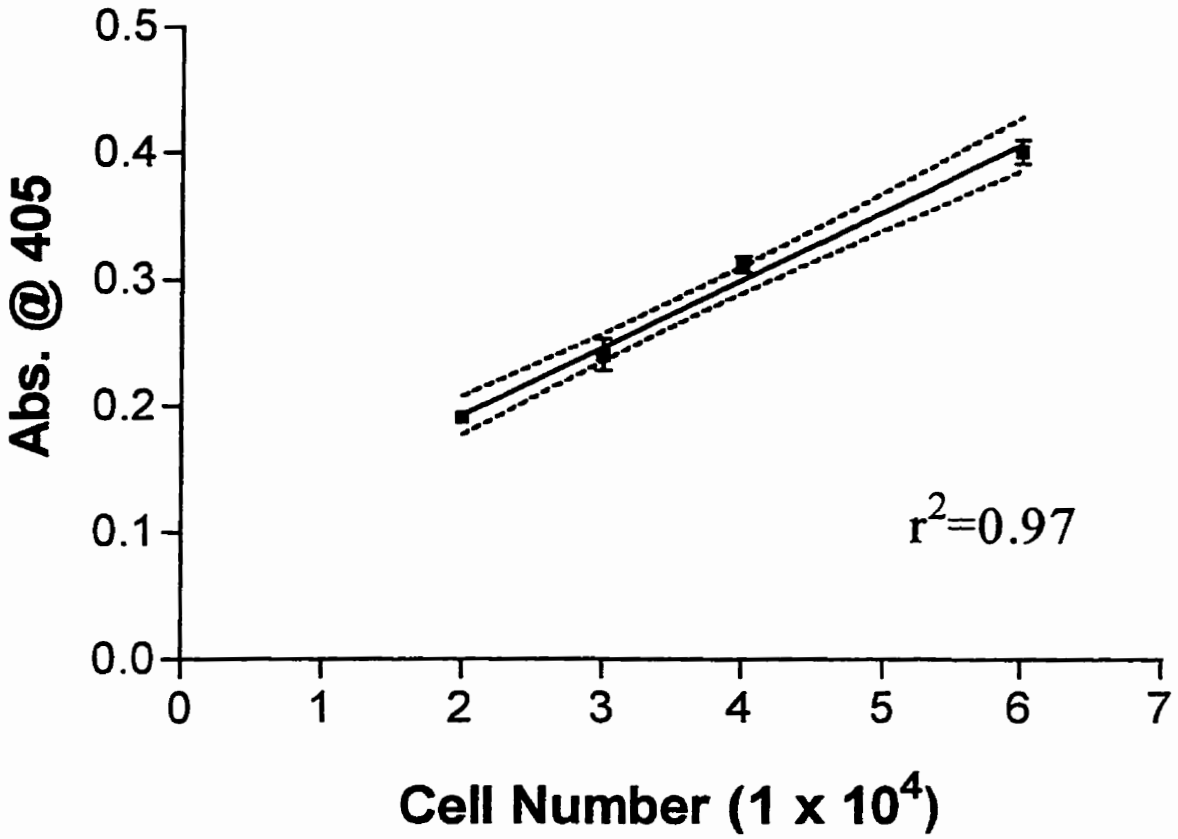


Figure 21. Graph of absorbance (acid phosphatase activity) versus fibroblast cell number. Data presented as mean ± SD for N =3 experiments carried out in 3 sister cultures. Dashed lines = 95% confidence interval.

dependant manner in both the NR1a/2A and NR1a/2B expressing cells (Figures 22, 23). Interestingly, there is a significant difference in potency of the antagonist at the NR2B containing NMDA receptors, compared to the NR2A containing receptors. In the NR2A expressing cells, complete inhibition of NMDA receptor mediated toxicity was achieved with 0.50 mM D,L-AP-5, while in the NR2B expressing cells, a concentration of 2mM D,L-AP5 was required for complete antagonism of toxicity (Figure 23). In both the NR2A and NR2B expressing cells, complete antagonism of NMDA receptor mediated toxicity results in cell viability equal to that of sham washed mouse fibroblasts which do not express NMDA receptors. Complete antagonism of NMDA receptor mediated toxicity was also achieved with 0.5 mM ketamine, a non-competitive NMDA receptor antagonist. These results are the same as those obtained by Grimwood et al. (1996) using this mouse fibroblast model to investigate electrophysiological properties of recombinant NMDA receptors.

3.10 Pregnanolone Sulfate and Pregnenolone sulfate Modulation of Excitotoxicity in Murine Fibroblasts Expressing NMDA Receptors

Using the mouse hippocampal culture model, it was determined that PREG-S might positively modulate NMDA receptor mediated excitotoxicity, while PREGNAN-S negatively modulates this toxicity. Mouse fibroblasts were used to examine the ability of PREGNAN-S and PREG-S to modulate NMDA receptor mediated toxicity through direct actions of the steroids at the NMDA receptor.

