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A model of acute glutamate- and domoate-induced toxicity was developed and characterized in cultured rat cerebellar granule cells (CGCs) using experimental conditions which preserve the voltage-dependency of NMDA receptor function. Glutamate, which is normally non-toxic to CGCs in physiologic media (pH 7.4), was shown to induce a cytotoxic response after 2 hours when the exposure temperature was reduced from 37° to 22°. Pharmacological characterization of this response demonstrated that cytotoxicity is mediated by the activation of NMDA receptors, while non-NMDA receptors produce a depolarizing stimulus that enhances release of the voltage-dependent Mg^{2+} blockade of NMDA receptor ion channels. Reduced temperature was shown to facilitate NMDA receptor activation by compromising the ability of CGCs to maintain normal electrochemical gradients during glutamate-induced ion flux. When compared to glutamate, the non-NMDA receptor agonist, domoate, demonstrated an acute cytotoxic response in CGCs that was also mediated predominantly by NMDA receptors. NMDA receptor activation was produced secondary to a domoate-induced release of glutamate and aspartate from CGCs; therefore, domoate synergistically potentiates glutamate/aspartate-mediated neurotoxicity. Domoate-

induced excitatory amino acid (EAA) release was investigated and found to occur almost exclusively through reversal of the high affinity Na+-coupled glutamate transporter and by osmoregulatory mechanisms. CGCs also responded to domoate-induced depolarization by releasing adenosine which suppresses exocytotic EAA release through A1 receptor activation.

The functional and pharmacological characteristics of NMDA receptors were characterized in 12 DIC CGCs using the channel blocking compound [^{3}H]MK-801 (dizocilpine). Kinetic analysis of [^{3}H]MK-801 binding indicated the possible existence of at least two NMDA receptor populations on 12 DIC CGC membranes, and the equilibrium competition binding of MK-801 and other channel blocking compounds was consistent with the presence of high and low affinity binding sites. The neuroprotective potencies of NMDA receptor channel blockers correlated significantly with their affinities for the NMDA receptor derived from equilibrium competition analysis of [^{3}H]MK-801 high-affinity binding. Thus, whereas 12 DIC CGCs express a pharmacologically heterogeneous population of NMDA receptors, it is the high-affinity component of [^{3}H]MK-801 binding that mediates glutamate toxicity.

Development and Characterization of a Model of Glutamate and Domoate Toxicity in Cultured Rat Cerebellar Granule Neurons

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Chapter 1
Introduction

L-glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system (CNS). It excites virtually all central neurons and is present within neuronal synapses at millimolar concentrations (1). In addition to its involvement in rapid excitatory neurotransmission, glutamate plays a central role in synaptic plasticity, an activity-dependent process involved in synapse formation and memory aquisition (2,3), and in the neurodegeneration associated with a variety of acute and chronic neurological disorders (4). The neurotoxic potential of glutamate was first demonstrated in 1957 by Lucas and Newhouse (5), who showed that retinal neurons degenerate after prolonged periods of glutamate exposure. Subsequent experiments by Olney (6,7) established that this toxicity not only applied to glutamate (and retinal neurons), but was an effect common to the actions of a variety of excitatory amino acids on central neurons. This effect, which Olney called excitotoxicity, was postulated to be the cause of neuronal loss that occurs in a variety of neurological diseases (6). Indeed, with the ongoing elucidation of glutamate receptor structure and function, the development of a large pharmacopoeia of glutamate receptor agonist and antagonist drugs, and the establishment of numerous in vivo and in vitro experimental systems; this hypothesis has gained strong support (4). Moreover, the evidence increasingly suggests that glutamate neurotoxicity is involved in slowly progressive neurodegenerative diseases such as Huntington's, Alzheimer's and ALS (8).

Because glutamate has been implicated in a number of important CNS pathologies, the mechanisms by which it produces neurotoxicity have been extensively studied. Less well characterized, however, are the mechanisms associated with the toxicity of a variety of exogenous excitatory amino acids. One such toxin of recent importance to human health is domoic acid, a tricarboxylic amino acid produced by the red seaweed *Chondria armata* that is well known in the Japanese pharmacopoeia for its anthelmintic properties (9) (figure 1.1). In November/December, 1987, domoate was the agent responsible for an unusual intoxication, called amnesic shellfish poisoning (ASP), among people who had eaten mussels (*Mitilus edulis*) grown commercially off Prince Edward Island in Eastern Canada (10,11). In this outbreak, however, domoic acid was found to have been synthesized by the pennate diatom, *Nitzschia pungens* (12). The neurological symptoms of ASP included headache, confusion, nausea, disorientation, seizures, loss of memory, coma and death, with the most serious problems occurring in older patients (10). Autopsy of the brains from four people who died due to domoate toxicity revealed neuronal damage in the amygdala, hippocampus and other limbic areas (11) in a pattern similar to that observed experimentally in animals after the administration of the structurally related analog, kainic acid (figure 1.1) (13,14). Since its first documented appearance in seafood in 1987, domoate has continued to appear not only in cultured mussels, but in other filter feeding species such as clams, crabs and anchovies on both the east and west coasts of the US and Canada (15,16). Moreover, a number of other species of unicellular algae which synthesize domoate have been identified in recent seafood contaminations (17,18).

In this thesis project, cultured rat cerebellar granule neurons were used as an in vitro model to study cellular mechanisms involved in domoate- and glutamate-mediated neurotoxicity. Given the increasing occurrence of domoic acid contamination in shellfish, knowledge of the cellular and biochemical pathways involved in domoate excitotoxicity would be extremely important to the development of therapeutic strategies for future

human intoxications. Moreover, excitatory amino acids such as domoate may be useful tools for the modeling of siezure disorders, inasmuch as the glutamate receptor subtypes with which domoate interacts play an important role in epilepsy (19,20). Most importantly perhaps, an understanding of domoate excitotoxic mechanisms may broaden our understanding of the many neurological diseases thought to involve abnormal glutamate receptor function. The following sections will review glutamate receptor structure and function, the important cellular components involved in glutamate excitotoxicity, and the considerations in selecting cultured cerebellar granule neurons to model domoate and glutamate toxic mechanisms.

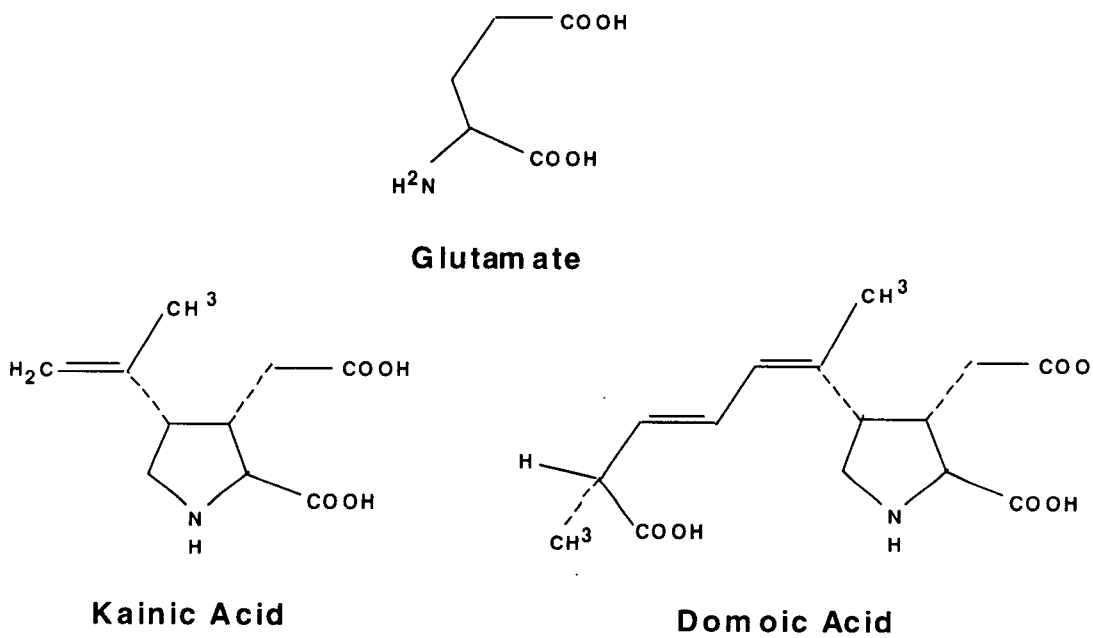

Fig. 1.1 Chemical structure of glutamate, domoate and kainate

1.1 Glutamate Receptors

Glutamate interacts with two major receptor families: ionotropic, ligand gated ion channels and metabotropic G-protein coupled receptors (21). Ionotropic glutamate receptors have been classified pharmacologically into N-methyl-D-aspartate (NMDA) and non-NMDA receptors, with the latter being further differentiated pharmacologically into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate preferring subtypes (AMPA/kainate receptors) (21-23). Physiologically, rapid glutamatergic neurotransmission is mediated by non-NMDA receptors, which in general are selectively permeable to Na^+ and K^+ (24,25). NMDA receptors form Na^+- and Ca^{2+}-permeable ion channels that are activated in both a voltage and ligand dependent manner (21,26,27). Because NMDA receptors are blocked by Mg^{2+} in a voltage dependent manner, calcium influx increases in response to increasing levels of membrane depolarization such as that which occurs during high frequency synaptic activity. In this context, calcium activates processes that are fundamental to long term changes in synaptic strength (2,3). Metabotropic glutamate receptors are coupled to the activation of phospholipase C (PLC) and release of Ca^{2+} from intracellular stores, and to inhibition of adenylyl cyclase (28,29). Thus, metabotropic glutamate receptors can produce both prolonged excitatory and inhibitory effects in the CNS and are involved in numerous neuronal functions including synaptic plasticity and neuronal development (29, 30).

1.1.1 Ionotropic Glutamate Receptors

All ionotropic glutamate receptors exist as oligomeric proteins comprised of several subunits which coassemble to form functional cation channels (21-25). Glycosylation analysis and electrophysiological testing of mutant and chimeric receptors indicate that each subunit contains multiple membrane spanning domains (31-34).

General features of ionotropic glutamate receptor subunit structure include: (i) a large extracellular N-terminal domain comprising approximately one-half of the subunit (21,36-41); (ii) three membrane spanning domains (TM1, TM3 and TM4)(31-35); (iii) a fourth channel-lining reentrant loop (TM2) that enters and exits the cell membrane from the intracellular side (31-35); and (iv) an intracellular C-terminal domain (31-35, 42-44). Other general features include: a Q/R/N site in TM2 which governs the ion permeability characteristics of all ionotropic glutamate receptors (23,24,45-51); amino acid sequences in the extracellular loop segment between TM3 and TM4 and in the N-terminus that share sequence homology with bacterial periplasmic amino acid-binding proteins and which together form the glutamate binding domain of the receptor (33,52); and intracellular phosphorylation consensus sequences (44,57-61).

Non-NMDA receptors. Non-NMDA receptor subunits are encoded by nine different genes, and have been subclassified into three groups based on their sequence similarity and agonist selectivity (23,24). Sequence homology of subunits within each group generally exceeds 80 %, whereas inter-group similarity is low (≤40 %) (39,70). Thus, GluR1 through GluR4 subunits comprise the AMPA-preferring receptors (62). AMPA receptor subunits have been shown to assemble into functional homomeric or heteromeric oligomers with different functional properties (21-24,46,62,63). GluR5 through GluR7 and KA-1/KA-2 subunits constitute low and high affinity kainate receptors, respectively (64-71). KA-1 and KA-2 subunits by themselves do not form functional receptors, but instead combine with GluR5-7 to form high affinity kainate receptors (70,71). Evidence from in vitro studies indicates that both homooligomeric GluR5-GluR7 and heteromeric GluR5-6/KA1-2 channels may exist in vivo, whereas kainate and AMPA subunits do not heterooligomerize (68,70,72,73) .

Non-NMDA receptors display considerable diversity in activation and channel gating characteristics, due largely to the variety of possible receptor subunit combinations

that exist within the CNS; however, a greater level of molecular and functional diversity is created through non-NMDA receptor subunit pre-mRNA editing mechanisms (51). An early discovery was that the genomic sequences of all non-NMDA receptors encode for glutamine (Q) at the Q/R/N site of TM2, whereas arginine (R) is found in virtually all GluR2, and at varying developmentally controlled ratios in GluR5 and GluR6 subunit cDNAs (25,74-76). It is now known that pre-mRNA editing of glutamine codons (CAG) to arginine (CGG) codons occurs by a process of adenosine deamination in GluR2, GluR5 and GluR6 subunits (51,77). In general, it is the presence of this positively charged arginine residue in the channel lining domain that determines the selective permeability of non-NMDA receptors for monovalent cations (74). The absence of arginine renders non-NMDA receptors permeable to both mono- and di-valent cations. Thus, although non-NMDA receptors had originally been thought to selectively conduct monovalent cations, it has now been shown that glutamate can trigger Ca^{2+}-dependent intracellular events directly through Ca^{2+}-permeable AMPA/kainate receptors in some neuronal and glial cells (78,79). Because of the wide distribution of edited subunits, however, the vast majority of CNS neurons contain Na^{+}/K^{+} permeable AMPA and kainate receptors (74).

The molecular complexity of AMPA receptors is increased further by alternative splicing of a functional module of 38 amino acids in one of two sequence versions, termed flip and flop (80), located in the extracellular loop between TM3 and TM4. Coupled with this feature is the presence of an R/G site in GluR2, GluR3 and GluR4, which directly precedes the flip/flop module, and is edited in similar fashion to the Q/R/N site of TM2 (81). Both of these post-transcriptional processing features appear to be active during CNS development for the fine tuning of the kinetic properties of AMPA receptors. Flip versions of AMPA receptor subunits with unedited R/G sites (R is present) are generally more numerous at earlier developmental stages (81). Functionally, homooligomeric AMPA receptors containing the flip module have been shown to

desensitize more slowly than flop-containing receptors (82). Moreover, the timecourse of recovery from desensitization is affected by R/G site editing such that R-containing AMPA receptors recover more rapidly. Thus, developmentally interrelated flip/flop splicing (83) and R/G site editing (81) affect the size and shape of the fast component of excitatory post-synaptic currents and control the ability of neurons to convey rapid trains of synaptic activity.

Among the kainate-preferring non-NMDA receptors, the GluR6 subunit displays additional unique sites for pre-mRNA editing. Two sites, both located in transmembrane segment TM1, are diversified by RNA editing to generate either isoleucine (I) or valine (V) in one and tyrosine (Y) or cysteine (C) in the other TM1 position (23,50). In contrast to AMPA receptor channels, the presence of Q in the TM2 Q/R/N site of GluR6 results in channels with low Ca^{2+} permeability, whereas an R residue produces a higher Ca^{2+} permeability if the I/V and Y/C sites of TM1 are fully edited (V and C residues present). If the TM1 sites are unedited, Ca^{2+} permeability is less dependent on the presence of either Q or R in TM2 (23,50). Thus the Ca^{2+} permeability of kainate receptor channels can vary depending upon editing within both the TM1 and TM2 domains. Moreover, the pairing of RNA editing with specific heteromeric subunit assemblies might be used to regulate and diversify Ca^{2+} permeability properties, since combinations of edited and unedited subunits expressed in vitro appear to create a wide spectrum of Ca^{2+} permeabilities among kainate receptors (23,50).

Finally, alternatively spliced C-terminal sequences have been found in GluR2 and GluR4 subunits (84,85). These long and short molecular forms have been demonstrated in murine brain, however their functional and developmental significance have not yet been studied.

NMDA receptors. NMDA receptors are composed of two distinct types of subunits that are encoded by five separate genes; one type is termed NMDAR1 (NR1) and

the other type is comprised of four subunits termed NMDAR2A to NMDAR2D (NR2A-D) (21,23,24). The mouse homologues have been designated ζ and ε for NR1 and NR2, respectively (36,39). NR1 is capable of assembling as a homooligomer that displays properties characteristic of native NMDA receptors when expressed in *Xenopus* oocytes (21,36). NR2 subunits, however, do not form functional receptors but instead potentiate NMDA receptor-gated currents and confer specific functional properties to NMDA receptors when in heteromeric assembly with NR1. (41,86). Because NR1 subunits are required for the formation of functional NMDA receptors, they are expressed in virtually all neurons of the CNS, whereas NR2 subunits display distinct regional and developmentally regulated expression patterns (38,87). Recent data suggest that NMDA receptors contain at least two different NR2 subunit types in combination with NR1 (88,89). Thus, a large variety of possible NR1/NR2 subunit combinations would account for the regionally heterogenous functional and pharmacologic diversity of NMDA receptors in the CNS (90-93).

The functional and molecular diversity of NMDA receptors is enhanced even further, in a fashion similar to non-NMDA glutamate receptors, by post-transcriptional RNA editing of the NR1 subunit. Differential splicing of three different exons generates eight possible isoforms, seven of which have been identified in vivo (21,40,43,86,94,103). Each isoform is expressed in a regionally and developmentally characteristic fashion (86). One alternatively spliced exon casette, located in the N-terminal extracellular domain (N1), encodes a 21 amino acid sequence, while adjacent 37 and 38 amino acid sequences (C1 and C2) exist in the C-terminus domain. Splicing out the distal C-terminal cassette removes a stop codon, which exposes a new open reading frame that encodes 22 amino acids (C2') before a second stop codon is encountered (43). Thus, NR1 subunit splice variants differ in two regions: the distal end of the amino-terminus domain with two configurations; and the carboxy-terminus with four configurations.

The NMDA receptor-channel complex is distinct among glutamate receptors in that it is highly permeable to Ca^{2+} and blocked in a voltage-dependent manner by Mg^{2+} (21,26,27). These properties underlie the ability of NMDA receptors to mediate the induction of long-term potentiation of synaptic efficacy, a form of activity-dependent synaptic plasticity that underlies learning, memory and CNS development (2,3). The property of Ca^{2+} permeability is conferred to the NMDA receptor-channel complex by an asparagine (N) residue at the TM2 Q/R/N site, which is present in all NMDA receptor subunits (48,49,74). Mutations at the site of the conserved asparagine residue reduce or abolish Ca^{2+}-permeability and have been shown to alter the sensitivity to channel blockade by Mg^{2+} and the prototypical channel blocking drug MK-801 (48,49,74).

In addition to the fundamental properties of Ca^{2+} permeability and voltage-dependent Mg^{2+} blockade, NMDA receptors are modulated by a variety of endogenous and exogenous inhibitory and potentiating agents. The presence of glycine, which binds to a distinct strychnine-insensitive coagonist site, is required for glutamate to activate NMDA receptors (95-98). The glycine binding site has been shown to exist within an extracellular domain that shares sequence similarity with a periplasmic amino acid-binding protein from *Salmonella typhimurium* (33,52). Glutamate and glycine reciprocally enhance one another's affinity for binding to the NMDA receptor (99). NMDA receptors also possess a pharmacologically distinct modulatory site for the binding of Zn^{2+} (94,100). Zn^{2+} at high concentrations inhibits agonist-induced responses, whereas at low concentrations it potentiates responses at certain splice variants of the NMDA receptor (94). The Zn^{2+}-induced potentiating responses appear to occur without increasing NMDA receptor agonist or glycine potency and are mimicked by several heavy metal cations (94). As many as three sites exist on NMDA receptors for the binding of polyamines (94,99,100). Polyamines exert multiple effects on the NMDA receptor, which include an increase in the magnitude of agonist-induced currents in the presence of saturating concentrations of glycine, an increase in the affinity of the receptor for glycine,

and voltage-dependent inhibition (101). These effects vary depending on the specific subunit composition of the NMDA receptor. Several extracellular redox-sensitive amino acid residues modulate receptor activation properties (53-56). Overlapping the polyamine sites within the lumen of the cation channel are sites for non-competitive NMDA receptor antagonist drugs such as phencyclidine (PCP), which block NMDA-induced currents in a voltage- and use-dependent manner (49,90-93).

Electrophysiological experiments on recombinantly expressed NMDA receptors indicate that each of the NR1 splice variants is capable of assembling into functional homomeric channels (36,40,41,44,57,59,94,102), and each variant differs with respect to agonist affinity, current amplitude, modulation by Zn^{2+}, potentiation by polyamines and regulation by PKC. For example, NR1 homomers containing the N1 cassette ($NR1_{1XX}$)[1] generally produce larger mean current amplitudes but have a lower affinity for glutamate than do receptors without the N1 cassette (40,102). Moreover, Zn^{2+} and spermine potentiate responses at $NR1_{0XX}$ but not $NR1_{1XX}$ homomers (40,94,102) The potentiating effect of PKC phosphorylation appears to be modulated by both the N- and C-terminal cassettes (57,59). Interestingly, PKC phosphorylation has been shown to occur predominantly on serine residues located entirely within the C1 cassette, which suggests that sensitivity of the NMDA receptor to phosphorylation can be regulated through alternative splicing of the C-terminal domain (44).

Various binary combinations of NR1 and NR2 subunits have been recombinantly expressed and investigated for their functional properties as well. In general, many of the functional and pharmacologic characteristics of native NMDA receptors are reproduced by these combinations. For example, NMDA-activated currents in *Xenopus* oocytes

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[1]At this time, a unified nomenclature for the various NR1 isoforms has not been established. The eight splice variants have been denoted by Durand, et al. (1993) with subscripts which indicate the presence (1) or absence (0) of the three alternatively spliced exons from the 5' to 3' end. Thus, $NR1_{011}$ designates a subunit that lacks the N1 but has both C1 and C2 cassettes. An 'x' indicates that the presence of an exon is indeterminate.

expressing NR1 and NR2B or NR1 and NR2C subunits have been demonstrated to be 2 to 25 times smaller than those produced by recombinantly expressed NR1-NR2A subunits (38,39), and the affect of Mg^{2+} to block inward currents is weaker in NR1-NR2C and NR1-NR2D as compared to NR1-NR2A and NR1-NR2B heteromers (103). Moreover, the rate of current decay is 3 to 30 times more rapid for NR1-NR2A receptors than for any of the other heteromeric subunit combinations (38,103). Regarding ligand affinities, NR1-NR2A and NR1-NR2B recombinants display a higher affinity for glutamate than NR1-NR2C recombinants, whereas the latter displays up to ten-fold higher glycine affinity (104). A similar profile exists for the channel blocking compound, MK-801, in which the affinity at NR1-NR2A and NR1-NR2B receptors has been demonstrated to be as much as thirty times higher than for NR1-NR2C recombinants (88,89,104). Thus, recombinant NMDA receptors reproduce many of the functional characteristics of native NMDA receptors found in brain regions where these subunits have been detected.

1.1.2 Metabotropic glutamate receptors

To date, eight different metabotropic glutamate receptors (mGluR 1-8) have been identified and numbered according to the order in which their cDNA's were cloned (28). mGluRs have been classified into 3 groups with approximately 70% intra-group and 45% inter-group sequence identity (21,28). Group I receptors, comprised of mGluR 1 and mGluR 5, are coupled through PLC to the hydrolysis of inositol phospholipids, yielding inositol triphosphate (IP3) and diacylglycerol (DAG). Thus Group I metabotropic glutamate receptors mobilize intracellular Ca^{2+} and stimulate protein kinase C activity (21,28,105). Group II (mGluR 2-3) and III (mGluR 4,6-8) metabotropic glutamate receptors are negatively coupled to adenylate cyclase through inhibitory G-proteins (28,105). Interestingly, although mGluRs couple to the same G-proteins as other members of the G-protein coupled receptor superfamily, they do not share sequence similarity with

other G-protein coupled receptors (106). Instead, mGluRs appear to be more closely related to a Ca^{2+}-sensing receptor recently isolated from bovine parathyroid gland (107). Metabotropic glutamate receptors therefore define a new family of G-protein coupled receptors.

1.2 Mechanisms of Neuronal Excitotoxicity

1.2.1 Glutamate Toxicity

Of all excitatory amino acids (EAAs) known to produce toxic effects in the central nervous system, the neurotransmitter glutamate has been the most extensively characterized because of its involvement in CNS pathologies associated with hypoxia, ischemia, trauma, hypoglycemia and epilepsy (4). As previously described, rapid glutamatergic neurotransmission is mediated by non-NMDA receptors (AMPA/kainate receptors) (1-3), whereas NMDA receptors serve as coincident detectors requiring concurrent depolarizing stimuli, such as that which occurs during high frequency synaptic activity, to relieve the voltage-dependent Mg^{2+} blockade of the ion channel (21,24). In this physiologic context, NMDA receptors increase cytoplasmic Ca^{2+} concentrations at discreet, active synapses, and thereby activate processes fundamental to long term changes in synaptic strength (2,3). In certain pathological conditions, however, excessive glutamate receptor stimulation induces large and sustained NMDA receptor-mediated increases in cytoplasmic Ca^{2+} concentration that ultimately cause neuronal death (108-110).

Normally, extracellular glutamate concentrations rise to high levels only briefly, and in a spatially localized manner. This occurs because glutamate is rapidly removed from the extracellular space by a high affinity Na^+-coupled glutamate transporter, which is present in both neurons and glia (111-114). Three Na^+ are co-transported inward with

each glutamate molecule against one outwardly transported K^+ for a net influx of one positive charge (114). Thus, the glutamate transporter is indirectly driven by Na^+/K^+ ATPase, which utilizes approximately 60% of available cellular ATP to maintain neuronal transmembrane electrochemical gradients (115). The efficiency of glutamate reuptake is high enough that extremely large doses of intracerebrally injected glutamate are required to induce CNS lesions (116). During acute CNS pathologies, however, disruptions in oxygen or glucose availability deprive neurons and glia of the energy required to sustain normal Na^+/K^+ ATPase activity (117-119). The resultant neuronal depolarization increases synaptic glutamate release and impairs or even induces a reversed mode of glutamate transporter function, with the consequence that neurons incur prolonged exposures to high concentrations of extracellular glutamate. (120-123).

In neurotoxicological experiments utilizing dissociated hippocampal and cerebral cortical neuronal cultures, brief exposures to elevated glutamate concentrations have been shown to produce two distinct phases of neuronal injury (4,124). An early phase, mediated by the depolarization-dependent influx of Na^+, Cl^- and water, manifests as a rapidly developing neuronal swelling that is reversible if either glutamate concentration or exposure duration are limited (125,126). This early component has been linked to the activation of AMPA/kainate receptors, inasmuch as non-NMDA receptor antagonists are able to prevent this early phase of glutamate toxicity. A later phase of glutamate-induced excitotoxicity is associated with the excessive and unregulated influx of Na^+ and Ca^{2+} through NMDA receptor ion channels, which also induces a mild early neuronal swelling (125-127), but is primarily characterized by a slowly developing neuronal degeneration that occurs within hours of glutamate exposure (108,119). This delayed component of glutamate-induced injury can be completely prevented by the presence of NMDA receptor antagonists or by the removal of extracellular Ca^{2+} from the exposure buffer (126). Subsequent experiments have directly linked $^{45}Ca^{2+}$ accumulation to the resultant delayed neurodegeneration observed after glutamate exposure in cortical neurons (108). Thus,

these data show that glutamate-induced neurodegeneration is produced by prolonged, generalized increases in cytoplasmic calcium which accumulates subsequent to excessive NMDA receptor activation (117-119).

Not all neurons undergo an excitotoxic response when exposed to exogenously applied glutamate, however. Experiments utilizing cultured cerebellar granule cells (CGCs) indicate that even high concentrations of glutamate are non-toxic when exposure occurs in a physiologic milieu. CGCs apparently have the ability to maintain ionic homeostasis in the face of ion flux induced by glutamate stimulation (118,128,129). Non-physiologic conditions which simulate those occurring in CNS pathologies, such as depletion of neuronal energy reserves (117,118) or depolarization induced by K^+ or veratridine (130-132), are therefore required to produce glutamate-induced neurodegeneration in CGCs. Glutamate toxicity is also facilitated in CGCs by the omission of Mg^{2+} from the exposure medium (133,134). Thus, a critical element in the transformation of glutamate from neurotransmitter to neurotoxin is the ability to produce a depolarizing stimulus of sufficient magnitude to induce release of the voltage-dependent Mg^{2+} blockade of NMDA receptor ion channels (117,118,129) The ability to maintain membrane potentials is therefore an important determinant of neuronal survival, for example, in the penumbra of a stroke focus where oxygen availability and neuronal energy reserves are partially compromised.

As mentioned previously, the delayed neuronal degeneration that occurs subsequent to pathologic NMDA receptor activation is mediated by sustained increases in free cytosolic Ca^{2+} (108,126,132) Homeostatic mechanisms which normally control $[Ca^{2+}]_i$ may therefore be overloaded or compromised during cerebral pathological states. These mechanisms include: Ca^{2+} extrusion by plasma membrane Ca^{2+}-ATPase (135) and the Na^+/Ca^{2+} exchanger (136), Ca^{2+} buffering by Ca^{2+}-binding proteins (137), and Ca^{2+} uptake into endoplasmic reticulum (138) and mitochondria (139-142). Of the Ca^{2+} extrusion mechanisms, Ca^{2+}-ATPase is a high affinity, low capacity system (142),

whereas the Na^+/Ca^{2+} exchanger manifests lower affinity but possesses a much higher capacity for Ca^{2+} extrusion (142). Therefore, the Na^+/Ca^{2+} exchanger is likely the major route for extrusion of the micromolar levels of $[Ca^{2+}]_i$ that accumulate during intense glutamate receptor stimulation (143,144). In cultured cerebellar granule neurons, cytosolic Na^+ concentrations have been estimated to increase to as high as 60-100 mM during excitotoxic glutamate exposures (144,145). Alterations of this magnitude in the Na^+ gradient are known to impair or even reverse Na^+/Ca^{2+} exchanger function (142); therefore, a net Ca^{2+} influx may actually occur through the Na^+/Ca^{2+} exchanger during toxic glutamate exposures. With Ca^{2+} extrusion mechanisms impaired, cytosolic Ca^{2+} must instead be handled through buffering and sequestration mechanisms. Of these two mechanisms, mitochondrial Ca^{2+} uptake has been shown to play a dominant role in calcium buffering during excitotoxic glutamate exposures (110,146). Although mitochondrial metabolism is stimulated by the physiological $[Ca^{2+}]_i$ increases encountered during normal cellular functioning, prolonged exposures to high $[Ca^{2+}]_i$ are known to uncouple electron transfer from ATP synthesis (147,148) and induce free radical production (149). Ca^{2+}-induced free radicals oxidatively damage mitochondria (and other cellular components), ultimately reducing neuronal energy reserves (109,110,150) and impairing active Ca^{2+} extrusion and sequestration mechanisms. Thus, early mitochondrial dysfunction plays a key role in glutamate neurotoxicity (110).

In addition to inducing oxidative mitochondrial damage and inhibiting energy metabolism, sustained elevations in cytoplasmic Ca^{2+} are thought to produce cytotoxicity through mechanisms which reflect disruption of intracellular processes normally regulated by Ca^{2+} availability (151). Several simultaneous injury processes are therefore likely to occur. Among these processes, activation of catabolic enzymes may play a major role. For example calpain, a neutral protease, is involved in the enzymatic cleavage of several structural proteins including tubulin, microtubule-associated proteins, spectrin and neurofiliment polypeptides (152). Calpains have been shown to be involved in

postischemic neuronal degeneration in the hippocampus (153-155). Moreover, the involvement of proteases in excitotoxicity is supported by the suppressive effects of protease inhibitors, such as calpistatin, on postischemic hippocampal damage (156,157). Elevated cytosolic Ca^{2+} may also activate phospholipases, resulting in cell membrane degradation and liberation of arachidonic acid (158). Arachidonic acid metabolism by oxidases leads to the production of oxygen free radicals and peroxidative degradation of lipids and other cellular elements (159). Oxygen free radicals are also generated subsequent to the conversion of xanthine dehydrogenase to xanthine oxidase by a Ca^{2+}-activated protease (109,160). Furthermore, sustained cytosolic Ca^{2+} elevations produce neurodegeneration through the activation of nitric oxide synthase (161). NO rapidly reacts with oxygen free radicals to form the peroxynitrite radical ($ONOO^-$). Subsequent interaction with hydrogen peroxide, a product of superoxide dismutase activity, produces highly toxic hydroxyl radicals. The notion that NO mediates neuronal injury is supported by the recent finding that cortical neurons cultured from neuronal nitric oxide synthase-deficient mice are resistant to glutamate-induced cytotoxicity (162).

A number of studies have shown that Ca^{2+} overload can trigger endonuclease activation. Endonucleases play a central role in programmed cell death, or apoptosis (163,164). Recent studies have shown that some neuronal subpopulations may die via apoptosis following activation of this endogenous cell death program by glutamate (165-169). Moreover, in cultured cerebellar granule neurons, neuronal death has been demonstrated to result from either apoptosis or necrosis, depending on the severity of neuronal injury and extent of mitochondrial dysfunction induced by excitotoxin exposure (170).

1.2.2 AMPA/kainate Receptor-Mediated Toxicity

Some exogenous EAAs, such as kainate and domoic acid, interact specifically with non-NMDA receptors yet induce a calcium-dependent CNS toxicity similar to glutamate (171,172). The mechanisms by which these AMPA/kainate receptor agonists produce Ca^{2+}-mediated neurotoxicity are as yet uncertain. In a number of vitro systems AMPA/kainate receptor-mediated neurotoxicity is largely dissociated from the involvement of NMDA receptors, since NMDA receptor antagonists fail to protect against non-NMDA receptor agonist challenge (173,174). Moreover, only a prolonged exposure to AMPA/kainate receptor agonists produces neuronal degeneration in some experimental systems (4,174), while acute exposures are sufficient to kill neurons in others (175-177). Thus, whereas the toxicity of glutamate can be clearly linked to NMDA receptor activation and subsequent calcium entry, the mechanisms underlying AMPA/kainate receptor-mediated, calcium-dependent neurodegeneration have not been well defined in cell culture systems.

In contrast to the in vitro data, however, studies in animals indicate that AMPA/kainate receptor agonist-induced neurotoxicity is mediated by NMDA and non-NMDA receptors. Furthermore, domoic acid administered systemically to rats and monkeys produces acute neurotoxicity and is rapidly cleared from the circulation (178,179). In the rat, kainic acid produces siezures followed by necrosis of neurons in the hippocampus, amygdala, piriform cortex and other limbic areas (13,14,180). Several classes of competitive and non-competitive NMDA receptor antagonists have been shown to prevent the majority of this seizure related toxicity (14,180-182). Moreover, chemical denervation of hippocampal mossy fiber tracts from granule neurons of the dentate gyrus substantially attenuates domoate (182) and kainate-induced (183,184) toxicity in CA3 neurons. These observations provide evidence consistent with the hypothesis that synaptic

activity and NMDA receptor activation play a role in non-NMDA receptor agonist-mediated neurotoxicity.

Given the discrepancies between the in vitro and in vivo data, it is probable that AMPA/kainate receptor agonists produce cytotoxicity through several possible mechanisms. One route by which AMPA/kainate receptors could elevate cytosolic Ca^{2+} to toxic levels is through a depolarization-dependent activation of voltage operated Ca^{2+} channels (186). The involvement of voltage activated Ca^{2+} channels is supported by the finding that the dihydropyridine, nifedipine, substantially attenuates slow AMPA/kainate receptor-mediated neurotoxicity in cultured mouse cortical neurons (187), and nimodipine has been shown to significantly attenuate lesions produced by kainate injected intracerebrally in rats (188). Large cytosolic Na^+ accumulations that occur subsequent to AMPA/kainate receptor agonist exposure may also induce a reversed mode of operation of the Na^+/Ca^{2+} exchanger (144). This route of Ca^{2+} entry could be particularly important during kainate and domoate exposures, inasmuch as these agonists induce mostly non-desensitizing electrical responses in neurons, depending on which non-NMDA receptor subunits are expressed (68). Recent reports have demonstrated the presence of Ca^{2+}-permeable AMPA/kainate receptors in a subpopulation of neurons and glia (74,189). These neurons have been shown to be particularly vulnerable to non-NMDA agonist-induced toxicity (189). Thus, although NMDA receptor-mediated increases in cytosolic Ca^{2+} concentration are a major cause of excitatory amino acid-induced injury and death in neurons, non-NMDA receptors alone may produce cytotoxic increases in intracellular Ca^{2+} concentration.

As alluded to previously, one other potential route for AMPA/kainate receptor-mediated Ca^{2+} entry into neurons is through the NMDA receptor ion channel. NMDA receptors would be activated subsequent to non-NMDA receptor agonist-induced glutamate release (190,191). Moreover, because some AMPA/kainate receptor agonists are capable of activating non-desensitizing currents in neurons, in contrast to the rapidly

desensitizing currents induced by glutamate, release of the Mg^{2+} blockade of NMDA receptor ion channels would be facilitated (68). In support of this notion, extracts from mussels contaminated with subtoxic concentrations of domoic acid have been shown to potentiate synergistically the excitotoxic effect of glutamate and aspartate in cultured cerebellar granule neurons (174).

1.3 Development of an *In Vitro* Model of Glutamate- and Domoate-Induced Toxicity Using Cultured Rat Cerebellar Granule Neurons

Primary cultures of rat cerebellar granule neurons represent a valuable model for studying the physiology, biochemistry and toxicology of excitatory amino acids (EAAs) because they can be cultured as a relatively homogenous cell population of greater than 90% purity (192), express both metabotropic (193) and ionotropic (194) glutamate receptors, and form an extensive network of neurites and synaptic contacts (192). Cerebellar granule neurons are also sensitive to EAA-mediated cytotoxicity (116-118,128).

When grown in a serum-containing medium, cerebellar granule neurons develop a survival requirement for depolarizing conditions between two and four days in vitro which, if not met by elevated extracellular concentrations of K^+ or NMDA, results in a significant cell loss by the end of one week in culture (192,195). Neuronal depolarization is believed to mimic the trophic influence of the first innervation received by post-migratory granule cells from glutamatergic mossy fibers in vivo (195,196). Depolarizing conditions also affect the regulation and developmental timecourse of glutamate receptor expression (197-201) with the consequence that the pharmacology of agents acting on glutamate receptors changes both as a function of growth conditions and age of neurons in culture (197,198,200,202). In this thesis project, cerebellar granule neurons were utilized at a single age of 12 days in culture (DIC). Neurons at this age express both

NMDA and non-NMDA receptor subtypes and respond to excitatory amino acids in a characteristic and reproducible manner (203,204). In Chapter 5, the pharmacologic and toxicologic significance of NMDA receptors expressed in 12 DIC cerebellar granule neurons (CGCs) is investigated, and the effect of chronic depolarization on the developmental regulation of NMDA receptor subunit expression is discussed (204).