PREGNAN-S at a concentration of 0.1 mM in 1% DMSO produced significant protection

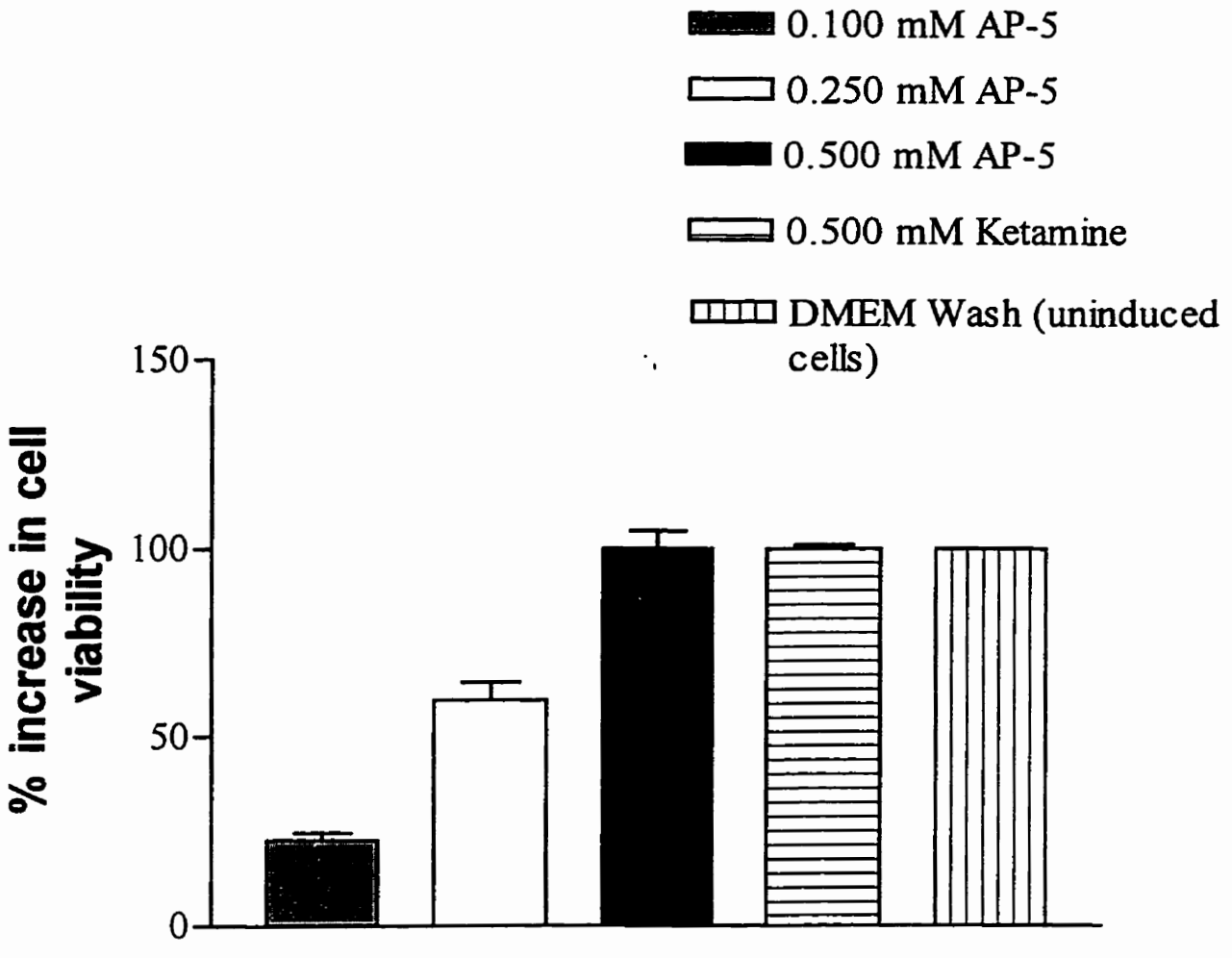


Figure 22. Concentration dependence for the effect of D,L-AP-5 on NR1a/2A-expressing fibroblast viability. Data presented as mean ± SD, N=3 carried out in 3 sister cultures, of % increase in cell viability relative to DMEM treatment, after NMDA receptor induction (Figure 20).

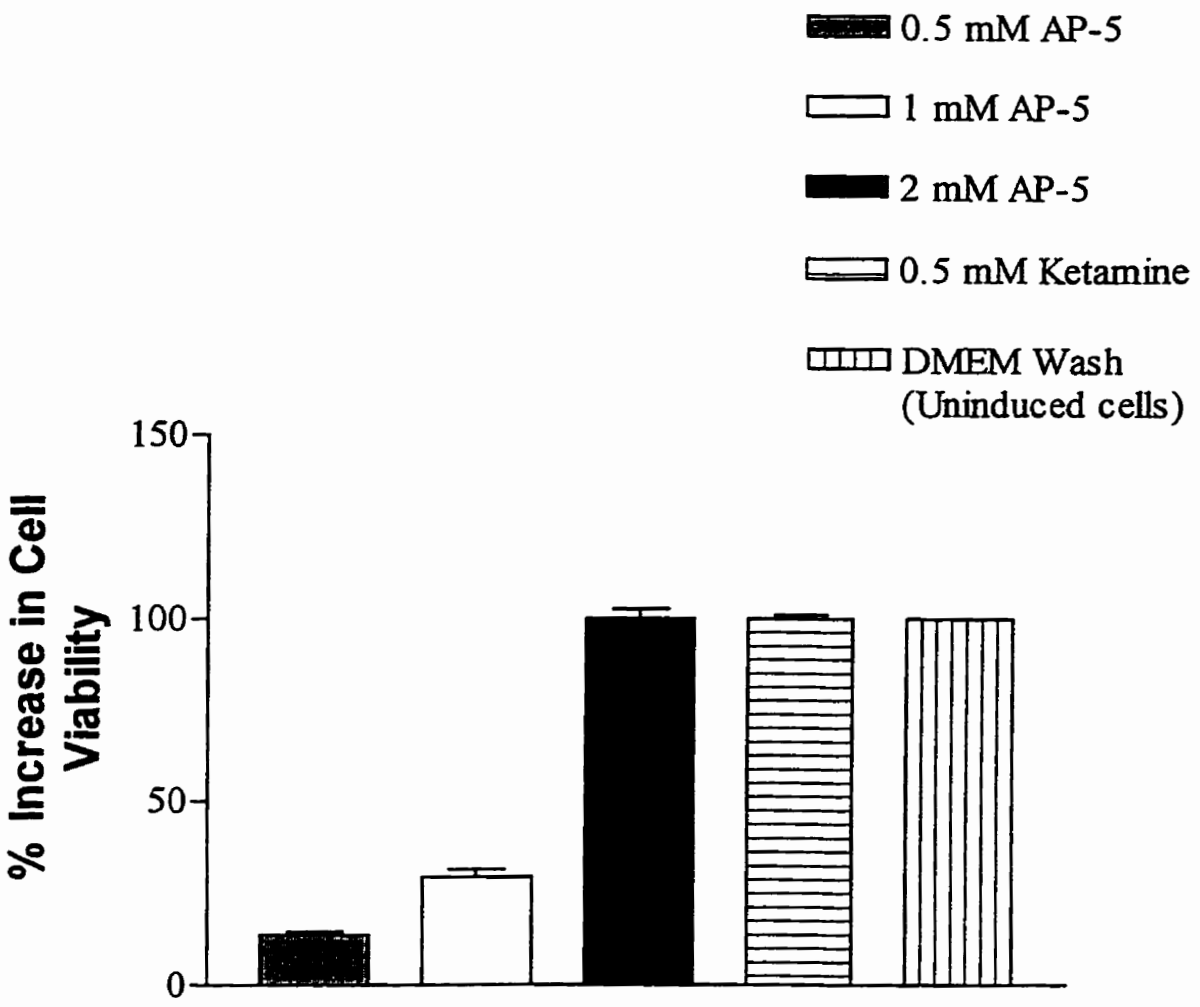


Figure 23. Concentration dependence for the effect of D,L-AP-5 on NR1a/2B-expressing fibroblast viability. Data presented as mean ± SD, N=3 carried out in 3 sister cultures, of % increase in cell viability relative to DMEM treatment, after NMDA receptor induction (Figure 20).

of mouse fibroblasts expressing both the NR2A and NR2B subunits (Figure 24). There was an increase in cell viability of 28.7 ± 5.4 % relative to vehicle control, in fibroblasts expressing NR1a/2A receptor, while a similar increase in cell viability (24.4 ± 4.9%) was observed in NR2B expressing cells. There was no difference in steroid modulation of the NR2A and NR2B subunit containing receptors.

The modulation of NMDA receptor mediated toxicity in the mouse fibroblast model by PREG-S was different from that seen in the primary culture model. The maximum soluble concentration of PREG-S (using DMSO as vehicle) exhibited no modulation of toxicity in either the NR2A or NR2B expressing cell lines (Figures 25, 26).