The effect of chronic depolarization on AMPA receptor subunit expression and the functional activity of AMPA receptors has also been investigated in CGCs (201), although not as rigorously as with NMDA receptors. In granule cells of the adult rat cerebellum, AMPA receptors have been shown to be composed primarily of GluR2 flip and GluR4 flop subunits (23). When grown under standard culture conditions (with 25 mM K^+), however, CGCs have been shown to express high levels of GluR1 mRNA and protein in addition to GluR2, GluR3 and GluR4 subunits (85,201). Therefore, AMPA receptors on CGCs may contain various combinations of all four AMPA receptor subunits. The effect of growth conditions on kainate receptor subunit expression in CGCs has not yet been characterized; however, GluR6 and KA2 subunits are known to be expressed in cerebellar granule neurons in vivo (23), and pharmacological studies show kainate receptors to be present in CGCs (194). Thus, CGCs express both AMPA and kainate receptor subtypes.

An important consideration when using CGCs for toxicologic experimentation is that exposure conditions must be manipulated to overcome the inherent resistance that CGCs have to the cytotoxic effects of excitatory amino acids (EAAs) (117,118,129,133). Moreover, the specific experimental conditions employed may affect the intensity of neuronal injury induced by EAAs and the pathways through which the neuronal damage occurs (4,109,110,115,117,118). To induce glutamate toxicity, for example, non-physiological exposure conditions have been employed which mimic those occurring in CNS pathologies. In this experimental context, CGCs provide an appropriate model for studying the cytopathologic mechanisms of such diseases as stroke, trauma and hypoxia

(117,118,129,133,144-146,170,191,203). For non-NMDA receptor agonists such as domoate, however, an appropriate model should utilize a physiologic exposure milieu, inasmuch as domoate intoxications are known to occur in initially healthy individuals (10,11). A physiologic milieu would preserve both normal cell signaling mechanisms by maintaining the voltage-dependence of NMDA receptor currents, and the functional interrelationships that exist between NMDA and AMPA/kainate receptors.

In the current project, it was intended that neurotoxicological experiments be designed so that NMDA and AMPA/kainate receptor agonist-induced toxicity occur under identical exposure conditions, thereby allowing direct comparisons to be made between domoate excitotoxic mechanisms and the well characterized mechanisms of glutamate-induced toxicity. This required that the resistance possessed by CGCs to glutamate challenge in a physiologic exposure milieu (i.e. non-depolarizing K^+ concentration with Mg^{2+} and glucose present) be overcome by reducing the exposure temperature from 37° to 22°. The facilitation of acute glutamate toxicity in CGCs by reduced temperature is characterized in Chapter 2 of this manuscript (203). In Chapter 3, this characterization is extended by examining how reduced temperature affects CGC membrane potential during glutamate challenge. In Chapter 4, glutamate- and domoate-induced neurotoxicity are compared directly in CGCs using the previously defined exposure conditions, and the cellular mechanisms of domoate-induced cytotoxicity are addressed.

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Chapter 2

Characterization of Glutamate Toxicity in Cultured Rat Cerebellar Granule Neurons at Reduced Temperature

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2.1 Abstract

We have defined conditions whereby glutamate becomes toxic to isolated cerebellar granule neurons in a physiologic salt solution (pH 7.4). Under these conditions, acute glutamate excitotoxicity manifest only when the temperature was reduced from 37° C to 22°C. N-methyl-D-aspartate (NMDA) was non-toxic at either temperature at concentrations as high as 1 mM. Glycine strongly potentiated both the potency and efficacy of glutamate, but revealed only a modest NMDA response. The non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxalinedione (CNQX), potently protected against glutamate challenge, although the contribution of antagonism at strychnine-insensitive glycine sites could not be excluded. To further characterize the non-NMDA receptor contribution to the excitotoxic response, the promiscuity of glutamate interaction with ionotropic receptors was simulated by exposing neurons to NMDA in the presence of non-NMDA receptor agonists. NMDA toxicity was potentiated four to seven fold when non-NMDA receptors were coactivated by a subtoxic concentration of AMPA, kainate or domoate. These results suggest that non-NMDA receptor activation participates in the mechanism of acute glutamate toxicity by producing neuronal depolarization (via sodium influx) which in turn promotes the release of the voltage-dependent magnesium blockade of NMDA receptor ion channels.

2.2 Introduction

The mechanisms responsible for the transition of glutamate from excitatory CNS neurotransmitter to neurotoxin have been studied extensively in various in vitro systems. In cultured murine cerebral cortical neurons, two distinct components of this glutamate-induced excitotoxicity have been elucidated (1,2). An early component, mediated by the depolarization-dependent influx of Na^+, Cl^- and water, manifests as a rapidly developing

neuronal swelling that is reversible if either glutamate concentration or exposure duration are limited (3,4). The early component has been linked to activation of non-N-methyl-D-aspartate (non-NMDA) receptors and can therefore be mimicked by non-NMDA receptor agonists such as kainate, domoate or AMPA; however, prolonged exposures are required for these compounds to induce significant lethality (2,5). The second component of glutamate-induced cytotoxicity has been associated with the excessive and unregulated influx of Ca^{++} through NMDA receptor ion channels, which also induces a mild early neuronal swelling (3,4,6), but is primarily characterized by a slowly developing neuronal degeneration that occurs within hours of a brief (5 minute) exposure to high concentrations of glutamate or NMDA (7,8).

Glutamate, which displays activity at all excitatory amino acid receptor subtypes, stimulates both the early and delayed components of the excitotoxic response. Non-NMDA receptor antagonists confer protection against the majority of the early neuronal swelling but not the later neuronal degeneration (9). Conversely, NMDA receptor antagonists only partially reduce the acute neuronal swelling, yet protect against the delayed neuronal degeneration (5,10).

In cultured rat cerebellar granule neurons, glutamate excitotoxicity is also mediated by Ca^{+} entry through NMDA receptors (11,12). However, cerebellar granule neurons differ in their relative resistance to these glutamate-induced effects. When cerebellar granule neurons are exposed in a physiologic milieu at 37°C, glutamate is non-toxic even at high concentrations, revealing an apparent ability of these neurons to maintain ionic homeostasis in the face of ion flux induced by agonist stimulation (13-15). Specific manipulations, such as the omission of Mg^{++} from the exposure medium, are therefore required for glutamate to induce toxicity (16,17). Excitotoxicity in cerebellar granule neurons is also facilitated by the depletion of neuronal energy reserves (14,18), or by depolarization induced by elevated K^{+} or exposure to veratradine (19-21). The neurons then undergo either rapid or delayed neuronal degeneration, depending upon the

duration and intensity of glutamate exposure. These data indicate that in a physiologic salt solution cerebellar granule cell excitotoxicity manifests only when presented in the context of an independent depolarizing stimulus sufficient to allow release of the voltage-dependent Mg^{++} blockade of NMDA receptor ion channels, thereby resulting in excessive influx of Na^{+} and Ca^{++} (22-24).

Questions remain however, as to how NMDA and non-NMDA receptor mechanisms might be functionally integrated in the excitotoxic response to glutamate and other excitotoxins, since exposure conditions utilized in studies involving cerebellar granule neurons are often permissive to the direct activation of NMDA receptors by excitatory amino acid agonists. In the present study, we define conditions that unmask glutamate toxicity in a physiologic salt solution, and in the absence of additional depolarizing stimuli. These conditions leave receptor and homeostatic regulatory functions intact. We report herein that glutamate becomes acutely toxic to cerebellar granule neurons when the exposure temperature is reduced. Moreover, we have delineated the NMDA and non-NMDA receptor mediated components of this acute glutamate-induced neurotoxicity.

2.3 Materials and Methods

2.3.1 Materials

(RS)-alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), D(-)-2-amino-5-phosphonopentanoic acid (D-AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and domoic acid were purchased from Tocris Cookson (Bristol, U.K). 3-amino-1-hydroxy-2-pyrrolidone (HA-966) was purchased from Research Biochemicals Inc. (Natick, MA). L-glutamate, glycine, kainic acid, N-methyl-D-aspartic acid (NMDA),

bovine serum albumin (BSA), trypsin, DNAse and soybean trypsin inhibitor (SBTI) were purchased from Sigma (St. Louis, MO). Basal Eagle's Medium (BME) was purchased from Gibco Labs (Grand Island, N.Y.).

2.3.2 Cell Culture

Primary cultures of cerebellar granule neurons were obtained from 8 day old Sprague Dawley rats as described previously (18), with minor modifications. Cerebella were isolated and placed in Kreb's buffer containing 3 mg/ml bovine serum albumin and 1.2 mM $MgSO_4$ (KB/BSA), minced by mild trituration, washed once in KB/BSA and incubated with shaking for 15 minutes at 37°C in 30 ml KB/BSA containing 2200 U/ml trypsin. The digestion was terminated by addition of 15 ml KB/BSA containing 26 µg/ml DNAse, 166 µg/ml soybean trypsin inhibitor (SBTI) and 1.7 mM $MgSO_4$. Following low speed centrifugation for 1 minute, the tissue was resuspended in KB/BSA containing 80 µ g/ml DNAse, 500 µg/ml SBTI and 2.8 mM $MgSO_4$, triturated with a Pasteur pipette and the suspension allowed to settle. The supernatant, containing dissociated cells, was transferred to another tube and the trituration repeated on the remaining undissociated tissue. The supernatants were combined, centrifuged at low speed for five minutes and the cell pellet resuspended in basal Eagle's medium containing 25 mM KCl, 2 mM glutamine, 10% fetal calf serum and 100 µg/ml gentamicin. The cells were seeded onto poly-l-lysine coated (5 µg/ml) 35mm polystyrene culture dishes (Corning) at a density of 2.5 x 10^5 cells/cm^2. Care was taken to ensure a uniform neuron distribution by thorough mixing of the suspension after addition to each plate. Cells were incubated at 37°C in a 5% CO_2, 95% humidity atmosphere. Cytosine arabinoside (10 µM) was added 18-24 hrs later to inhibit the replication of non-neuronal cells. At 8 days in culture, 100 µl of a 25 mg/ml D-glucose solution was added to each plate to replenish energy resources and to compensate for growth media evaporative losses (25).

2.3.3 Excitotoxicity Assays

Cerebellar granule neurons were utilized at 12 days in culture. Excitotoxicant exposure was conducted at a temperature of 22°C. Prior to agonist exposure, growth medium was removed and the cultures exposed for 5 minutes to 1 ml Locke's incubation buffer containing (in mM): 154 NaCl; 5.6 KCl; 1 MgCl; 2.3 CaCl; 5.6 glucose; 8.6 HEPES; pH 7.4. The Locke's buffer was then replaced with 1 ml of fresh Locke's solution containing experimental compounds and the cultures were then incubated for 2 hrs. At the termination of the exposure, 0.6 ml of the incubation buffer was collected for analysis of LDH activity, and the neurons were washed three times in fresh Locke's solution. The neurons were then maintained for a one hour post-exposure incubation prior to a five minute staining with the vital dye fluorescein diacetate (5 μg/ml) (26). Ethidium bromide (10 μl of a 50 μg/ml solution) was added four minutes after the fluorescein diacetate to stain the nuclei of nonviable neurons. The dye solution was replaced with fresh Locke's and the neurons placed on an inverted microscope (Zeiss model IM35) equipped with fluorescence optics. Under fluorescence, soma and neurites of live neurons appear bright green in color whereas dead neurons do not accumulate fluorescein. Nuclei of dead neurons, that are not washed away, appear red due to ethidium bromide staining.

2.3.4 Assessment of Neuronal Viability

Five to six randomly selected fields from each plate were photographed at 80x magnification. Live neurons were counted from the photographic slides and the results averaged for each concentration. Results were referenced to the average control culture populations processed in parallel to experimental plates.

LDH activity of the incubation buffer was assayed as described previously (27). Aliquots (0.2 ml) of incubation buffer were added to 2.3 μmol sodium pyruvate and 0.2

mg NADH, each in 0.5 ml of 0.1 M $KHPO_4$ buffer (pH 7.5), for a total assay volume of 1.2 ml. The change in absorbance at 340 nm was measured with a UV spectrophotometer and LDH activity calculated from the linear portion of the slope of the absorbance curve. LDH concentration was expressed in units/ml, where one unit is the amount of LDH that produces a decrease of 0.001 absorbance units per minute per ml of incubation medium. LDH efflux at each drug concentration was determined from triplicate plates and the results averaged.

2.3.5 Data Analysis

EC_{50} values and maximum responses for excitotoxicants were determined by nonlinear least squares fitting of a logistic equation to concentration response data. Nonlinear regression analysis was performed with Graphpad Inplot software (San Diego, CA). These data were analyzed according to the following equation where Y equals the response, C is the EC_{50}, [X] is the agonist concentration, D the slope factor and A and B the minimum and maximum plateaus of the concentration-response curve: $Y = A + (B-A)/1+(C/[X])^D$

2.4 Results

Initial experiments confirmed that glutamate and NMDA are nontoxic even at millimolar concentrations when tested at 37°C in a physiologic salt solution (data not shown). In contrast, at 22°C glutamate induced a concentration dependent reduction in the number of viable neurons (EC_{50} = 84.2 μM), while NMDA remained non-toxic at concentrations as high as 1 mM (Fig. 2.1). The maximal neurotoxic response to glutamate represented a 50% reduction in the number of viable neurons. The initial cytotoxic response, as evidenced by neuronal swelling, could be visualized by light microscopy as

early as ten minutes after exposure to glutamate; however, the maximal excitotoxic response appeared only after a 90 to 120 minute incubation. A one hour incubation in fresh Locke's solution subsequent to glutamate exposure improved the ability of surviving neurons to be stained with fluorescein diacetate, thereby improving visualization and quantification of viable neurons. The results of LDH efflux assays correlated closely with data obtained by the direct counting method and provided independent confirmation of excitotoxic cell injury (fig.2.1)

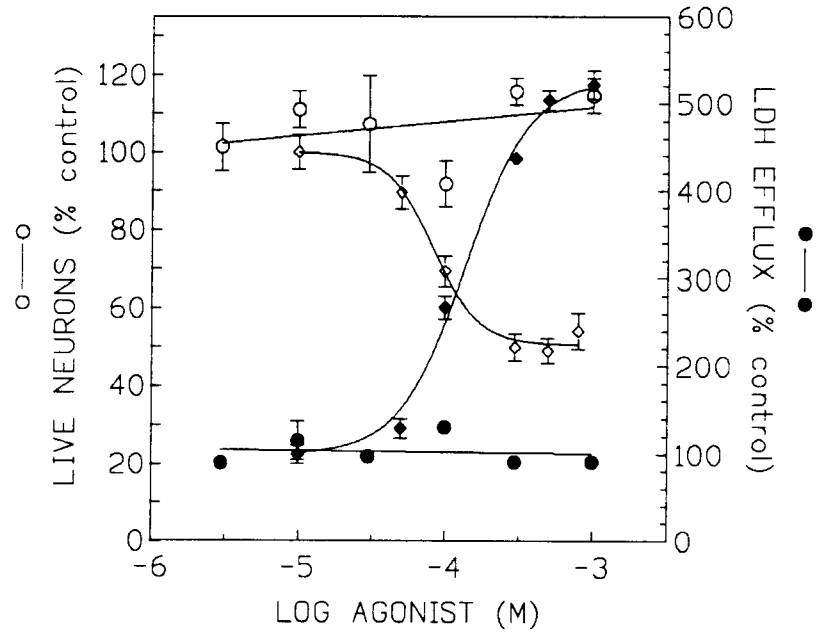

Fig. 2.1 Comparison of the excitotoxic concentration-response relationship for glutamate (◊) and NMDA (O)(◊,O = live count; ◆,● = LDH efflux) at 22°C. Incubations were carried out in Locke's buffer containing 5mM K^+ without added glycine. Individual points represent mean ± SEM from a representative experiment for NMDA and three separate experiments for glutamate. Individual experiments were performed in triplicate.

Inasmuch as glycine is known to be required as a coagonist for glutamate activation of the NMDA receptor, the effect of exogenous glycine on the response to

glutamate and NMDA was investigated. In the presence of 10 μM glycine, the potency of glutamate was increased approximately two-fold; from a control EC_{50} of 84.2 μM to a value of 43.4 μM in the presence of glycine. A maximal five-fold augmentation of glutamate potency occured when 100 μM glycine was included in the incubation buffer (glutamate EC_{50} = 17.9 μM). Glycine also increased modestly the efficacy of glutamate from a maximum neuronal loss of 50% to 65% (Table 2.1). Similarly, the toxicity of NMDA was enhanced in the presence of 100 μM glycine, but remained at a much lower potency (EC_{50} = 354 μM) and efficacy (30% neuron loss) than glutamate (Fig.2.2a and 2.2b).

TABLE 2.1 The effect of glycine on the excitotoxic potency and efficacy of glutamate.

Glycine (μM)	Glutamate EC_{50} (μM)[a]	Efficacy (% neuron loss)[b]
0	84.2 (75.8-93.5)	50 (47.8-52.4)
10	43.4 (40.8-46.2)	55 (53.9-56.1)
100	17.9 (15.8-20.4)	65 (59.1-70.3)

[a]EC50 values are based upon direct neuron counts. [b]Values in parentheses represent 95% confidence intervals. Data represent the mean of 3-4 separate experiments performed in triplicate.