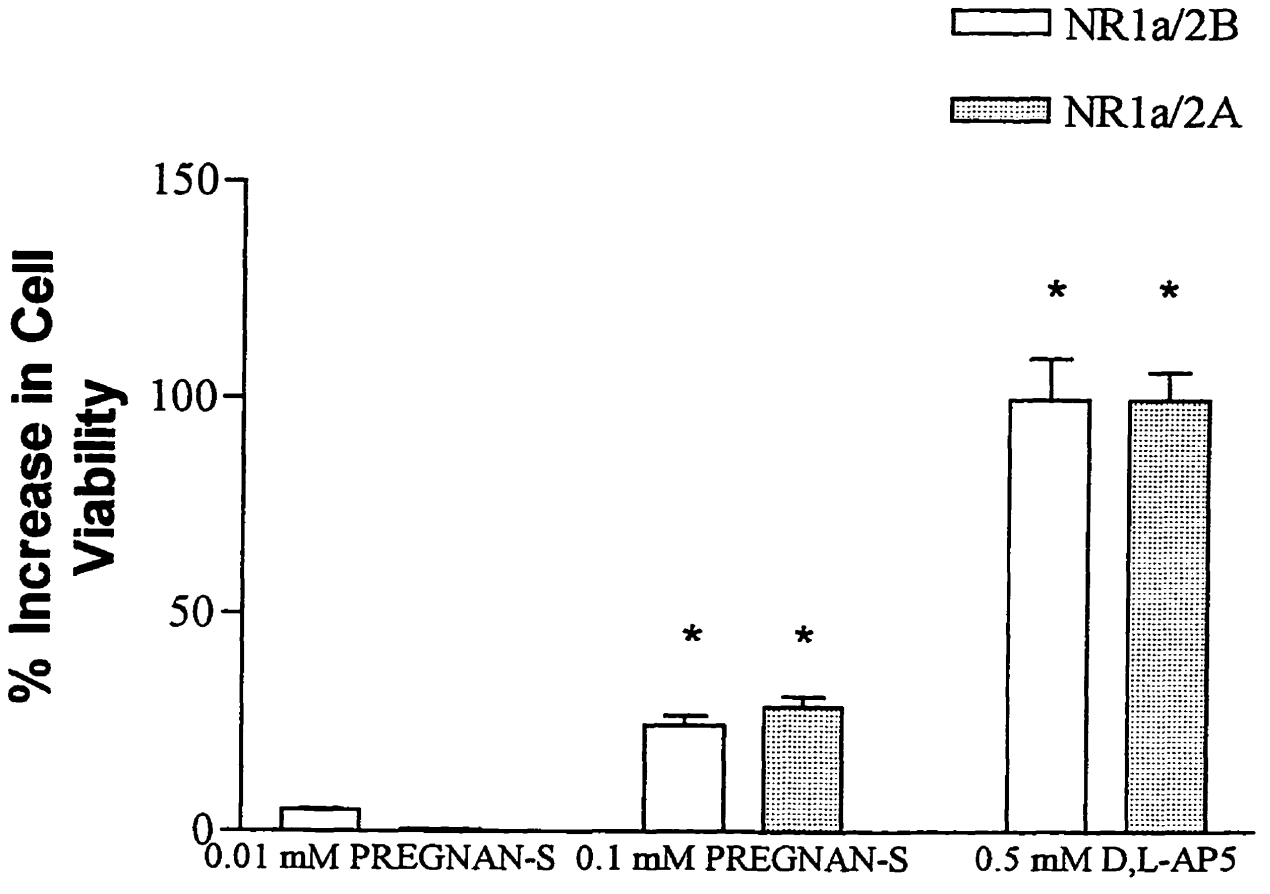


Figure 24. Concentration dependence for the effect of pregnanolone sulfate (PREGNAN-S) on NMDA receptor mediated toxicity in NR1a/2A and NR1a/2B expressing fibroblasts. Data presented as mean ± SD, N=3 carried out in 3 sister cultures, of % increase in cell viability relative to DMEM treatment, after receptor induction (Figure 20). * = significantly different from 0.01 mM PREGNAN-S using 1-way ANOVA and Newman-Keuls post-hoc test.

Figure 25

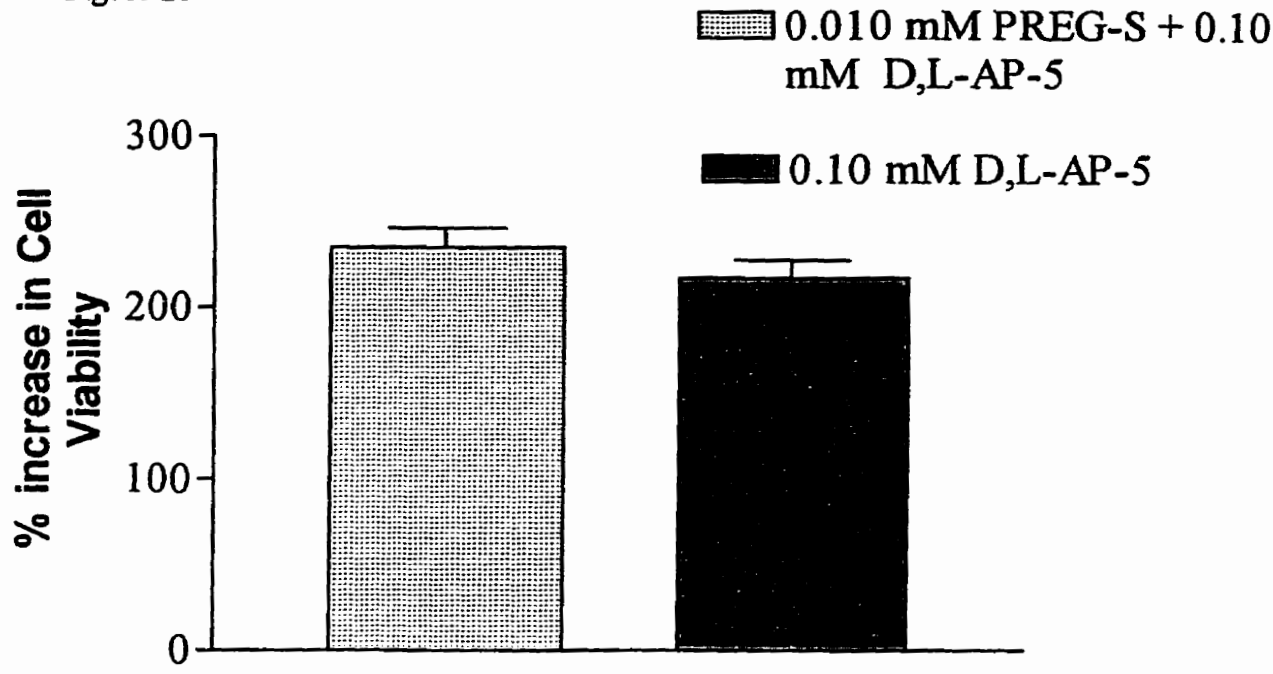


Figure 26

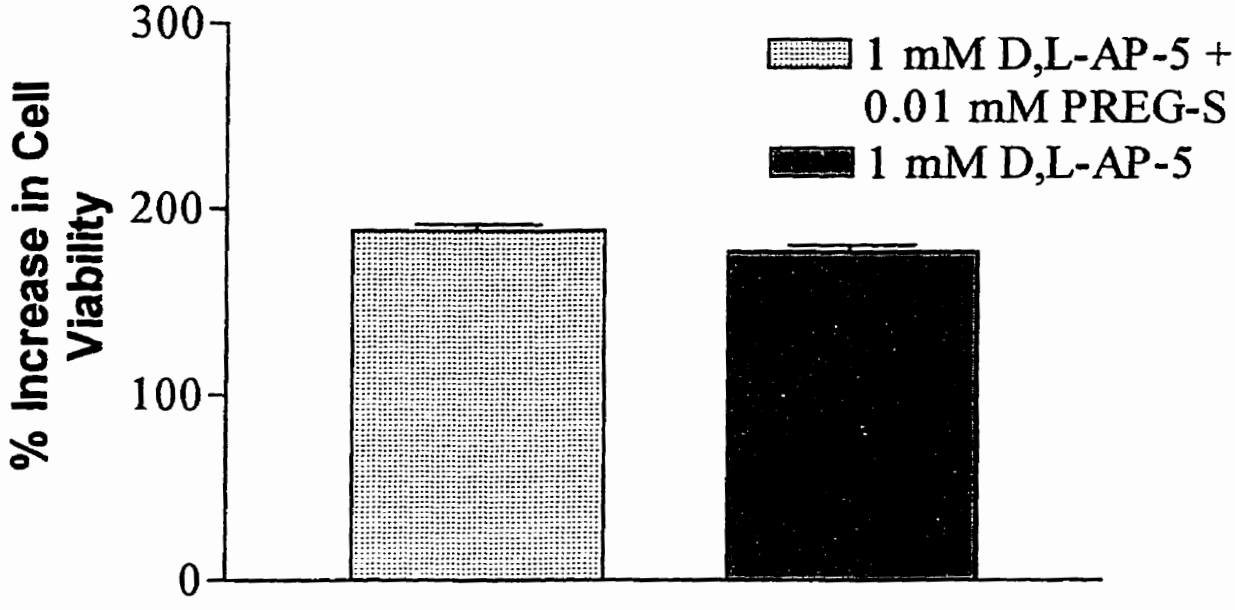


Figure 25, 26. Effect of pregnenolone sulfate (PREG-S) on NMDA receptor-mediated toxicity in NR1a/2A (Figure 25) and NR1a/2B (Figure 26) expressing fibroblasts. Data presented as mean ± SD, N=3 carried out in 3 sister cultures, of % increase in cell viability relative to DMEM treatment, after NMDA receptor induction (Figure 20).

Chapter 4

General Discussion

The main objectives of this study involve: (i) the establishment of in vitro models of NMDA receptor mediated excitotoxicity and (ii) the investigation of toxicity modulation by the neurosteroids PREGNAN-S and PREG-S. While the development of the mouse fibroblast model was accomplished with few problems, development of the primary culture models was not completely accomplished. Primary cultures of hippocampal neurons were cultivated, and were shown to elicit NMDA and L-glutamate-mediated excitotoxicity. However, the lack of reproducibility in the primary rat hippocampal culture model presented a problem and this factor prevented the evaluation of PREG-S and PREGNAN-S modulation of NMDA excitotoxicity. Subsequently, murine hippocampal cultures, generated in Dr. John Macdonald's laboratory at the University of Toronto, were used as an alternate primary culture model of excitotoxicity. Unfortunately, neurosteroid modulation of NMDA excitotoxicity in the murine model was difficult to establish, due to marked toxicity of the vehicle. In contrast to the primary culture models, the mouse fibroblast model exhibited reproducible NMDA receptor mediated cell death, which permitted the evaluation of PREG-S and PREGNAN-S modulation of NMDA excitotoxicity. The two neurosteroids were thus selected for examination on the basis that they are both capable of modulating NMDA channel conductance. Specifically, PREG-S positively modulates conductance, while PREGNAN-S negatively modulates conductance.

4.1 Primary Culture Immunohistochemistry

Immunohistochemical detection of neurons and glia, using antibodies which were capable of selectively staining neurons and glia in rat brain tissue slices ,was difficult to establish in primary culture. Cell cultures contained cells which resembled, morphologically, both neurons and glia. For example, cells with triangular cell bodies (characteristic of certain hippocampal neurons), were observed to grow over a confluent layer of cells which resembled glia. However, immunohistochemical identification of the two cell types was not accomplished. Several modifications were made to the immunostaining protocol in an attempt to: (i) achieve GFAP glial staining without background neuronal staining and (ii) to improve NFP staining of neurons. However, none of these improved GFAP or NFP staining. For example, increasing the time of the initial peroxidation period did not reduce background neuronal staining in GFAP stained cultures. This suggests that endogenous peroxidase activity in the cultured neurons did not contribute to their non-specific staining. Furthermore, reducing the secondary antibody concentration in GFAP stained cultures from 1:200 to 1:500 had no effect on background neuronal staining. This observation suggests that the primary GFAP antibody was cross-reacting with neuronal protein, as the absence of primary anti-GFAP resulted in the complete loss of neuronal or glial staining. There were also difficulties encountered in staining neurons for NFP. The only alteration made to the NFP staining protocol to improve neuronal staining was the reduction in primary antibody concentration to eliminate possible stearic hindrance and inhibition of primary antibody binding to the NF epitoipe. Unfortunately, this change also

produced no improvement in NF staining of neurons. In conclusion, the GFAP and NF primary antibodies used in these studies could not specifically stain neurons and glia in vitro. However, in future work, other primary antibodies could be investigated to characterize the relative presence of neurons and glia in culture.

4.2 NMDA Receptor- Mediated Toxicity in Rat Hippocampal Cultures

Characterization of NMDA receptor mediated excitotoxicity in primary rat hippocampal cultures was difficult to achieve. While reproducible measurement of excitotoxicity was not possible, viable cells could be maintained in culture for 12 DIV. Alterations made in the initial culture conditions considerably improved cell viability during the duration of the culture as indicated by visual inspection of the cultures. The most important modification to the culture conditions was the elimination of cell culture media change after 7 DIV. Adding new media at this time period significantly reduced the number of neurons present. It is quite possible that high levels of glutamate contained in the animal sera, a component of the tissue culture media, could produce excitotoxicity in cultures expressing NMDA receptors. In fact, glial conditioning of serum-containing medium reduces toxicity of the serum when used in neuronal cultures, possibly due to removal of L-glutamate (Ye and Sontheimer 1998).

Using the modified culture conditions, which were similar to those of Xiong and MacDonald (1997), viable neurons could be prepared to characterize NMDA excitotoxicity. One such experiment, shown in chapter 3, indeed demonstrated NMDA excitotoxicity . However, this experiment was not representative of other excitotoxicity assays carried out using the rat

hippocampal model. Specifically, most control cultures did not survive the 24 hour DMEM-base incubation, and exhibited levels of LDH release similar to those seen with NMDA exposure. Thus, these cultures were not considered suitable for investigations of the NMDA-mediated excitotoxicity and its modulation by neurosteroids.