To further the pharmacological characterization of glutamate toxicity in cerebellar granule neurons, the effects of NMDA and non-NMDA receptor antagonists against a maximal glutamate (300 μM) challenge were assessed (Fig. 2.3). The competitive NMDA receptor antagonist D-2-amino-5-phosphonopentanoic acid (D-AP5), completely and concentration dependently protected against glutamate toxicity (EC_{50} = 22.8 μM), thus demonstrating a requirement for NMDA receptor activation in the acute cytotoxic

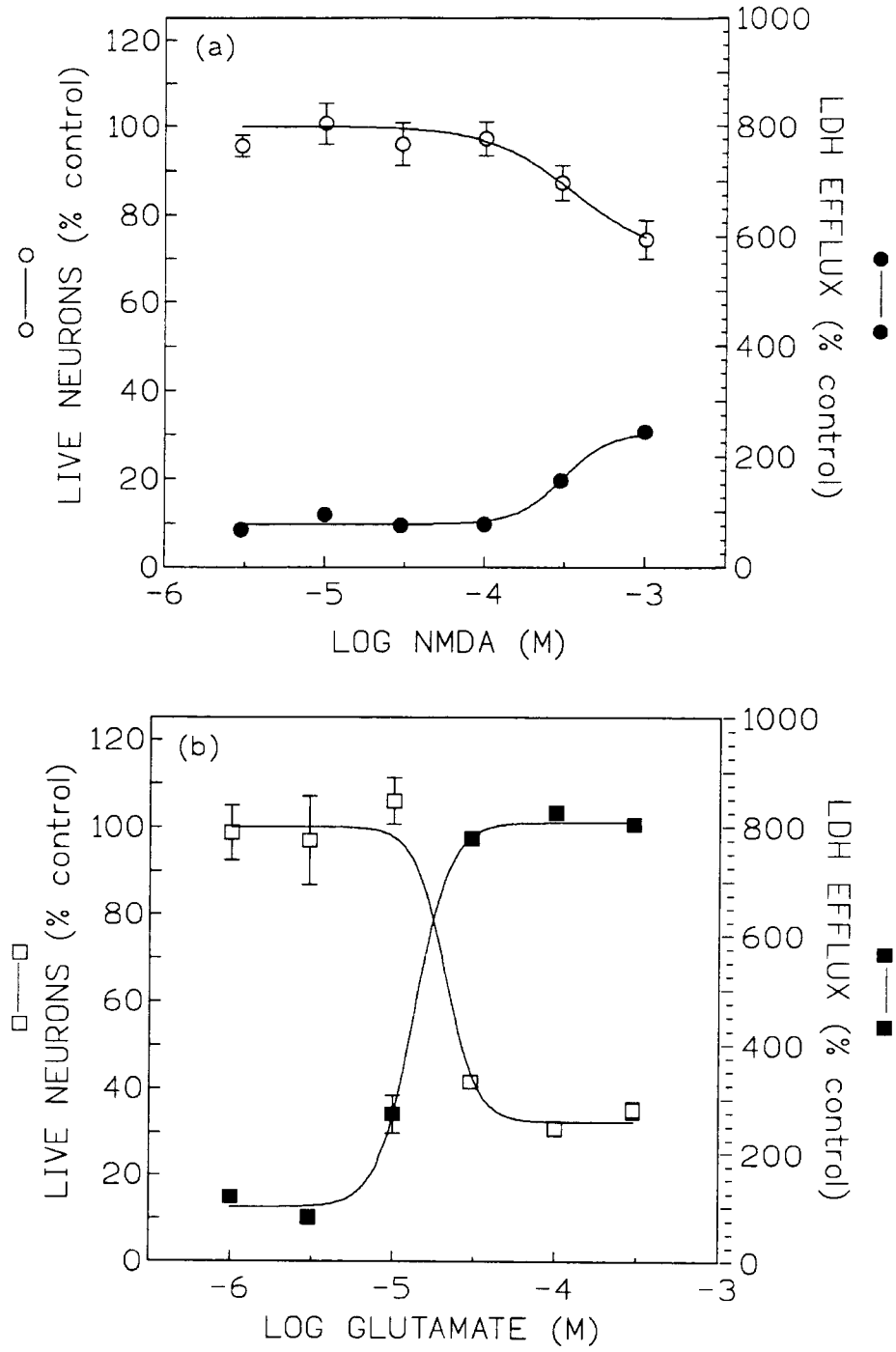

Fig. 2.2 (a) NMDA becomes toxic in the presence of 100μM glycine. Data points represent the mean ± SEM of three separate experiments performed in triplicate. EC_{50}=354μM (202-622μM); (b) Comparative glutamate concentration-response (100μM glycine present) from a representative experiment performed in triplicate. EC_{50}=17.9μM (15.8-20.4μM). The magnitude of LDH efflux corresponded to the maximum cell loss for each compound. Values in parentheses are 95% confidence intervals.

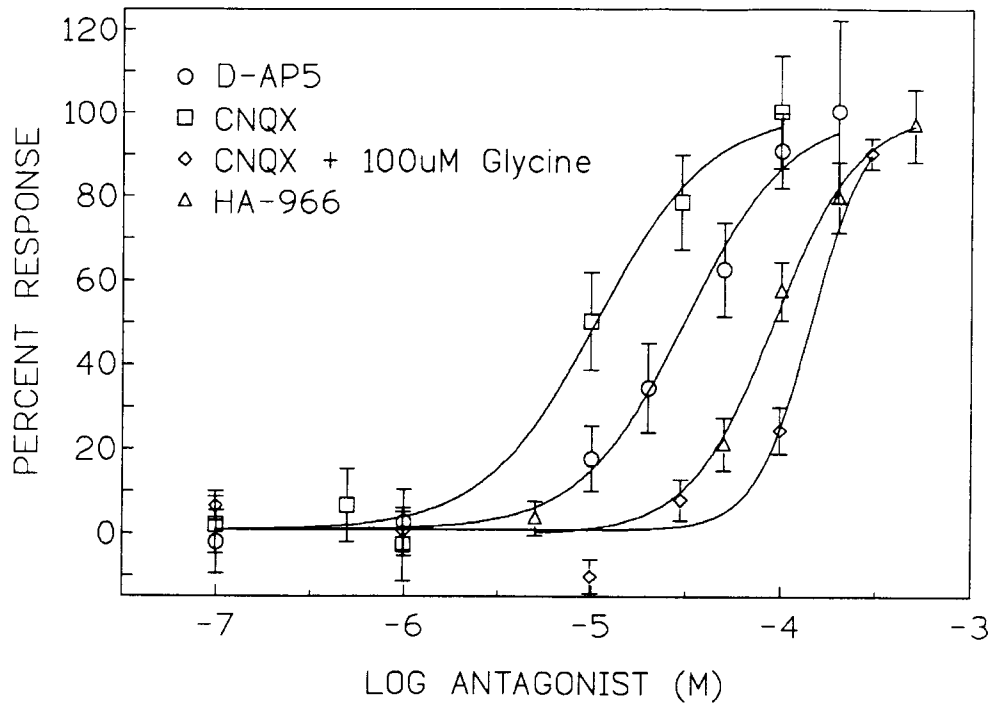

Fig. 2.3 Antagonist concentration-response against a 300μM L-glutamate challenge. The competitive NMDA receptor antagonist D-AP5 (o) and the strychnine insensitive glycine site antagonist HA-966 (Δ), were tested without the addition of glycine. CNQX was tested with (◊) and without (□) the addition of 100μM glycine. Data points represent the mean ± SEM of at least three separate experiments performed in triplicate.

response. In accordance with the neuroprotective actions of D-AP5, the strychnine-insensitive glycine site antagonist HA-966 was fully protective (EC_{50} = 95.7 μM). The addition of the non-NMDA receptor antagonist CNQX to cultures also afforded complete protection against glutamate challenge (EC_{50} = 9.1 μM). The CNQX neuroprotection concentration-response curve could be shifted rightward by exogenous glycine (Fig. 2.3), indicating that CNQX neuroprotection may derive partially from its known ability to displace glycine from strychnine-insensitive binding sites on NMDA receptors.

Since NMDA is a much less potent excitotoxin than glutamate under the conditions employed, we determined whether the toxicity of this selective agonist could

be enhanced in the presence of low concentrations of non-NMDA receptor agonists, in effect simulating the promiscuity of glutamate at NMDA and non-NMDA receptors. Concentration-response curves were generated for kainate, domoate and AMPA, and subtoxic concentrations for each compound were determined from these curves (Fig. 2.4).

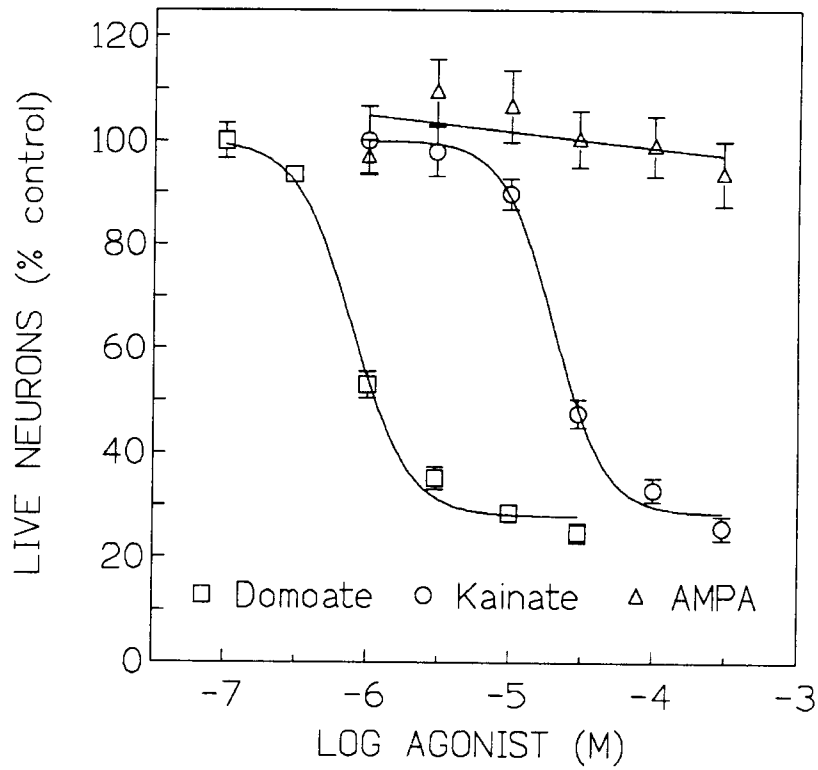

Fig. 2.4 Concentration-response relationships of non-NMDA receptor agonists. (□) Domoate EC_{50}=1.51μM (1.31-1.74μM); (o) Kainate EC_{50}=20.2μM (16.1-25.4μM); (Δ) AMPA. Experiments were carried out in the presence of 100μM glycine. Values in parentheses represent 95% confidence intervals.

The subthreshold concentrations chosen for kainate and domoate were 7 μM and 0.2 μM respectively. AMPA had no effect on neuron viability or LDH efflux at concentrations as high as 300 μM, however somal swelling was observed at concentrations of 100 μM or

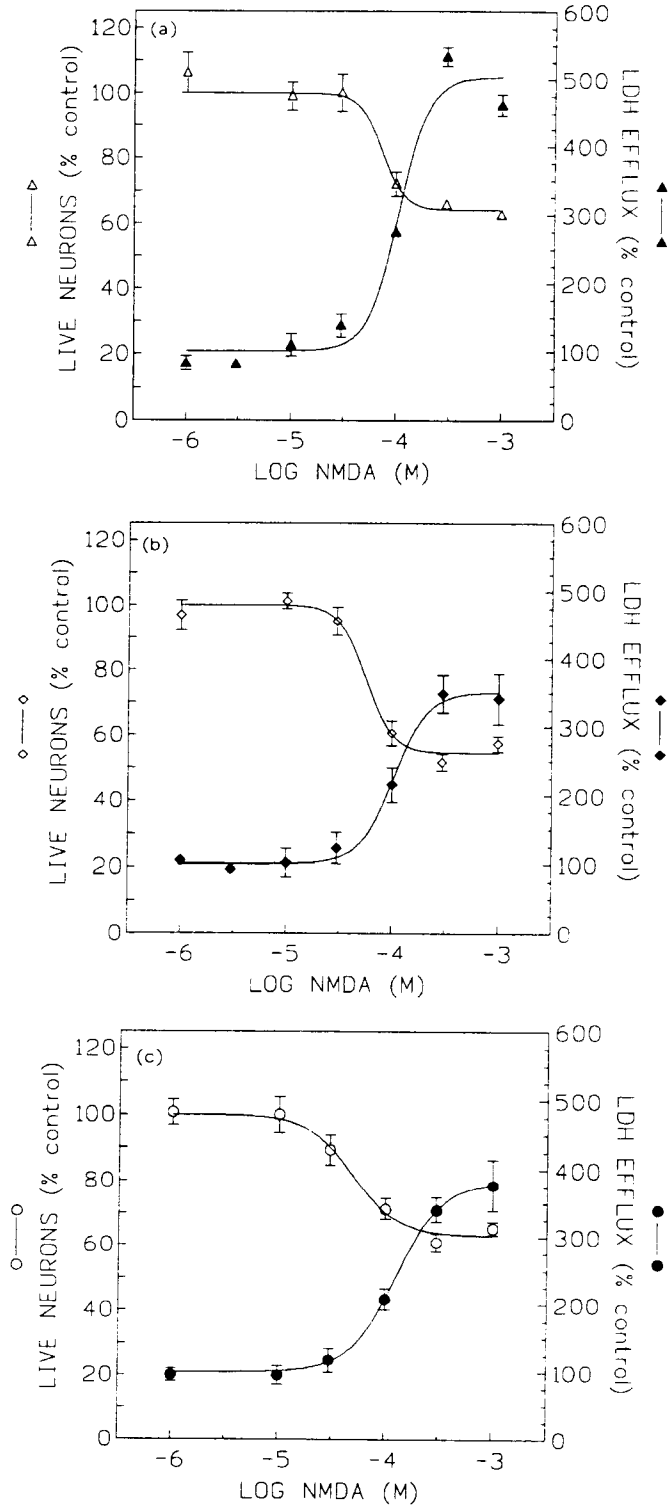

Fig. 2.5 NMDA concentration-responses in the presence of non-NMDA receptor agonists. Non-NMDA agonist concentrations were those for which little or no effect was observed alone: (a) 7μM kainate(Δ); (b) 0.2μM domoate(◊); (c) 30μM AMPA(o). Data points represent mean ± SEM of three separate experiments. LDH efflux performed in triplicate for each experiment.

greater. An AMPA concentration of 30 μM was therefore chosen for the NMDA interaction experiments.

NMDA-induced cytotoxicity, as measured by LDH efflux, was enhanced in the presence of these non-NMDA receptor agonists (Fig. 2.5a-c and Table 2.2).Kainate and domoate potentiated the EC_{50} values for NMDA cytotoxicity from a control value of 354 μM to EC_{50} values of 106 μM and 105 μM respectively. Kainate and domoate potentiated the EC_{50} values for NMDA cytotoxicity from a AMPA also increased the potency of NMDA, reducing the EC_{50} to 125 μM. When assessed by direct counting of fluorecsein stained neurons, the potentiation of NMDA-induced neurotoxicity by non-NMDA receptor agonists manifested as a four to seven-fold leftward shift in the

TABLE 2.2 The effect of non-NMDA receptor agonists on the excitotoxic potency and efficacy of NMDA.

Non-NMDA Agonist[a]	NMDA EC_{50} (μM)[b]	Efficacy (% neuron loss)[c]
Control	354.0 (201.5-621.8)	30 (≥30)
Kainate (7μM)	105.5 (71.6-155.5)	36 (28-44)
Domoate (0.2μM)	104.7 (80.4-136.4)	45 (38-52)
AMPA (30μM)	124.7 (104.9-148.3)	37 (31-43)

[a]Non-NMDA receptor agonists were present at concentrations that cause no loss of neurons. [b]EC50 values are based upon LDH efflux data. [c]Values within parentheses represent 95% confidence intervals. Data represent the mean of 3 experiments performed in triplicate.

concentration-response curve. Moreover, the efficacy of NMDA was also increased, although remaining somewhat lower than the maximal level of neuron loss occuring in response to glutamate.

2.5 Discussion

Previous reports have shown that cultured cerebellar granule neurons are completely resistant to high concentrations of glutamate when exposure occurs in a physiologic salt solution, but sensitive to low glutamate concentrations upon glucose deprivation or removal of Mg^{++} from the exposure medium. Thus a pivotal event in the induction of glutamate toxicity appears to be the release of the voltage-dependent Mg^{++} blockade of NMDA receptor ion channels (14-16). In agreement with these reports, we have confirmed that cerebellar granule neurons are resistant to glutamate when exposed in a physiologic salt solution at 37°C. In contrast, we have shown that toxicity to glutamate can be unmasked in a physiologic salt solution when the exposure temperature is reduced from 37° to 22°. Low temperature potentiation of glutamate excitotoxicity in cultured cerebellar granule neurons has been previously noted (28). In the present report, we have confirmed and extended these findings through a delineation of the roles of both NMDA and non-NMDA receptors in glutamate-induced neuronal death in a physiologic salt milieu.

Several mechanisms may account for the facilitation of excitatory amino acid toxicity by low temperature. One possibility is that a reduction in the rate of glutamate uptake, coupled with a reduction in the rate of enzyme activities involved in the maintenance of energy charge or ion homeostasis, would impair both the ability of the neurons to limit glutamatergic stimulation and to sustain a transmembrane electrochemical potential of sufficient magnitude to preserve the voltage-dependent Mg^{+}

$^{+}$ block. High affinity L-glutamate uptake in brain slices and synaptosomes is temperature sensitive (29). Inasmuch as the rate of enzyme catalyzed reactions generally decreases with reduced temperature, it is reasonable to propose that the observed sensitivity to glutamate may be caused primarily by a reduction in the activity of Na^+/K^+ ATPases, since the maintenance of Na^+ and K^+ gradients and therefore the neuronal resting potential is known to be a primary function of these enzymes. In sheep cardiac Purkinje fibers, the Q_{10} for Na^+/K^+ ATPase current transients has been shown to be between 1.6 and 2.3 over the temperature range of 26-46°C (30). Moreover, electrophysiological experiments in cultured rat skeletal myotubes demonstrated that Na^+/K^+-ATPase activity accounts for about 30% of the resting membrane potential (31). This component of the resting potential in skeletal muscle myotubes could be completely and reversibly eliminated by reducing the temperature from 35°C to 10°C, an effect equivalent to that observed when the myotubes were treated with ouabain. In hippocampal CA1 neurons, the sodium pump has been shown to contribute significantly to the hyperpolarization that occurs subsequent to glutamate-induced depolarizations (32). This hyperpolarization following glutamate challenge was inhibited to the same degree by ouabain or low temperature. Na^+/K^+ ATPase inhibition has also been shown to enhance excitotoxic responses in a variety of experimental systems. For instance, inhibition of the sodium pump by ouabain resulted in a >95% loss of cerebellar granule cells when challenged with either glutamate or NMDA in a physiologic medium containing Mg^{++} (18). This effect was also observed in rat cortical neurons whereby the degree of toxicity to glutamate varied inversely with the degree of chemical inhibition of Na^+/K^+-ATPase (33). We therefore propose that susceptibility to glutamate challenge in a balanced salt solution at 22°C is most likely due to an impaired ability of neurons to respond to disturbances in ion gradients, primarily as a result of the temperature-dependent reduction in Na^+/K^+-ATPase activity. The ensuing neuronal depolarization following glutamate exposure would facilitate release of the voltage-dependent Mg^{++} blockade of NMDA

receptor channels, permitting the excessive influx of Ca^{++} and Na^{+} with attendant excitotoxic cell injury.

Our initial experiments indicated that a significant neurotoxic response to glutamate required a 90 to 120 minute exposure duration and that NMDA was nontoxic under these conditions. Addition of glycine increased both the potency and efficacy of glutamate and enabled NMDA to evoke a neurotoxic response. These data may be compared to those of Novelli, et al. (18), in which virtually all cerebellar granule neurons displayed excitotoxic injury after a 30 minute exposure to either glutamate or NMDA at 37°C when Mg^{++} or glucose were omitted from the incubation medium. Complete inhibition of the sodium pump by ouabain similarly facilitated the loss of virtually all neurons by exposure to either glutamate or NMDA. Our results differ from these previous reports in that we observed NMDA to be substantially less effective than glutamate as an excitotoxin in a physiologic salt solution. This difference in efficacy between glutamate and NMDA suggests that agonist binding to NMDA receptors may be necessary but not sufficient to induce substantial cytotoxicity in the presence of Mg^{++}. The excitotoxic response to glutamate appears to require the concurrent activation of both NMDA and non-NMDA receptors.

The involvement of non-NMDA receptors in glutamate-induced neurotoxicity was explored by assessing the ability of the non-NMDA receptor antagonist CNQX to inhibit glutamate toxicity. CNQX afforded potent neuroprotectant effects in response to glutamate challenge. Interpretation of this effect of CNQX is complicated somewhat by the ability of this compound to act as an antagonist at the strychnine-insensitive glycine site on NMDA receptors (34-36). Thus in addition to acting as a non-NMDA receptor antagonist, CNQX is nearly equipotent as HA-966 in displacing [^{3}H]glycine from the strychnine-insensitive site of the NMDA receptor (37). CNQX was however approximately ten times more potent than HA-966 in attenuating neurotoxicity (fig. 2.3). This difference in the relative potency of CNQX and HA-966 as neuroprotectants as

compared to their affinity for the [^{3}H]glycine site may indicate that the observed neuroprotection is due, at least in part, to antagonism of glutamate at non-NMDA receptors. Similary, the glycine-induced rightward shift in the CNQX concentration-response relationship may be related to the ability of glycine to enhance the affinity of glutamate for NMDA receptors (38-41). The observation that glycine enhanced the excitotoxic potency of glutamate and NMDA provides evidence that the sensitivity of NMDA receptors to agonist activation was indeed augmented by this coagonist (fig. 2.2). This positive heterotropic effect of glycine on the interaction of glutamate with the NMDA receptor may underlie the glycine-induced rightward shift in the CNQX concentration-response relationship.