4.3 NMDA Receptor-Mediated Excitotoxicity in Mouse Hippocampal Cultures

Owing to problems with the rat primary hippocampal cultures, another primary culture model of excitotoxicity was investigated. Specifically, the primary mouse hippocampal cultures grown in Dr. John MacDonald's laboratory at the University of Toronto were employed. These cultures had been used previously in the electrophysiological investigation of the NMDA receptor, suggesting that they expressed this receptor and thus could be used to study excitotoxicity. Indeed, investigation of these cultures showed that they exhibited glutamate mediated excitotoxicity, suggesting that they constitute a good model with which to investigate the modulatory effects of neurosteroids on NMDA receptor mediated toxicity. However, the maximum toxicity elicited by 0.5 mM NMDA (31% release of total LDH activity) was less than that seen with 0.5 mM glutamate, which produced a 69% release of total LDH activity. One possible reason for this discrepancy may be that cultures prepared for the examination of NMDA mediated excitotoxicity contained less neurons than did the cultures prepared for the glutamate toxicity assays. Subsequently, the percent release of total LDH enzyme activity would be lower, since it is only neurons that die and release LDH enzyme in glutamate and NMDA mediated excitotoxicity. (Koh et al. 1988). (LDH activity released (neurons)\ Total LDH activity (neurons

+ glia) = % release of total LDH activity). Although, the magnitude of LDH release was less than that seen with glutamate toxicity, NMDA mediated excitotoxicity in this culture system was more reproducible than in the rat model.

4.4 Modulation of NMDA-Receptor Mediated Excitotoxicity by Pregnenolone Sulfate and Pregnanolone Sulfate

Using the previously described primary mouse hippocampal culture model, the ability of PREG-S and PREGNAN-S to modulate NMDA excitotoxicity was investigated. Unfortunately, the potential modulatory effects of the neurosteroids were obscured due to the toxicity of the drug vehicle, cyclodextran. A 20% cyclodextran solution produced toxicity similar to that of 0.05 mM NMDA, a concentration of the agonist against which the modulatory effects of the neurosteroids were to be evaluated . The highest concentration of PREG-S (0.1 mM) contained 20% cyclodextran, and produced a significant increase in NMDA excitotoxicity. However, it is uncertain whether PREG-S itself modulated NMDA excitotoxicity, or if the vehicle potentiated the NMDA toxicity. Concentrations of 0.01 and 0.001 mM PREG-S also positively modulated NMDA receptor dependant toxicity. However, even though these steroids were solvated in 10% cyclodextran, this concentration may have produced toxicity on its own. A 10% cyclodextran control was not used in these series of experiments, as the vehicle toxicity was not anticipated. Because of this complication with vehicle toxicity, the modualtory effect of PREG-S could not be established. Performing further studies with vehicle controls of 10% and 20% cyclodextran, and solvating NMDA in these different vehicle concentrations would allow appropriate

comparison of the PREG-S modulated NMDA mediated toxicity to the NMDA toxicity alone. Alternatively, dimethyl sulfoxide (DMSO) could be used as a steroid vehicle, as it exhibited no toxicity in the subsequent fibroblast model of NMDA excitotoxicity (see below). However, there was no opportunity to pursue these studies further, as only one set of cultures could be prepared in the MacDonald laboratory.

PREGNAN-S modulation of NMDA mediated toxicity was less ambiguous. PREGNAN-S reduced NMDA mediated toxicity from 28 % of total LDH release to 17% of total LDH release, a level which is comparable to that seen with exposing the culture to DMEM-base alone. In this case, vehicle toxicity may have been avoided, as the 0.05 mM PREGNAN-S was solvated in 10% cyclodextran. As mentioned previously a 10% cyclodextran control was not included in the study to validate this assumption. The possibility that PREGNAN-S protects against NMDA toxicity is strengthened by the report that pregnanolone hemmisuccinate, a synthetic analogue of PREGNAN-S, reduces cell death by 15 to 20% (Weaver et al. 1997) at a concentration of 0.01 mM. In summary, pregnanolone sulfate appears to reduce NMDA receptor mediated excitotoxicity, while pregnenolone sulfate possibly increases NMDA receptor mediated toxicity.

4.5 Mechanism of Pregnanolone Sulfate and Pregnenolone Sulfate Modulation of NMDA Toxicity

The mechanism of action of PREG-S and PREGNAN-S in the modulation of NMDA mediated excitotoxicity possibly involves modulation of $GABA_A$ receptor function as well as

modulation of the NMDA receptor. For example, PREG-S reduces $GABA_A$ receptor activity by reducing the frequency of channel opening, at micromolar concentrations (Meinville and Vicini, 1989), while at similar concentrations, PREGNAN-S potentiates $GABA_A$ receptor activation (Belleli et al. 1994). It is possible that an action at the $GABA_A$ receptor may contribute to the protective effects of PREGNAN-S and the enhancement of NMDA induced toxicity by PREG-S.

Both PREG-S and PREGNAN-S have been shown to directly modulate NMDA receptor channel conductance. While PREG-S increases channel conductance, PREGNAN-S decreases conductance through interactions at sites which are autonomous from other modulatory sites on the NMDA receptor (Park-Chung et al. 1997). For this reason, it is highly probable that direct modulation of the NMDA receptor is important in the modulation of NMDA excitotoxicity. Furthermore, the direct actions of PREG-S and PREGNAN-S at the NMDA receptor compliment their actions at the $GABA_A$ receptor. In this way, for example, PREGNAN-S positive modulation of the GABA receptor summates with its direct inhibition of the NMDA receptor to prevent neuronal depolarization and excitotoxicity.

Modulation of the NMDA receptor by any mechanism other than a direct action or action through the $GABA_A$ receptor is doubtful. For example, PREG-S has no activity at the sigma receptor, unlike DHEA-S which positively modulates the NMDA receptor by binding to and activating the sigma receptor (Debonnel et al. 1996). Modulation of the NMDA receptor and NMDA receptor mediated toxicity by genomic actions of the neurosteroids is also unlikely. PREG-S and PREGNAN-S are both capable of binding to the progesterone receptor, after conversion back to progesterone inside the neuron. (Robel et al. 1994). However, progesterone

has not been shown to be neuroprotective in the glutamate-mediated excitotoxicity model (Singer et al. 1996), suggesting that progesterone produced from PREG-S and PREGNAN-S is not modulating the NMDA receptor function.

4.6 Characterization of the Mouse Fibroblast Model of Excitotoxicity

In contrast to the technical problems observed with the primary culture system, the mouse fibroblast model of excitotoxicity was successfully established. Measurement of toxicity was performed using the acid phosphatase assay, which permitted reproducible quantitation of viable cells in culture. This method was determined to be more effective in the fibroblast model system than the LDH assay, which was used in assessing excitotoxicity in primary culture. Using the acid phosphatase assay, both the NR1a/2A expressing cells and NR1a/2B expressing cells were shown to exhibit spontaneous cell death in the absence of NMDA receptor antagonist. These results confirm observations made by Grimwood et al. (1996), that mouse fibroblasts expressing NMDA receptors undergo excitotoxic cell death when NMDA receptor antagonists are absent from the culture medium. Pharmacological characterization of the recombinant NMDA receptors was also performed by Grimwood et al.(1996). They observed that antagonism of NMDA receptor conductance by D,L-AP-5 was dependent on subunit composition. For example, the dissociation constant (K_D) for D,L-AP-5 at the NR2A containing receptors was two-fold lower than that observed at the NR2B expressing receptors. Therefore, the binding affinity for AP-5 at the NR2A receptor is greater than that at the NR2B receptor. Additionally, it was determined that ketamine, a non-competitive NMDA receptor antagonist

exhibited no difference in K_D between the two receptor populations. In the present study, the differences in D,L-AP-5 and ketamine at the NR2A and 2B receptors was manifested in their ability to modulate NMDA receptor mediated excitotoxicity. D,L-AP-5 exhibited greater potency at the NR2A subunit containing receptor than at the NR2B containing receptor, while ketamine was equipotent at both types of receptor. The ability of D,L-AP-5 to prevent cell loss suggests that this toxicity is due to selective activation of NMDA receptors by endogenously released excitatory amino acids. Indeed, preliminary results from our laboratory show that high levels of glutamate and glycine (a known NMDA receptor co-agonist) are released by the fibroblasts into the culture media (data not shown).

Based on these observations from our laboratory, it would appear that the mouse fibroblast model is ideal for examining the modulation of NMDA receptor-dependent cell death by the two neurosteroids, pregnanolone sulfate (PREGNAN-S) and pregnenolone sulfate (PREG-S). In primary culture, modulation of NMDA receptor mediated toxicity by PREG-S and PREGNAN-S possibly results indirectly from the action at $GABA_A$ receptors and directly from actions at the NMDA receptor, as these neurosteroids interact with both classes of receptors. In contrast, in the mouse fibroblast model, which does not express $GABA_A$ receptors, modulatory effects of PREG-S and PREGNAN-S on NMDA receptor mediated cell death would only result from a direct action of steroid at the NMDA receptor.

4.7 Pregnenolone Sulfate and Pregnanolone Sulfate Modulation of NR1a/2A or NR1a/2B Subunit Containing NMDA Receptors.

The effect of PREG-S and PREGNAN-S on NMDA receptor conductance has been extensively investigated by Park-Chung et al.(1997). In chick spinal cord neuronal cultures, PREGNAN-S decreased single channel conductance, PREG-S had an inverse effect, increasing single channel conductance in chick spinal cord neurons. These findings suggest that PREGNAN-S and PREG-S should modulate NMDA receptor-mediated toxicity in the mouse fibroblast model in opposite fashion. In this study, PREGNAN-S negatively modulated the NMDA receptor dependent cell death, however, PREG-S had no effect on cell viability. At the single dose used here, there was no difference between the action of PREGNAN-S on fibroblasts expressing the two type of NMDA receptors. However, such a difference may be revealed by dose response studies, as is the case for the D,L-AP-5 experiments. A single dose comparison suggests that the steroid modulatory site is present on both receptor isoforms.

In contrast with PREGNAN-S, PREG-S produced no modulation of NMDA excitotoxicity. According to reported studies (Park-Chung et al. 1996), PREG-S is a positive modulator of NMDA receptor function, and thus was expected to augment NMDA receptor dependant cell death in the fibroblast model. It could be that the dose of this agent used was insufficient to potentiate cell death. In previous studies, ten-fold higher concentrations have been used to demonstrate potentiation of NMDA receptor currents (Park-Chung et al. 1996). Thus, higher concentrations should be tested, although the dose of PREG-S used in this study approaches its solubility limit in the drug vehicle.

4.8 Summary

Experimental observations from the present research project can be summarized in the following points.

1. Rat hippocampal cultures were successfully generated, and cultivated for 12 DIV.

2. Excitotoxicity was not reproducible in these rat hippocampal cultures. Non specific glutamate toxicity was evident in most cultures, making them unsuitable models for the examination of PREG-S and PREGNAN-S modulation of NMDA mediated excitotoxicity.

3. Mouse hippocampal cultures displayed reproducible NMDA mediated excitotoxicity. It was determined, using this culture system, that PREGNAN-S negatively modulates NMDA receptor mediated excitotoxicity. PREG-S may positively modulate NMDA receptor mediated toxicity in this system, however, vehicle toxicity may account for the modulatory effect.