Another approach to assess the participation of non-NMDA receptors in the acute neurotoxic response to glutamate was to ascertain the influence of concurrent non-NMDA receptor activation on the response to NMDA, thereby simulating the promiscuity of glutamate at both classes of receptor. Although the subtoxic concentrations of non-NMDA receptor agonists employed produced only minor somal swelling in the absence of NMDA, they reduced NMDA LC_{50} values by more than three fold and increased neuronal cell loss 20 to 50 percent. Thus concurrent non-NMDA receptor activation enables NMDA receptor stimulation capable of producing a neurotoxic response.

It is noteworthy that AMPA alone did not cause neuronal death, but rather produced only mild neuronal swelling at high concentrations. Expression of AMPA receptor subunits has been shown in cultured rat cerebellar granule neurons by Northern and Western blotting techniques (42). Therefore, we conclude that either (a) the level of AMPA receptor expression is insufficient for AMPA to cause significant toxicity, or (b) rapid desensitization of AMPA receptors precludes production of significant toxicity under the conditions of these experiments when AMPA is used alone (43,44). Neither of these two possibilities necessarily precludes the potentiation of NMDA toxicity by AMPA receptor activation. Nonetheless, AMPA also exhibits a low affinity for kainate

receptors (45,46) and elicits small currents in certain heteromeric assemblies of kainate receptors (47,48). Thus, interaction with kainate receptors may also contribute to the observed effect of AMPA on NMDA-induced neurotoxicity.

The results described herein emphasize the influence of non-NMDA receptors on the excitotoxicity induced by glutamate. This influence is relevant to neuropathologies which result from the excessive activation of glutamate receptors. Activation of non-NMDA receptors would be expected to produce neuronal depolarization, which would facilitate cation influx through NMDA receptor ion channels due to relief of the Mg^{++} blockade. Such a depolarizing influence may be particularly detrimental when neuronal function has been compromised by hypoxia or hypoglycemia. Non-NMDA receptor antagonists may therefore provide valuable therapeutic benefits against neuropathology induced by such neuronal compromise, a notion supported by in vivo studies. Thus, the AMPA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX), produced a protective effect against post-ischemic behavioral pathology and hippocampal damage in gerbils when administered as late as one hour after the onset of global ischemia (49,50). NBQX was also protective against damage to hippocampal CA1 pyrimidal neurons (51) and cerebellar purkinje cells (52) in a rat model of ischemia. Striatal and cortical injury after insulin-induced hypoglycemic coma in rats was also found to be due to stimulation of both AMPA and NMDA receptors (53). The data presented in this report provide in vitro results which support the conclusions of in vivo studies.

In conclusion, our results document the effect of reduced temperature to augment excitatory amino acid mediated toxicity in cultured cerebellar granule neurons when exposure occurs in a physiologic milieu. Moreover, this has allowed us to clearly demonstrate that glutamate-induced excitotoxicity is dependent on the functional integration of non-NMDA and NMDA receptor activation. These findings suggest a mechanism by which non-NMDA receptors contribute to glutamate-induced toxicity and

are consonant with the results of in vivo studies detailing the neuroprotective effect of non-NMDA receptor antagonists against CNS pathology induced by ischemia and hypoglycemia.

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Chapter 3

Glutamate-Induced Reduction of Membrane Potential in Cultured Rat Cerebellar Granule Neurons Occurs Only in the Presence of Reduced Temperature

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3.1 Introduction

The membrane potential of cells that are too small to be measured with a microelectrode can be determined by methods that use lipophilic ions such as tetraphenylphosphonium (TPP^+) (1). These compounds permeate freely through biological membranes, have the propensity for charge delocalization, and therefore passively equilibrate with electric potential (2). TPP^+ has been used for measuring membrane potentials ($\Delta\Psi$) in neural preparations such as neuroblastoma cells (3), neuroblastoma-glioma hybrid cells (4), and in synaptosomes (5-8). In the present study, we have estimated $\Delta\Psi$ in cultured rat cerebellar granule neurons (CGCs) in physiologic media at 37° and 22° using a variation of the method of Lichtshtein, et al. (4). Inasmuch as CGCs are known to be resistant to the toxic effects of glutamate in physiologic media at 37° (9-11) but vulnerable at 22° (12), we have investigated whether reduced temperature undermines the ability of cultured neurons to maintain normal resting membrane potential during glutamate exposure. Large decreases in $\Delta\Psi$ would have several deleterious effects, including a reduction of the voltage-dependent Mg^{2+} blockade of NMDA receptor ion channels with attendant passage of toxic quantities of Ca^{2+} into the neurons (13,14).

3.2 Materials and Methods

3.2.1 Materials.

Tetraphenylphosphonium bromide (TPP^+) was purchased from Acros Organics (Geel, Belgium). [^{3}H]TPP^+ (34 Ci/mmol), [^{14}C]D-sorbitol (217 mCi/mmol) and [^{3}H]H2O (1 mCi/ml) were purchased from Dupont NEN (Boston, MA). All other chemicals were purchased from Sigma (St. Louis, MO).

3.2.2 Uptake of [^{3}H]TPP$^+$

CGC cultures at 12 DIC were divided into two treatment groups: one was maintained throughout the experiment at 37° and the other at 22° C. The culture plates were washed three times with 1.0 ml Locke's buffer containing (in mM): 154 NaCl; 5.6 KCl; 1 MgCl; 2.3 CaCl; 5.6 glucose; 8.6 HEPES; pH 7.4. To prevent depolarization-induced cell volume changes from occurring, 100 mM sucrose was included in all buffers. The neurons were incubated for 5 min., after which the wash buffer was aspirated completely and replaced with 500 µl Locke's exposure buffer containing 5-10 µM [^{3}H] TPP$^+$ (0.01 - 0.03 Ci/mmol). High K^+ Locke's buffer containing 40mM NaCl and 120mM KCl was used to maximally depolarize neurons and determine $\Delta\Psi$-independent [^{3}H]TPP$^+$ uptake. After a 60 min incubation during which the radiolabel was allowed to equilibrate between intra- and extracellular compartments, 5 µl of either L-glutamate (300 µM final concentration) or vehicle were added and the cells incubated for a period of 5 or 20 min. The exposure buffer was then aspirated completely from the culture plates and analyzed for extracellular [^{3}H]TPP$^+$ content. Intracellular [^{3}H]TPP$^+$ content was determined after solubilization of the neurons in 500 µl 1% Triton X-100. All assays were performed in triplicate.

3.2.3 Determination of Intracellular Space.

Intracellular volume (ICV) was determined in both non-depolarized and K^+-depolarized neurons. Growth media was aspirated from the culture plates and the neurons washed three times in 22° C Locke's buffer containing 100 mM sucrose. The wash buffer was aspirated completely and replaced with 500 µl (4°C) Locke's containing [^{3}H]H_2O and [^{14}C]D-sorbitol. After gentle agitation for 1 min, 100 µl of the supernatant was removed and the volume of distribution of [^{3}H]H_2O (total intra- and extracellular space)

and [^{14}C]D-sorbitol (extracellular space) determined. ICV was taken as the difference between total and extracellular spaces.

3.2.4 Determination of $\Delta\Psi$.

For each condition, intracellular TPP^+ concentration was calculated from intracellular volume and [^{3}H]TPP^+ determinations. The TPP^+ distribution ratio (R_{TPP^+}) was determined from the following equation:

$$R_{TPP+} = \left(\left[TPP^+\right]_{in\ lowK^+} - \left[TPP^+\right]_{in\ hiK^+}\right) \Big/ \left[TPP^+\right]_{out}$$

R_{TPP^+} was inserted into the Nernst equation to obtain $\Delta\Psi$ as follows:

$$\Delta\Psi = -\frac{RT}{nF} \log R_{TPP^+}$$

where $\frac{RT}{nF}$ = -61 at 37° and -58 at 22°

3.3 Results

In the present study, 100 mM sucrose was present in all exposure buffers to prevent glutamate- and K^+-induced neuronal swelling, which would otherwise confound the ability to determine [^{3}H]TPP^+ concentrations in CGCs. Under these hyperosmotic conditions, the intracellular volume was calculated to be 2.71 ± 0.13 µl/10^6 cells and 2.68 ± 0.84 µl/10^6 cells in low and high K^+ media, respectively.

As shown in figure 3.1, neuronal membrane potential did not change significantly when CGCs maintained at 37° were exposed to 300 µM glutamate. $\Delta\Psi$ at 37° was

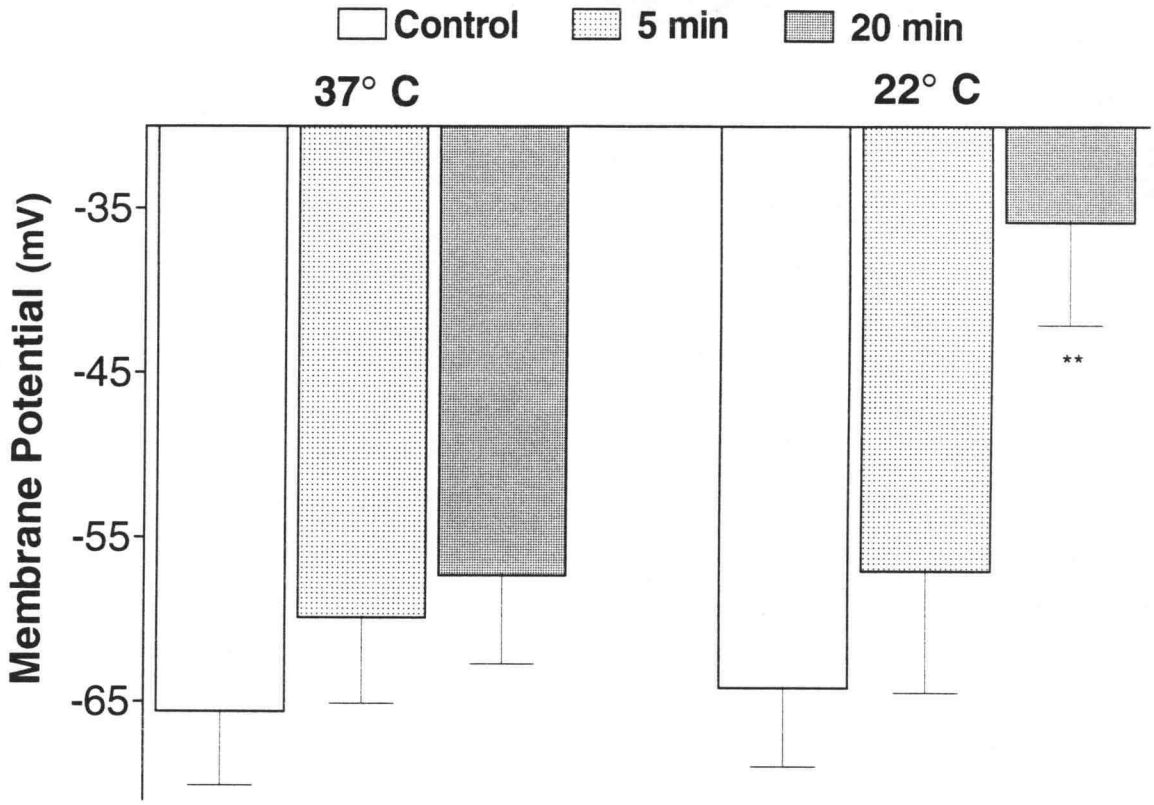

Fig. 3.1 Change in the membrane potential of 12 DIC cerebellar granule neurons exposed to 300 μM glutamate in physiologic media. Neurons were incubated in the presence of $[^3H]TPP^+$ for one hr at the indicated temperatures before the addition of glutamate. Neurons and media were analyzed for $[^3H]TPP^+$ content at the indicated times after glutamate addition. Determination of membrane potentials was performed as described in Materials and Methods. Values represent the mean ± SEM from two experiments performed in triplicate. ** $P < 0.003$

-65.6 ± 4.5 mV in control neurons, and -59.9 ± 5.2 and -57.3 ± 5.4 mV in glutamate-exposed neurons after 5 and 20 min, respectively. In contrast, CGCs maintained at 22° were unable to sustain normal resting membrane potentials when exposed to 300 μM glutamate. Although $\Delta\Psi$ in 22° control neurons (-64.1 ± 4.8 mV) was not significantly

different from controls at 37°, membrane potentials were reduced by 300 μM glutamate exposure to -57 ± 7.4 and -35.8 ± 6.3 mV after 5 and 20 min, respectively (fig. 3.1).

3.4 Discussion

The present results show that CGCs are able to maintain their membrane potentials close to resting levels when exposed to glutamate in physiologic media at 37°, but not when exposure occurs at 22°. These results are consonant with a previous report demonstrating that glutamate only induces excitotoxicity in rat CGCs in physiologic media when the temperature is reduced from 37° to 22° C (12). This inherent resistance of CGCs to acute glutamate-induced toxicity under physiologic conditions was thought to be due to their ability to maintain transmembrane electrochemical gradients at levels sufficient to preserve the voltage-dependent Mg^{2+} blockade of NMDA receptor ion channels. Excessive NMDA receptor activation would otherwise permit large amounts of Ca^{2+} and Na^{+} to enter the neurons, producing neuronal depolarization and Ca^{2+}-mediated delayed neurotoxicity (15). Moreover, the ability to maintain resting membrane potential would limit the duration of glutamate exposures by preserving glutamate transport function, inasmuch as the glutamate transporter is driven by the co-transport of Na^{+} down its concentration gradient (16-18). Large reductions in the Na^{+} gradient can induce a reversed mode of glutamate transporter operation, thus increasing extracellular glutamate concentrations (17,18). Furthermore, maintenance of low intracellular Na^{+} concentrations would promote optimal functioning of the high capacity, low affinity Na^{+}/Ca^{2+} exchanger, the activation of which becomes critical for limiting intracellular Ca^{2+}

concentrations during intense glutamate exposures (19-21). Loss of the ability to extrude intracellular Ca^{2+} would promote mitochondrial Ca^{2+} sequestration and toxicity, thereby reducing neuronal energy levels (22). The current results therefore support the notion that reduced temperature facilitates the induction of glutamate neurotoxicity in CGCs by compromising their ability to maintain normal electrochemical gradients during glutamate-induced ion flux.

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Chapter 4

Domoic Acid Neurotoxicity in Cultured Cerebellar Granule Neurons Is Mediated Predominantly by NMDA Receptors That Are Activated as a Consequence of Excitatory Amino Acid Release

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4.1 Abstract

The participation of NMDA and non-NMDA receptors in domoic acid-induced neurotoxicity was investigated in cultured rat cerebellar granule cells (CGCs). Neurons were exposed to 300 μM L-glutamate or 10 μM domoate for two hours in physiologic buffer at 22° followed by a 22 hr incubation in 37° conditioned growth media. Excitotoxic injury was monitored as a function of time by measurement of lactate dehydrogenase (LDH) activity in both the exposure buffer and conditioned media. Glutamate and domoate evoked respectively, 50% and 65% of the total 24 hr increment in LDH efflux after 2 hrs. Hyperosmolar conditions prevented this early response, but did not significantly alter the extent of neuronal injury observed at 24 hrs. The competitive NMDA receptor antagonist, D(-)-2-amino-5-phosphonopentanoic acid (D-AP5), and the non-NMDA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), reduced glutamate-induced LDH efflux totals by 73% and 27%, respectively, whereas together, these glutamate receptor antagonists completely prevented neuronal injury. Domoate toxicity was reduced 65-77% when CGCs were treated with competitive and non-competitive NMDA receptor antagonists. Unlike the effect on glutamate toxicity, NBQX completely prevented domoate-mediated injury. HPLC analysis of the exposure buffer revealed that domoate stimulates the release of excitatory amino acids (EAAs) and adenosine from neurons. Domoate-stimulated EAA release occured almost exclusively through mechanisms related to cell swelling and reversal of the glutamate transporter. Thus, while glutamate-induced injury is mediated primarily through NMDA receptors, the full extent of neurodegeneration is produced by the coactivation of both NMDA and non-NMDA receptors. Domoate-induced neuronal injury is also mediated primarily through NMDA receptors, which are activated secondarily as a consequence of AMPA/kainate receptor-mediated stimulation of EAA efflux.

4.2 Introduction

Among the excitatory amino acids (EAAs) known to produce toxic effects in the central nervous system, the neurotransmitter glutamate has been the most extensively characterized because of its involvement in CNS pathologies such as ischemia, hypoglycemia, trauma and epilepsy (1). Physiologically, rapid glutamatergic neurotransmission is mediated by non-N-methyl-D-aspartate (non-NMDA) receptors (AMPA/kainate receptors), which are sodium-permeable ligand gated ion channels (2). Glutamate also interacts with NMDA receptors, which form calcium and sodium-permeable ion channels that are activated in both a voltage and ligand dependent manner (3). NMDA receptors serve as coincidence detectors requiring concurrent depolarizing stimuli, such as occurs during high frequency synaptic activity, to relieve the voltage-dependent Mg^{2+} block of the channel. In this context, NMDA receptor signaling produces incremental increases in cytoplasmic calcium which activates processes that are fundamental to long term changes in synaptic strength (4,5). In certain pathological conditions, however, neurons possess insufficient metabolic energy to sustain normal resting transmembrane electrochemical gradients, and NMDA receptors are excessively activated. This produces large sustained increases in cytoplasmic calcium that ultimately cause neurodegeneration (6-8).

In dissociated hippocampal and cortical cultures, glutamate-mediated injury has been shown to progress through two distinct phases: an early neuronal swelling that is dependent on extracellular sodium and appears to be mediated by AMPA/kainate receptors, and an NMDA receptor-mediated delayed neurodegeneration that is dependent on extracellular calcium (1). Naturally occuring excitotoxic compounds, such as kainate and domoic acid, are known to interact specifically with AMPA/kainate receptors yet induce a calcium-dependent CNS toxicity similar to glutamate (9,10). The mechanisms by which AMPA/kainate receptor agonists cause neurotoxicity remain uncertain. In a

number of vitro systems AMPA/kainate receptor-mediated neurotoxicity is largely dissociated from the involvement of NMDA receptors, since NMDA receptor antagonists fail to protect against non-NMDA receptor agonist challenge (11,12). Moreover, only a prolonged exposure to AMPA/kainate receptor agonists produces neuronal degeneration in some experimental systems (1,12), while acute exposures are sufficient to kill neurons in others (13-15). Thus, whereas the toxicity of glutamate can be clearly linked to NMDA receptor activation and subsequent calcium entry, the mechanisms underlying AMPA/kainate receptor-mediated, calcium-dependent neurodegeneration have not been well defined in cell culture systems.