4. Murine fibroblasts expressing either the NR1a/2A or NR1a/2B receptor subunits exhibited NMDA receptor mediated excitotoxicity, which was antagonized by the competitive antagonist D,L-AP-5 and the non competitive antagonist ketamine.

5. In the fibroblast culture model of excitotoxicity, PREGNAN-S reproducibly potentiated NMDA receptor induced excitotoxicity, while PREG-S had no effect on NMDA receptor induced excitotoxicity.

4.9 Future Studies.

Further characterization of the modulation by PREG-S and PREGNAN-S of NMDA excitotoxicity must be pursued. As mentioned previously, vehicle toxicity might be avoided by utilizing DMSO as the vehicle. Such experiments would unequivocally show whether PREG-S positively modulates NMDA toxicity or whether PREGNAN-S negatively modulates this toxicity. Furthermore, PREG-S modulation of NMDA toxicity should be investigated in the mouse fibroblast model using concentrations of neurosteroid in excess of 0.01 mM. At such concentrations, PREG-S significantly potentiates the NMDA receptor conductance, and thus should positively modulate NMDA excitotoxicity.

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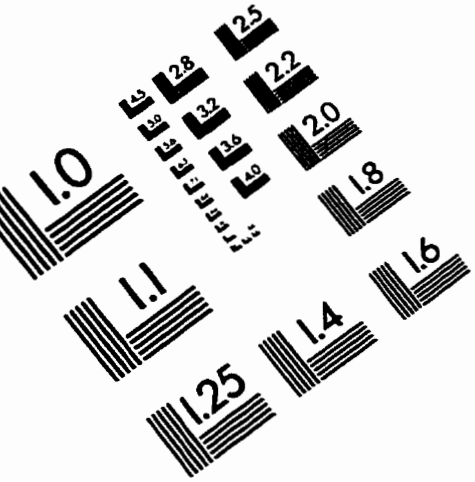
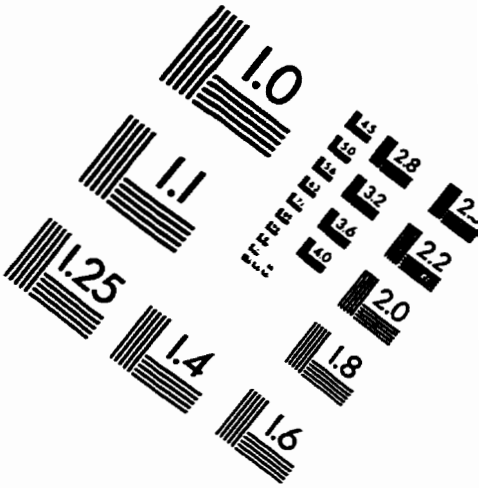
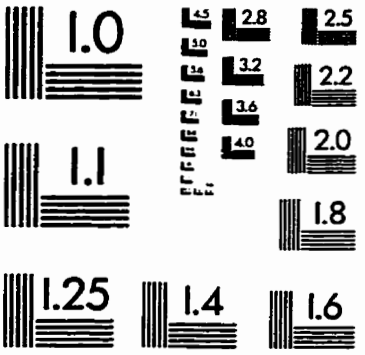
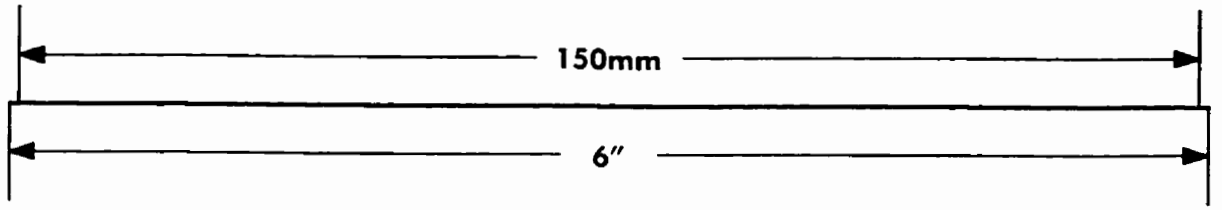
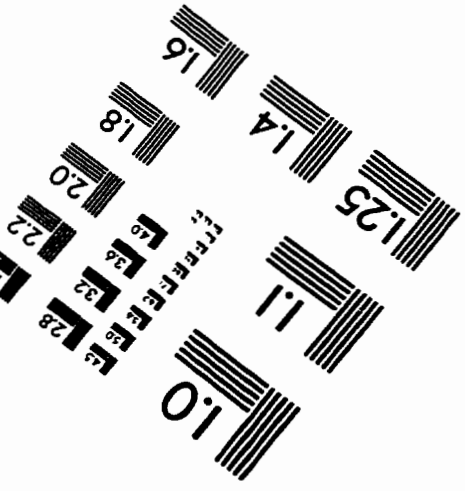
IMAGE EVALUATION TEST TARGET (QA-3)

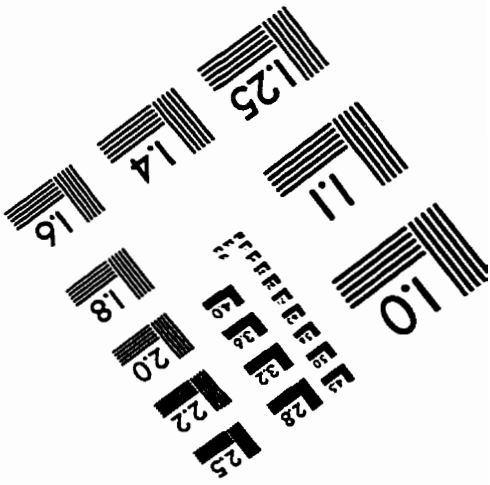












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