Studies in the adult animal show that AMPA/kainate receptor agonist-induced neurotoxicity is mediated by both NMDA and non-NMDA receptors. In the rat systemically administered kainic acid produces siezures followed by necrosis of neurons in the hippocampus, amygdala, piriform cortex and other limbic areas (16-18). Both competitive and non-competitive NMDA receptor antagonists have been shown to prevent the majority of this seizure related toxicity (16,17,19,20). These observations provide evidence consistent with the hypothesis that NMDA receptor activation plays a central role in neurotoxicity resulting from activation of AMPA/kainate receptors. It is also apparent from studies documenting the neuroprotective effects of non-NMDA receptor antagonists in animal models of focal and global ischemia, that both classes of receptor play important roles in glutamate-induced neurotoxicity (21-27).

In the present work, we have compared the mechanisms underlying glutamate- and domoate-induced neurotoxicity in cultured rat cerebellar granule cells (CGCs). Domoic acid was chosen as a model AMPA/kainate receptor agonist because it is a potential seafood contaminant with excitotoxic properties similar to kainate (28). Glutamate was studied for comparison because of its well documented ability to induce NMDA receptor-mediated toxicity in a number of in vitro systems. CGCs are a useful

system for this study because they express both NMDA and non-NMDA receptors (29) and can be grown in > 90% pure culture (30).

An important consideration when using CGC's for toxicologic experimentation is that exposure conditions must be manipulated to overcome the inherent resistance CGCs have to the cytotoxic effects of excitatory amino acids (EAA) (6,7,31,32). Thus, the specific experimental conditions employed affect both the intensity and pathways by which EAA-induced neuronal damage occurs (1,33-35). For agonists such as domoate, an appropriate model should utilize a physiologic medium, inasmuch as domoate intoxications are known to occur in initially healthy individuals (36,37). A physiologic medium preserves normal cell signaling mechanisms by maintaining the voltage-dependence of NMDA receptor activation and thus any functional interrelationships that exist between NMDA and AMPA/kainate receptors.

In the present report, CGCs were exposed to glutamate and domoate in physiologic medium at a temperature of 22°. Reduced temperature conditions have previously been shown to increase the neurotoxic potency of glutamate in cultured neurons (13,38). This has allowed us to directly compare glutamate and domoate-mediated toxicity under identical conditions and in the same temporal context (38). The present results confirm that glutamate and domoate toxicity are mediated by both NMDA and AMPA/kainate receptors. Moreover, we have shown in dissociated neuron cultures that acute domoate-induced neurotoxicity is largely mediated through NMDA receptors, which are activated by endogenous EAAs released into the media. This domoate-stimulated EAA efflux was pharmacologically characterized and shown to occur by mechanisms other than the vesicular release of excitatory neurotransmitter stores.

4.3 Materials and Methods

4.3.1 Materials

D(-)-2-amino-5-phosphonopentanoic acid (D-AP5), L-trans-pyrrolidine-2,4-dicarboxylic acid (PDA), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), and domoic acid were purchased from Tocris Cookson (Bristol, U.K). N^6-cyclopentyladenosine (CPA), 8-(p-sulfophenyl)theophylline (8-pSPT), 3-[(R)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP), N-(1- [2-thienyl] cyclohexyl)piperidine (TCP), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate(MK-801), dextrorphan (DX) and dextromethorphan (DXM) were purchased from Research Biochemicals Inc. (Natick, MA). Tetrodotoxin (TTX) was purchased from Sankyo Co. LTD. (Tokyo). Acetonitrile, L-glutamate, glycine, N-methyl-D-aspartic acid (NMDA), bovine serum albumin (BSA), trypsin, DNAse, poly-l-lysine (M.W. 393,000), cytosine arabinoside and o-phthaldialdehyde (OPT), soybean trypsin inhibitor (SBTI) were purchased from Sigma (St. Louis, MO). Basal Eagle's Medium (BME) was purchased from Gibco Labs (Grand Island, N.Y.). Chloracetaldehyde was purchased from Fluka Chemicals (Switzerland).

4.3.2 Cell Culture

Primary cultures of cerebellar neurons were obtained from 8 day old Sprague Dawley rats as described by Novelli et. al. (6), with minor variations. In brief, cerebella were dissected and the cells dissociated and suspended in basal Eagle's medium supplemented with 25 mM KCl, 2mM glutamine, 10 % fetal calf serum, and 100 µg/ml gentamicin. Cells were seeded onto poly-l-lysine coated (5 µg/ml) 6-well (35 mm dia.) culture dishes at a density of 2.5-3.5 $\times$ 10^5 cells/cm2. Care was taken to ensure a uniform plating density. The neurons were incubated at 37° in a 5 % CO_2, 95 % humidity

atmosphere. Cytosine arabinoside (10 μM) was added after 18-24 hours to inhibit the replication of non-neuronal cells. At 8 days in culture, 100 μl of a 25 mg/ml D-glucose solution was added to each plate to replenish energy resources and compensate for evaporative losses (6).

4.3.3 Excitotoxicity Assays

Neurons were utilized for toxicological assays at 12 days in culture. Growth medium was collected and saved, and the neurons washed twice in 1 ml Locke's incubation buffer containing (in mM): 154 NaCl; 5.6 KCl; 1.0 $MgCl_2$; 2.3 $CaCl_2$; 5.6 glucose; 8.6 HEPES; 0.1 glycine; pH 7.4. For experiments in which a hyperosmolar medium was used to inhibit neuronal swelling, 100 mM sucrose was added. 1.0 ml of fresh Locke's buffer containing the various test compounds was added to cultures followed by an incubation at 22° for 2 hrs. Control sister cultures were run in parallel with each treatment group. At the termination of incubation, the exposure buffer was collected and saved for later analysis of lactate dehydrogenase (LDH) activity and the cultures washed twice in 1 ml fresh Locke's followed by replacement with 2.0 ml of the previously collected and saved growth medium which was supplemented with 2.5 mg/ml D-glucose. Neurons treated for 2 hrs in the presence of 100 mM sucrose were maintained with the same concentration of sucrose in the conditioned growth medium. The cell cultures were returned to the 37° incubator. At 4, 8 and 24 hours after the beginning of the 2hr exposure, 0.25 ml aliquots of growth medium were collected and saved for analysis of LDH activity. LDH activity was assayed as described by Koh and Choi (39).

4.3.4 Measurement of Excitatory Amino Acids and Adenosine Release

Exposure conditions in release studies were identical to those used in excitotoxicity assays. Exposure buffer was collected at various timepoints and assayed for excitatory amino acid (EAA) content by HPLC after precolumn derivitization with o-phthaldialdehyde (OPT) (40). In general, 40 µl borate buffer (saturated solution, pH 9.5), 20 µl of an OPT solution (50 mg in 4.5 ml 100% methanol, 0.5 ml borate buffer, 50 µl ethanethiol) and 100 µl 100% methanol were added to a 40 µl aliquot of exposure buffer. Twenty microliters of the derivatized sample was applied to the column 2 min after the addition of OPT. A reverse phase column (Supelco LC-18 250 × 4.5 mm i.d.) was employed with a guard column (15 × 4.6 mm i.d.), both packed with 5 µm dia. particles. The effluent was monitored fluorimetrically using a model FS-970 Kratos fluorometer with the following settings for detection: excitation monochronometer 229 nm, a 470 nm cutoff filter for emission measurement, a 1.0 µA full scale range setting with a time constant of 0.5 s, and a sensitivity setting of 5.42 units. The mobile phase was 0.0125 M Na_2HPO_4 (pH 7.2) and acetonitrile in a gradient from 9 to 17 percent over 8 minutes followed by an increase to 49 % over 2 min, a hold for 3 min, and reduction to 9 % over 2 min to purge the column. Aspartate and glutamate were detected at retention times of 4.9 and 6.9 minutes, respectively.

Adenosine was measured fluorometrically after precolumn conversion to the N^6-etheno derivative (41). Equal volumes of chloracetaldehyde and exposure buffer were mixed (0.675 % final chloracetaldehyde concentration) and placed in a boiling water bath for 20 min. After cooling, 20 µl of the extract was injected onto the column using the same HPLC apparatus as for EAA measurement but with a 50 mM acetate (pH 4.5) and 6.5 % acetonitrile mobile phase (2 ml/min). For adenosine detection, the excitation monochronometer was set to 280 nm with a 418 nm emission cutoff filter, a 0.05 µA range setting with 5 s time constant, and sensitivity setting of 5.80 units.

4.4 Quantification of Results

4.4.1 Excitotoxicity assays

For each timepoint, total LDH activity in triplicate plates was calculated, the results averaged and LDH efflux in excess of control sister cultures run in parallel determined. The LDH efflux value obtained from exposure buffer at 2 hours was added to the 4, 8, and 24 hour growth medium LDH efflux values to give the total Δ LDH EFFLUX, which is a measure of the cumulative change in LDH activity over time. Results were normalized to the 24 hour LDH activity induced by either 300 μM L-glutamate or 10 μM domoic acid. Nonlinear regrassion analysis and graphs were made using Graphpad Prism software (San Diego, CA.).

4.4.2 Excitatory Amino Acid and Adenosine Efflux.

The fluorescent detection of adenosine, glutamate and aspartate derivatives was recorded on a Linear model 585 chart recorder. Peaks were excised and weighed, and the quantity of extract determined by comparison with external standards. External standards were expressed as a measurement of the area under the peak in picomoles. The quantity of amino acids or adenosine in sample extracts were then converted into units of concentration in the exposure buffer.

4.5 Results

In a previous report, we demonstrated that a 2 hr exposure to 300 μM L-glutamate or 10 μM domoate produced maximal neuronal losses of 65 and 79 %, respectively, in 12 DIC cerebellar granule neurons in a physiologic buffer at 22° (38). Moreover, this level of

EAA-induced neuronal injury was associated with a five- to eight-fold increase in lactate dehydrogenase (LDH) activity in the exposure buffer after two hours. Control neurons remained unaffected by these experimental manipulations. In the present experiments, identical exposure conditions were utilized and neuronal injury assessed by measuring LDH activity in the exposure buffer after 2 hrs and in conditioned growth media at 2, 4 and 22 hrs after the termination of excitotoxin exposure. A 24 hr profile of progressive neuronal injury was therefore derived from this cumulative change in LDH activity.

As shown in figure 4.1a, 50 ± 1.6 % of the 24 hr cumulative LDH efflux occured during the 2 hr glutamate exposure. Similarly, 64 ± 6.0 % of the domoate-stimulated LDH efflux was induced acutely (fig. 4.1b). Inasmuch as significant neuronal swelling was apparent within the first five minutes of EAA exposure, we investigated the extent to which osmotically driven swelling produced neuronal injury and LDH efflux. EAA-induced neuronal swelling was inhibited completely when 100 mM sucrose was added to both the exposure buffer and conditioned growth media. Moreover, LDH efflux at the 2 hr timepoint was reduced to 2.5 ± 0.8 % and 2.7 ± 0.7 % of 24 hr totals for glutamate and domoate, respectively. Therefore, the early leakage of LDH from neurons was generated by osmotic swelling (fig. 4.1a and 4.1b). After 24 hrs, however, there was no significant difference in total LDH activity in media from neurons exposed to EAAs under hyperosmolar as compared to isosmolar conditions (fig. 4.1a and 4.1b). Neuronal damage, which comprised a loss of approximately 70 % of the neurons with the disappearance of the majority of neurites, was virtually identical at 24 hrs under control and hyperosmolar conditions. This finding suggested that swelling by itself did not directly induce neuronal injury, but instead unmasked early damage to membranes and/or cytoskeletal elements. Neurons exposed to sucrose alone for 24 hrs were unaffected by the hyperosmolar conditions.

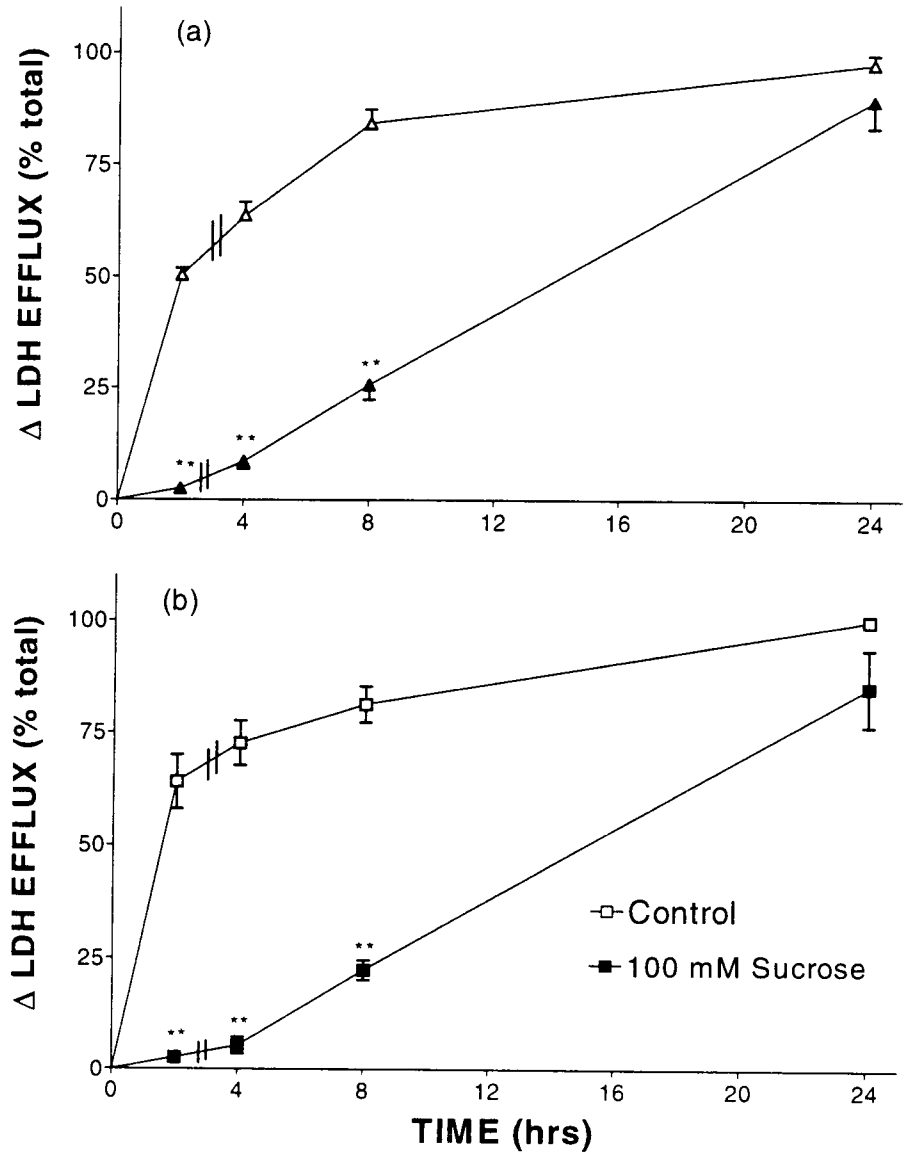
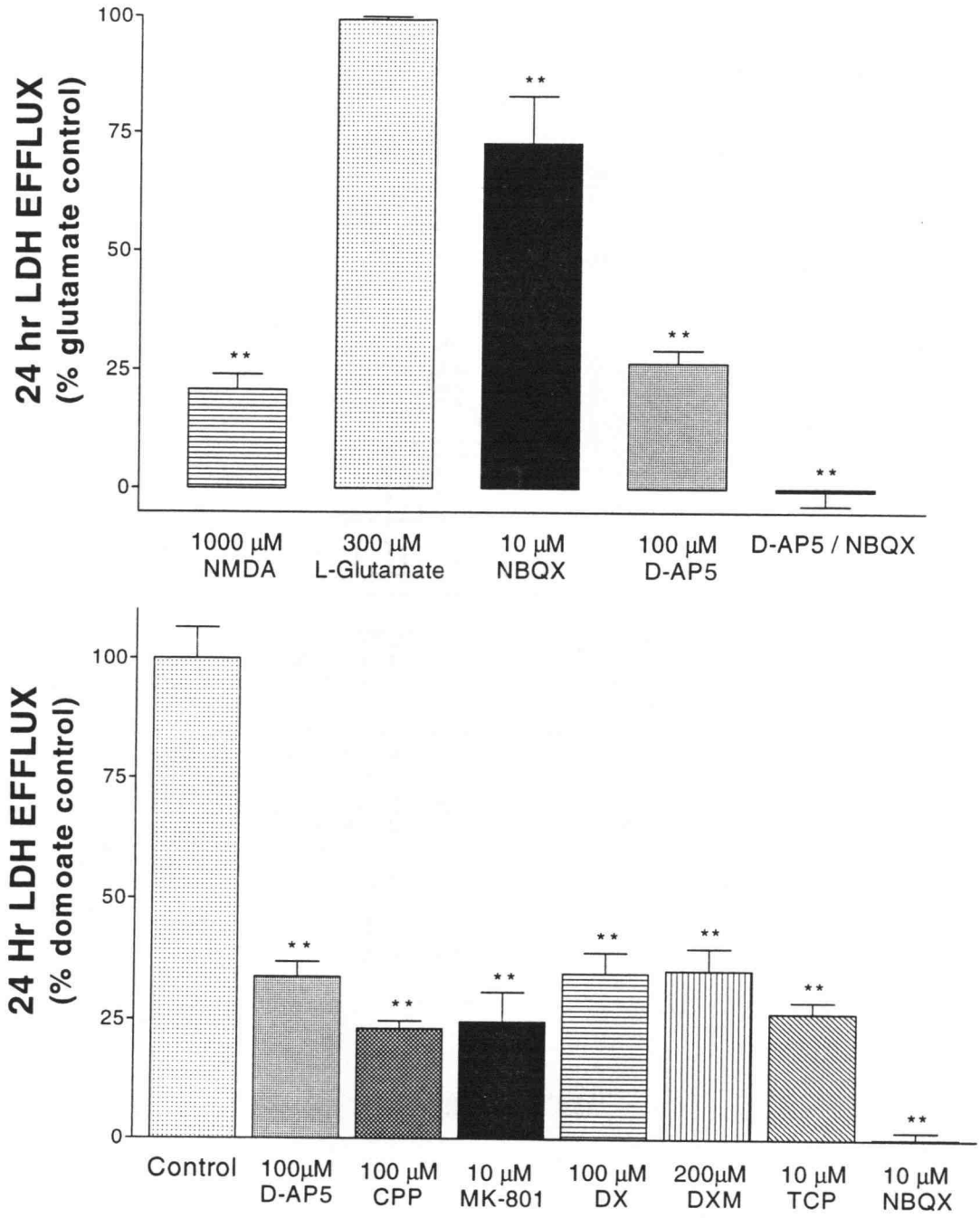


Fig. 4.1 Δ LDH efflux from 12 DIC rat cerebellar granule neurons exposed to 300 μM L-glutamate (a) or 10 μM domoate (b). Neurons were exposed to excitotoxins in 1 ml Locke's buffer for 2 hrs. At the termination of excitotoxin exposure, the Locke's buffer was replaced with 2 ml conditioned growth media supplemented with glucose and the neurons placed in the 37° / 5% CO_2 incubator. Aliquots of growth media were collected at the indicated times after excitotoxin exposure and the units of LDH activity determined. Values represent LDH activity in excess of non-intoxicated controls that were run in parallel with treated neurons. Slash marks indicate the change from exposure buffer to conditioned growth media. Each growth media LDH activity value was added to that determined from the exposure buffer to obtain a 24 hr profile of neuronal injury. Values are means ± SEM from at least 7 experiments run in triplicate. ** $P < 0.001$ as compared to controls.

In order to characterize NMDA and non-NMDA receptor contributions to glutamate- and domoate-induced cytotoxicity, the neuroprotective capacity of glutamate receptor antagonists was investigated. When glutamate receptor antagonists were utilized only during the 22 hr post-exposure period, no neuroprotection was afforded (results not shown). Moreover, EAA receptor antagonist neuroprotection was not significantly different between neurons treated with EAA receptor antagonists during the excitotoxicant exposure only or for the entire 24 hrs. Furthermore, cumulative 24 hr LDH activity was not significantly different between neurons treated in the presence or absence of 100 mM sucrose. Therefore, neurons were treated with glutamate receptor antagonists during EAA exposures only, and in the absence of sucrose.

Figure 4.2a shows the 24 hr cumulative LDH efflux levels in media from neurons exposed 2 hrs to 300 μM glutamate. In the presence of 10 μM NBQX, a concentration which would prevent the activation of AMPA and kainate receptors (24), glutamate-induced cytotoxicity was reduced modestly to 73 ± 10 % of control. Moreover, neurons exposed to glutamate in the presence of NBQX appeared as swollen after 2 hrs as neurons exposed to glutamate alone. By 24 hrs, neurite thinning and membrane blebbing were apparent and absolute neuron loss appeared somewhat reduced as compared to control. The competitive NMDA receptor antagonist D-AP5 reduced glutamate-induced LDH efflux more substantially, to 27 ± 3 % of control, and completely prevented neuronal swelling. Furthermore, little neuronal loss was apparent and neurites showed only minor thinning. When both EAA receptor antagonists were included in the exposure buffer, cerebellar granule neurons were afforded complete protection from neurotoxic injury (LDH efflux = -0.4 ± 3.1 % of control). As depicted in fig. 4.2a, NMDA was tested and found to produce only 21 ± 3 % of control glutamate cytotoxicity.

The EAA receptor antagonist neuroprotection profile against domate-exposed neurons contrasted sharply with that of glutamate. The non-NMDA receptor antagonist



4.2 Glutamate receptor antagonist neuroprotection profile in 12 DIC cerebellar granule neurons challenged with 300µM L-glutamate (a) and 10µM domoate (b). Bars represent the sum of the LDH activity, in excess of parallel-run controls, measured in the exposure buffer at 2 hrs and conditioned media at 24 hrs after initiation of glutamate exposure. Values are means ± SEM for at least 3 experiments run in triplicate. ** $P < 0.001$ as compared to glutamate control.

NBQX completely protected neurons from domoate-induced swelling and injury. Surprisingly, NMDA receptor antagonists also provided substantial neuroprotection against domoate-induced toxicity (figure 4.2b). The competitive NMDA receptor antagonists D-AP5 and CPP reduced domoate toxicity to 34 ± 3 and 23 ± 2 % of controls, respectively. Similarly, non-competitive NMDA receptor antagonists provided substantial neuroprotection. MK-801 reduced domoate toxicity to 25 ± 6 % of control, while dextrorphan, dextromethorphan and TCP reduced domoate-induced toxicity to 35 ± 4, 35 ± 5 and 26 ± 2 % of control LDH efflux, respectively. Although NMDA receptor antagonists afforded substantial neuroprotection after a 2 hr domoate exposure, it was noted that neurons appeared swollen to the same degree as glutamate control neurons yet underwent rapid recovery after replacement of the 37° conditioned growth media. By 24 hrs, neurites showed evidence of membrane blebbing and appeared thinner while the somata were unaffected by domoate treatment in the presence of NMDA receptor antagonists.

These results suggest that the majority of domoate-induced neurotoxicity is mediated by NMDA receptors, which may be activated as a consequence of domoate-stimulated release of endogenous EAAs. To test this hypothesis, cultures were exposed to10 μM domoate for 20 min and the incubation buffers assayed for the presence of glutamate and aspartate. The resultant EAA concentrations in exposure buffers from cultures containing approximately 2.5×10^6 neurons per plate were 10.0 ± 0.9 μM and 5.3 ± 0.76 μM for glutamate and aspartate, respectively. Although neuron densities were invariant between plates within a given culture, populations between cultures varied from 2.5 - 3.5 $\times$ 10^6 neurons per plate; therefore, results of EAA measurements were normalized to account for inter-experimental variability in amino acid concentrations resulting from neuron density variations. Figure 4.3 shows the concentrations of glutamate and aspartate in 1 ml of buffer after a 20 minute domoate exposure. NBQX completely prevented EAA efflux from neurons exposed to domoate. The competitive

NMDA receptor antagonist CPP reduced glutamate and aspartate efflux, respectively, to 45 ± 7 % and 15 ± 1 % of glutamate control. Similarly, the non-competitive NMDA receptor antagonists MK-801, dextromethorphan and dextrorphan reduced domoate-stimulated EAA efflux to 23 ± 8, 21 ± 3 and 29 % for glutamate and 9 ± 4, 15 ± 7 and 19 % for aspartate, respectively. These findings indicate that domoate, through the activation of non-NMDA receptors, induces the release of glutamate and aspartate from cultured cerebellar granule neurons. Glutamate and aspartate in turn activate NMDA receptors, which appear to promote further increases in EAA efflux into the exposure buffer.

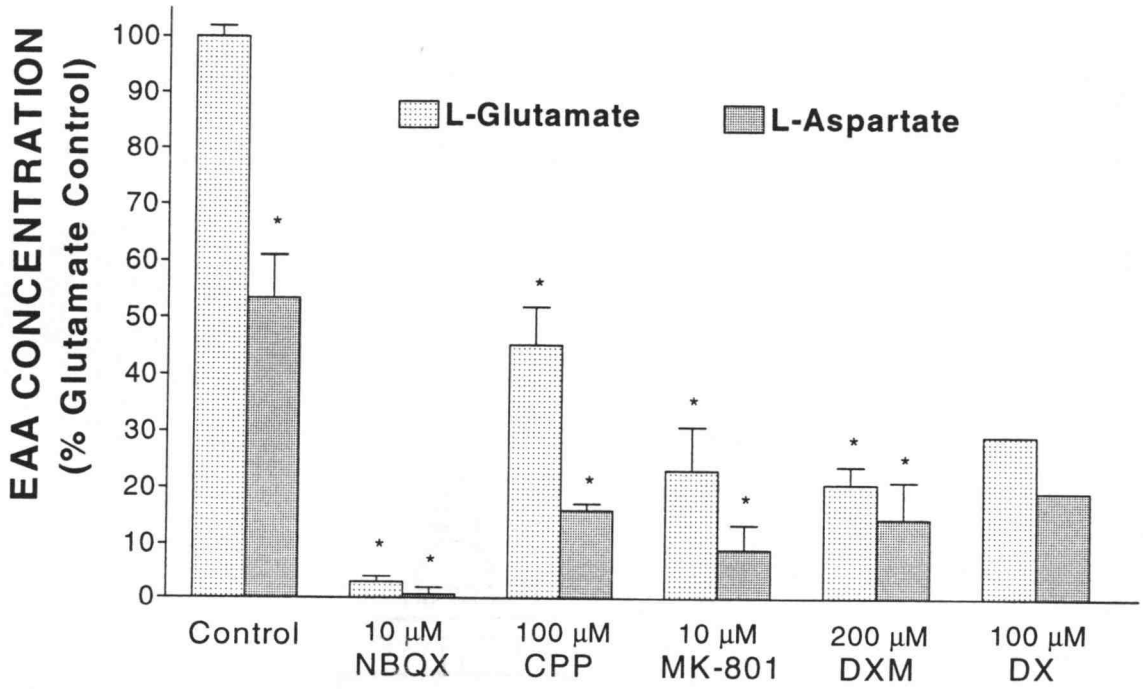


Fig. 4.3 Efflux of L-glutamate and L-aspartate from 12 DIC cerebellar granule neurons exposed to 10 μM domoate for 20 min. Neurons were exposed to domoate in 1 ml of Locke's buffer at 22° and aliquots analyzed for glutamate and aspartate by HPLC. Results were normalized to control L-glutamate concentrations. Values for all treatments except DX (N=1) represent means ± SEM from at least 3 experiments. CPP, 3-(R-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; DXM, dextromethorphan; DX, dextrorphan; Mk-801, methyl-10,11-dihydro-5-H-dibenzocyclohepten-5,10-imine NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline .* $P < 0.005$

To characterize the mechanism(s) of domoate-stimulated EAA release, cerebellar granule neurons were exposed to 10 μM domoate in a 1.0 ml volume and the buffer assayed for glutamate and aspartate at specific timepoints during the 20 minute exposure (figure 4.4). After a lag of 1 to 2 minutes, glutamate and aspartate concentrations increased biphasically up to the 15 minute timepoint, after which time EAA concentrations plateaued. When neurons were pre-incubated for 1 hr with 200 μM L-trans-pyrrolidine-2,4 dicarboxylic acid (PDA), a competitive and transportable inhibitor of the high affinity glutamate transporter, glutamate and aspartate concentrations were reduced by 47 ± 10 and 55 ± 15 %, respectively, at the 15 minute timepoint. Glutamate and aspartate efflux were reduced even further, by 90 ± 4 and 94 ± 5 % respectively, when swelling of PDA pre-exposed neurons was prevented with 100 mM sucrose. In this case, the EAA efflux profile appeared monophasic. The presence of sucrose alone reduced domoate-stimulated glutamate and aspartate efflux by 61 ± 3 and 80 ± 1 %, respectively.

From these results, it appears that domoate-exposed cultured cerebellar granule neurons release EAAs by two primary routes: reversal of the sodium-dependent glutamate transporter and via osmotically driven swelling with attendant EAA efflux. Further investigations were performed to ascertain whether a vesicular component of EAA release was also present. Tetrodotoxin (TTX) was utilized to inhibit neuronal depolarization resulting from Na^+ influx through voltage-dependent sodium channels. Neurons treated with 3 μM TTX showed a slight but significant reduction in the rapid phase of domoate-induced EAA efflux, but without a corresponding reduction in maximum EAA concentration (figure 4.5). When 10 μM CPA was used to activate A_1 adenosine receptors, and thereby inhibit vesicular release of EAAs, no change in

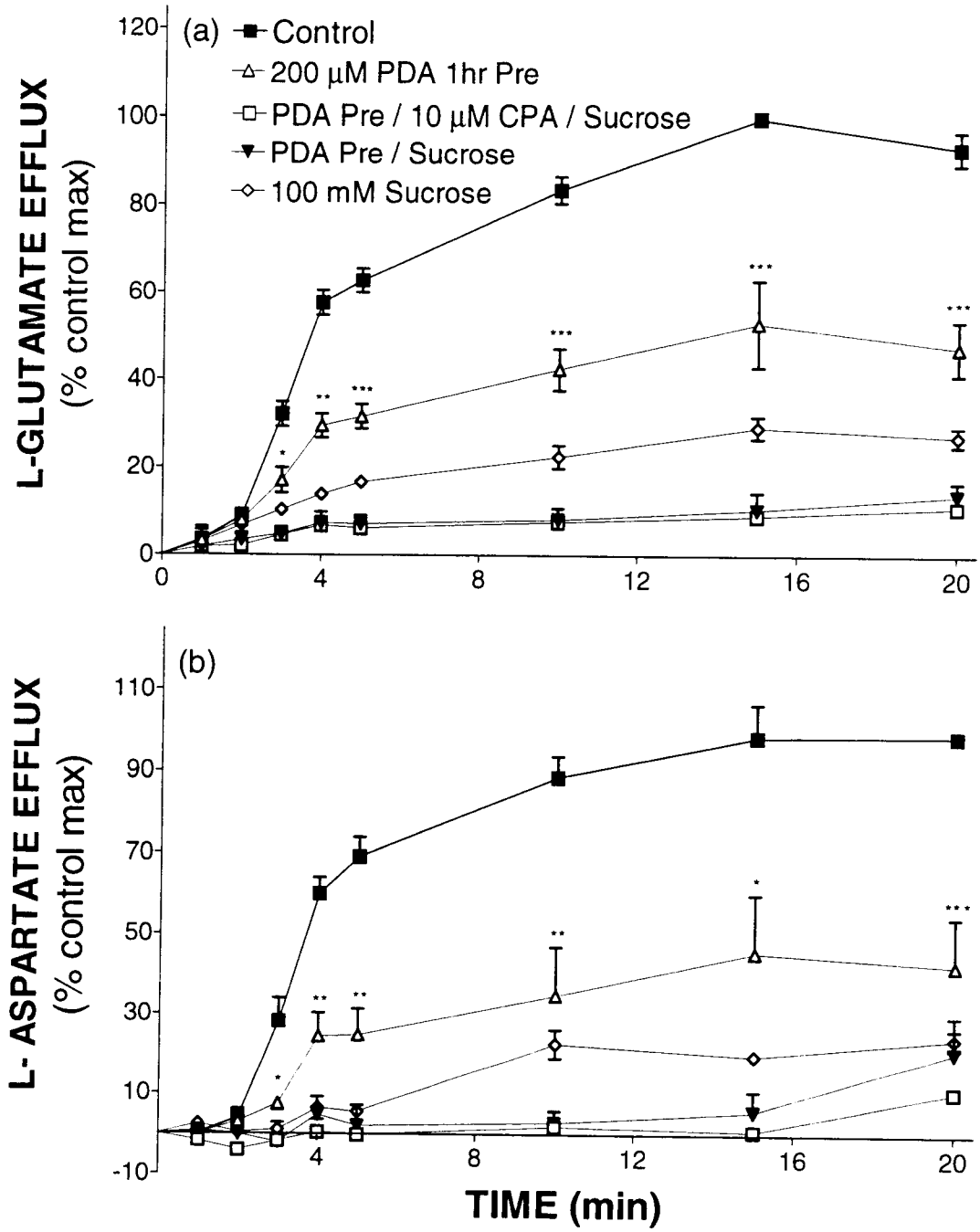


Fig. 4.4 EAA efflux from 12 DIC cerebellar granule neurons exposed to domoate. Cerebellar granule neurons were exposed to 10 µM domoate in a 1.0 ml volume and the buffer assayed for glutamate (a) and aspartate (b) at specific timepoints during the 20 minute exposure. Results were normalized to the maximum control value attained during the domoate exposure. Values represent means ± SEM from at least 3 experiments. * $P < 0.05$ ** $P < 0.005$ *** $P < 0.001$

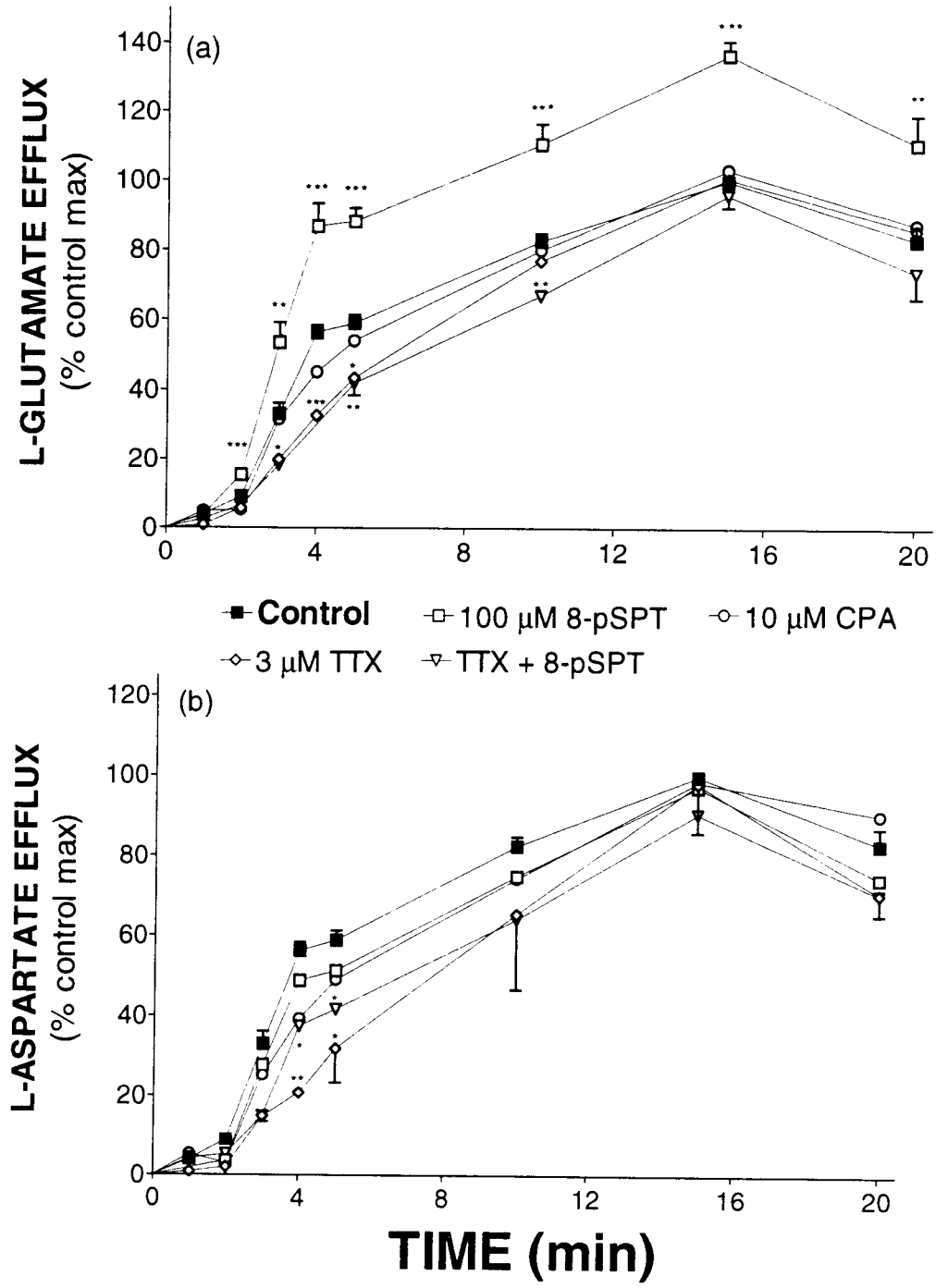


Fig. 4.5 EAA efflux from 12 DIC cerebellar granule neurons exposed to 10 µM domoate. Neurons were exposed to domoate in a 1.0 ml volume and the buffer assayed for glutamate (a) and aspartate (b) at specific timepoints during the 20 minute exposure. Results were normalized to the maximum control value attained during the domoate exposure. Values represent means ± SEM from at least 3 experiments. * $P < 0.05$ ** $P < 0.005$ *** $P < 0.001$

domoate-induced EAA efflux occured. The adenosine receptor antagonist 8-(p-sulphophenyl)theophylline (8- pSPT), however, elicited an increase in the rapid phase of domoate-induced glutamate efflux and elevated the maxumum glutamate concentration to 136 ± 4 % of domoate-stimulated controls (figure 4.5a). This 8-pSPT-induced increase in glutamate efflux was completely prevented by 3 μM TTX. 8-pSPT, however, was without effect on aspartate efflux (figure 4.5b). These results suggest that a small vesicular component of domoate-stimulated glutamate release is prevented by tonic adenosine receptor activation. The exposure buffer was therefore examined for the presence of adenosine. As shown in figure 4.6, 10 μM domoate caused the cerebellar granule neurons to release adenosine monophasically into the buffer. In three separate experiments (approx. 2.5×10^6 neurons/plate) the adenosine concentration in exposure buffer at 20 min reached a level of 1.25 ± 0.1 μM (fig. 4.6).

4.6 Discussion

A primary aim of this study was to characterize domoic acid-induced neurotoxicity in cultured cerebellar granule neurons under experimental conditions which simulate the neuronal milieu present in human intoxications. Inasmuch as the CNS pathology resulting from domoic acid intoxication occurs in normal healthy subjects, the model described herein employs a physiologic media which preserves normal cell signaling mechanisms. In a previous report, glutamate excitotoxicity was characterized in CGCs under identical exposure conditions (38). The present study extends those previous findings and directly compares glutamate- and domoate-induced neurotoxic processe

An initial concern was that the duration of excitotoxin exposure might induce an ion influx of sufficient magnitude to cause osmotic rupture of the neurons. Prevention of acute swelling with a hyperosmolar medium did prevent leakage of LDH from the

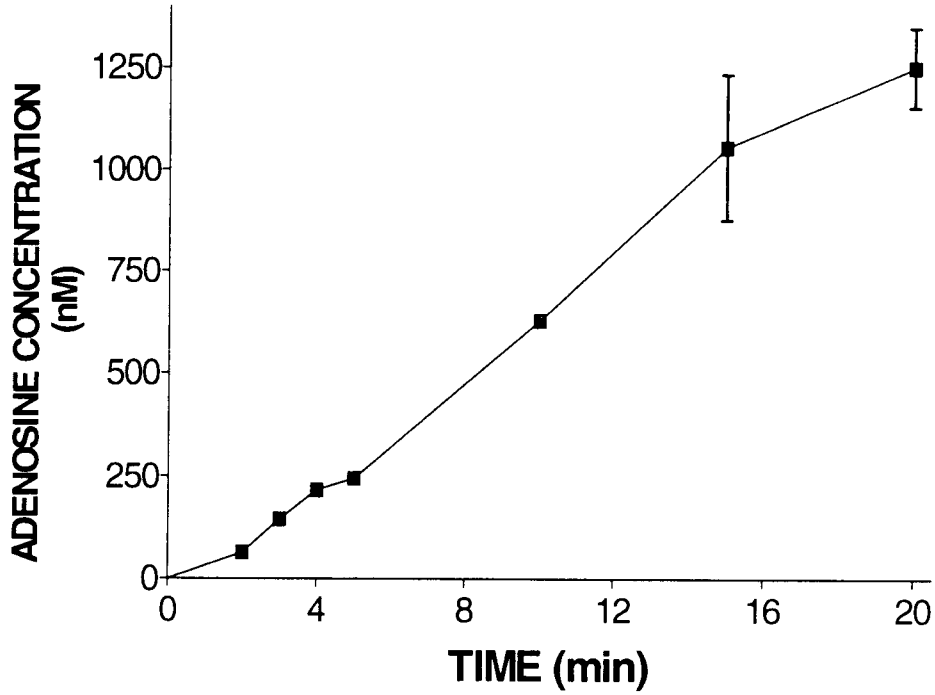


Fig. 4.6 Adenosine efflux from 12 DIC cerebellar granule neurons exposed to 10 μM domoate. Neurons were exposed to domoate in a 1.0 ml volume and the buffer assayed for adenosine at specific timepoints during the 20 minute exposure. Results represent means ± SEM from 3 experiments.

neurons. These findings are similar to those of Goldberg and Choi (8), who have shown that inhibition of swelling in cortical neuron cultures during oxygen-glucose deprivation protects against acute injury but not delayed neurodegeneration. In the present report, hyperosmotic conditions were expected to reduce total LDH accumulation by an amount equivalent to that released directly by osmotic rupture; however, this was not the case. We suggest that swelling produces acute neuronal injury only when membrane or cytoskeletal elements are damaged by processes related to NMDA receptor activation and Ca^{2+} entry into neurons. This conclusion is supported by the observation that neurons exposed to domoate in the presence of NMDA receptor antagonists appeared swollen to

the same extent as domoate-exposed controls after 2 hours, but recovered and were protected from subsequent neurodegeneration.

The present results indicate that NMDA and non-NMDA receptors functionally interact to produce acute glutamate- and domoate-induced excitotoxic responses, and both excitotoxins produce a primarily NMDA receptor-mediated cytotoxicity when exposed in a physiologic medium at 22°. These results differ from those previously reported by Kato et al. (15), who investigated kainate toxicity in CGCs. In their report, kainate-induced LDH release occurred within 30 minutes in 8 DIC and older CGCs, whereas glutamate was non-toxic at any age in culture. Moreover, neither acute nor delayed kainate toxicity appeared to involve the activation of NMDA receptors. There are several possible explanations which may account for these differences. First and foremost, 100 μM glycine was included in all exposure buffers in the present study. We have found that glycine enhances both the excitotoxic potency and efficacy of glutamate and augments NMDA-induced toxicity in this system (38). The inclusion of glycine renders this CGC medium physiologic inasmuch as glycine is present at micromolar levels in cerebrospinal fluid (41) and is required as a coagonist for NMDA receptor activation (42). The absence of glycine therefore will bias results away from the involvement of NMDA receptors. Second, neurons were subjected to a longer excitotoxin exposure period in the present study than in the report of Kato, et al. A longer excitotoxin exposure in conjunction with conditions that permit NMDA receptor signaling may explain why glutamate produced significant toxicity in this study. Third, kainate and domoate may not act in an identical manner to cause toxicity in CGCs. Domoate has been shown to induce larger currents through GluR5 homomeric receptors than kainate (43); therefore, differences in the ability to activate native AMPA/kainate receptors are likely to contribute to differences observed between kainate- and domoate-induced excitotoxicity. Moreover, the mechanisms of kainate- and domoate-induced EAA release from synaptosomes appear to be distinct (44). The presence of glycine in the exposure

buffer and distinct patterns of EAA release are therefore the most parsimonius explanations for the differences in the results with domoate, reported herein, and the previously described effect of kainate in CGCs (15).

Pharmacologic studies have shown that NMDA receptor-mediated increases in intracellular Ca^{2+} concentration are a major cause of excitatory amino acid-induced injury and death in neurons (1). Non-NMDA receptors, however, can also produce increases in intracellular Ca^{2+} by depolarizing neuronal membranes and activating voltage operated Ca^{2+} channels (45). AMPA/kainate receptor-mediated accumulation of intracellular Na^{+} may also allow Ca^{2+} entry through the Na^{+}/Ca^{2+} exchanger, which can operate in a reverse mode when the cell is depolarized (46). These mechanisms may account for the mild injury to CGCs induced by glutamate and domoate when NMDA receptor activation is blocked. Recent reports have also demonstrated the presence of Ca^{2+}-permeable AMPA/kainate receptors in specific neuronal and glial populations within the CNS; however, their existence on cerebellar granule neurons has not been reported (47). AMPA/kainate receptor-mediated depolarization may also increase free cytoplasmic Ca^{2+} by facilitating release of the Mg^{2+} blockade of NMDA receptor ion channels. This mechanism would explain why the AMPA/kainate receptor antagonist, NBQX, reduced the neurotoxic efficacy of glutamate in this study. Moreover, the absence of AMPA/kainate receptor involvement would partially account for the relatively mild injury incurred by CGCs during NMDA exposure. The lower efficacy of NMDA, as compared to glutamate in the presence of NBQX, may be explained by the partial agonist profile of NMDA at NMDA receptors (48,49).

An interesting finding was that domoate excitotoxicity is mediated predominantly by NMDA receptors under the conditions employed. This finding is in agreement with those of Favaron, et al. (13) and Manev, et al. (14), who demonstrated that kainate toxicity in CGCs could be reduced significantly by non-competitive NMDA receptor antagonists. Moreover, these findings agree with in vivo studies demonstrating that brain

damage induced subsequent to kainic acid-induced seizures is mediated by NMDA receptors (16-19). Novelli et al. (12) demonstrated that domoate-containing toxic mussels produce neurotoxicity in neuronal cultures through a synergism between excitatory amino acids. Their data show that subtoxic concentrations of domoic acid potentiate the excitotoxic effect of glutamate and aspartate, and that this neurotoxic synergism may occur through a reduction of the Mg^{2+} block at the NMDA receptor ion channel. Our data confirm and extend this earlier study by showing that the direct non-NMDA receptor-mediated effect of domoate produces only mild injury after a 2 hour exposure, whereas severe excitotoxicity results from the ability of domoate, through AMPA/kainate receptors, to stimulate the release of endogenous EAAs and effect a NMDA receptor-mediated neurotoxicity.

Competitive and non-competitive NMDA receptor antagonists reduced domoate-stimulated EAA release by 55 to 80 percent (fig. 3). Therefore, NMDA receptors mediate not only the majority of domoate-induced neuronal injury, but also a major component of EAA efflux. Kiedrowski et al., (50), demonstrated in cultured cerebellar granule neurons that transient applications of glutamate cause NMDA receptor-mediated increases in intracellular Na^+ that often exceed 60 mM. These large reductions in the Na^+ gradient were shown to impair the ability of neurons to extrude and/or buffer cytoplasmic Ca^{2+}, presumably due to a decrease in the efficiency of the Na^+/Ca^{2+} exchanger (50,51). We propose that, in addition to compromising the Na^+/Ca^{2+} exchanger, large NMDA receptor-mediated reductions in the Na^+ gradient inhibit or reverse the operation of the Na^+-dependent glutamate transporter. This possibility was investigated pharmacologically using PDA, a transportable, competitive inhibitor of the Na^+-dependent glutamate transporter (52). Indeed, when the reversed mode of glutamate transporter operation was inhibited by preloading neurons with PDA (53), domoate-stimulated glutamate and aspartate efflux were reduced by 50% (fig. 4). The remainder of non-vesicular EAA release evoked by domoate was almost completely accounted for by mechanisms related

to cell swelling. Neurons and astrocytes have been shown to counteract hyposmolarity-induced increases in cell volume by releasing taurine (54-58). Moreover, these regulatory volume decreases appear to involve chloride channels (59,60) with a molecular size exclusion limit of approximately the size of glutamine (61). Glutamate and aspartate are also released in response to cell swelling, and by a mechnism that has been shown to be inhibited by hyperosmotic sucrose (55). Sucrose alone in the present experiments prevented 61 and 80 percent of domoate-induced glutamate and aspartate release, respectively. The combination of sucrose and PDA reduced glutamate and aspartate efflux by 90 and 94 percent, respectively, indicating that domoate-stimulated EAA release occurs almost exclusively through mechanisms related to cell swelling and reversal of glutamate transport (fig. 4).

In a number of reports, the neuronal release of glutamate resulting from depolarization or ischemia has been shown to occur by two primary mechanisms: Ca^{++}-dependent vesicular release (62) or, in pathological conditions, by reversal of the high affinity Na^{+}-coupled glutamate transporter (53,62). The extent to which either mechanism predominates depends upon factors that affect neuronal energetics and ion homeostasis (62,63). Thus, experiments in cerebellar granule neurons show that the majority of EAA release induced by elevated K^{+} or veratridine originates from vesicular pools (64-66), whereas ischemia-induced EAA release has variably been shown to arise from vesicular (67), non-vesicular (68,69) or both vesicular and non-vesicular pools (70,71). In the present study little if any of the domoate-stimulated EAA accumulations could be accounted for by exocytotic mechanisms. Instead, we found that vesicular release of EAAs was inhibited by the concurrent release of endogenous adenosine from the neurons (fig. 6). Thus, a tonic inhibition of exocytotic EAA release was revealed by the adenosine receptor antagonist 8-pSPT (fig. 5a). 8-pSPT potentiated domoate-induced glutamate release in a TTX-sensitive manner, whereas aspartate accumulations were unaffected by this adenosine receptor antagonist (fig. 5b). This finding suggests that aspartate is not

contained in synaptic vesicles, a notion that is supported by a number of other in vitro studies (for review, see 72), including those on synaptosomes (63,73) and CGCs (74).

The demonstration that exocytotic glutamate release is inhibited by endogenous adenosine during domoate exposure is compatible with the results of Heron and Seylaz (75), who monitored the effects of veratradine and ischemia on intrahippocampal amino acid concentrations in rats by microdialysis. Under ischemic conditions, adenosine receptor agonists failed to modify EAA release, whereas theophylline significantly potentiated glutamate efflux, thus, indicating the presence of tonic adenosine receptor activation. Conversely, during veratridine-induced depolarization, adenosine receptor agonists reduced EAA release while theophylline was without effect. Thus, under the present conditions, cerebellar granule neurons appear to respond to domoate in a manner similar to ischemic neurons in vivo. Moreover, the present results are consonant with data showing that, not only is adenosine released in response to stimulation by NMDA and non-NMDA receptor agonists (76,77), but adenosine and its analogues also inhibit EAA release in a manner that is reversed by adenosine A_1 receptor antagonists (78,-80).

Recently, Tasker et al. (81), demonstrated that the competitive NMDA receptor antagonist CPP significantly attenuates CNS neurotoxicity when administered prior to domoate in rats, whereas MK-801 alone produced deleterious effects and AP5 was marginally protective. It was suggested that the failure to demonstrate a convinving neuroprotective response to AP5 is related to the poor bioavailability of this compound. In accordance with this interpretation our results with the direct application of NMDA receptor antagonists in vitro demonstrated an attenuation of domoate-induced neurotoxicity. Certainly, more detailed investigations will be required to ascertain the effectiveness of NMDA receptor antagonists against domoate neurotoxicity in vivo.

In conclusion, this report demonstrates that cultured cerebellar granule neurons in a physiologic milieu respond to NMDA and non-NMDA receptor agonists in a manner consistent with that reported in vivo. We have shown that the acute excitotoxic response

to both glutamate and domoate is predominantly mediated by NMDA receptors. NMDA receptors have been shown to be expressed in CGCs in primary culture (82). Glutamate activates NMDA receptors directly to produce a neurotoxic response. Concurrent non-NMDA receptor activation by glutamate produces an additional depolarizing stimulus which in turn potentiates excitotoxicity, either by increasing intracellular Ca^{2+} concentrations through voltage-sensitive Ca^{2+} channels, modulation of the Na^{+}/Ca^{2+} exchanger, or by reduction of the voltage-dependent Mg^{2+} blockade of NMDA receptor ion channels. In contrast, domoic acid produces a NMDA receptor-mediated excitotoxicity indirectly through the AMPA/kainate receptor-activated release of glutamate and aspartate into the medium. Domoate, therefore, synergistically potentiates glutamate/aspartate-mediated neurotoxicity. Cultured cerebellar granule neurons also respond to domoate-induced depolarization by releasing adenosine, which acts to suppress exocytotic release of glutamate; however, this compensatory response is offset by severe alterations in ion gradients which cause non-vesicular EAA release through both a reversal of high affinity glutamate transport and osmotically driven mechanisms.

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Chapter 5

Characterization of [^{3}H]MK-801 Binding to N-Methyl-D-Aspartate Receptors in Cultured Rat Cerebellar Granule Neurons and Involvement in Glutamate-Mediated Toxicity

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5.1 Abstract

The conditions required for growth and survival of cerebellar granule neurons in vitro are known to alter the developmental regulation of NMDA receptor subunit mRNA. In the present report, we have examined the functional and pharmacological characteristics of NMDA receptors on cerebellar granule neurons at 12 days in culture (12 DIC). Under open-channel conditions in extensively washed membranes, [^{3}H]MK-801 labeled a uniform population of sites (K_d = 3.2 ± 0.3 nM) in a saturable manner (B_{max} = 416 ± 18 fmol/mg); however, biexponential association and dissociation kinetics indicated the possible existence of at least two NMDA receptor populations which differ in pharmacological properties. The kinetically derived equilibrium dissociation constants for the high and low affinity binding components were 0.56 and 771 nM, respectively. The equilibrium competition analysis of MK-801 and other channel blocking compounds as displacers of [^{3}H]MK-801 revealed the presence of high and low affinity binding sites with relative apportionments of 70% and 30% respectively. The rank order potency profile of competitor binding at the high affinity site was (+)-MK-801 > TCP > dextrorphan > dextromethorphan > (+)-ketamine. When tested for the ability to protect 12 DIC cerebellar granule neurons from acute glutamate-induced toxicity, the neuroprotective rank order potency of these compounds was MK-801 > TCP > dextrorphan > (+)-ketamine > dextromethorphan, which correlated significantly with the high affinity competition binding profile and thus established the role of NMDA receptors in glutamate toxicity. The findings of these experiments indicate that NMDA receptors on 12 DIC cerebellar granule neurons are a heterogenous population that functionally mediate glutamate-induced neurotoxicity. The heterogenous [^{3}H]MK-801 binding sites may represent NMDA receptor channels composed of different subunits.

5.2 Introduction

Glutamate is believed to be the primary excitatory neurotransmitter in the mammalian central nervous system. It produces its actions through ionotropic NMDA, AMPA and kainate receptor subtypes and G-protein coupled metabotropic receptors, with each receptor subtype having a characteristic pharmacological profile and distinct physiological properties (1,2). Unique among ionotropic glutamate receptors is the NMDA receptor because of its high Ca^{++} permeability and involvement in synaptic plasticity, long term potentiation, learning and memory, and neurodegeneration. Regulation of NMDA receptor activity is complex. In addition to the binding of glutamate, NMDA receptor activation requires the binding of glycine at a distinct strychnine-insensitive coagonist site (3-6). The NMDA receptor ion channel is blocked in a voltage-dependent fashion by Mg^{++}, thus a concurrent depolarizing stimulus is required for the passage of ions to occur (7,8). In addition, pharmacologically distinct modulatory sites exist for the binding of Zn^{++} (9) and polyamines (10-12).

NMDA receptors have been characterized pharmacologically using a variety of compounds, among which are the dissociative anesthetics phencyclidine (PCP) and ketamine. The most potent of these channel blocking agents is the prototypical drug dizocilpine maleate (MK-801). These and related compounds are non-competitive NMDA receptor antagonists which act by binding to sites within the NMDA receptor ion channel in a voltage- and use-dependent manner. Their utilization in electrophysiologic and radioligand binding studies has revealed that NMDA receptor pharmacology is regionally heterogeneous within the CNS (13-17). The basis for this pharmacological heterogeneity has recently been confirmed by cloning studies showing that the NMDA receptor is composed of two distinct types of subunit encoded by five separate genes, one termed NMDAR1 (NR1) and four others termed NMDAR2A to NMDAR2D (NR2A-D) (18-20). Eight isoforms of NR1 cDNA generated by alternative mRNA splicing have also

been identified (9,21-23). NR1 is capable of assembling as a homooligomer that possesses many of the properties of the NMDA receptor. NR2 subunits however, do not form functional receptors but instead potentiate NMDA receptor activity when in heteromeric assembly with NR1. NR2 subunits also confer a functional variability to NMDA receptor channel complexes that is dependent upon specific NR1-NR2 heteromeric combinations (20,24). Because the NR1 subunit is essential to the formation of functional NMDA receptors, it is expressed in virtually all neurons of the CNS, whereas NR2 subunits display distinct heterogenous expression patterns (19,25).

NMDA receptor diversity is especially evident in the cerebellum, where studies have shown [^{3}H]MK-801 binding to be of lower affinity than in other brain areas (26-28). Earlier reports revealed either no detectable binding of [^{3}H]MK-801 to adult rat cerebellar membrane homogenates (29,30), or low levels of [^{3}H]MK-801 binding in extensively washed membranes (27). A unique cerebellar NMDA receptor pharmacology has also been demonstrated with the channel blocking compounds [^{3}H]dextrorphan (16) and [^{3}H] TCP (14,31). It is now known that cerebellar granule neurons are unique in that they express high levels of the NR2C subunit, thus contributing to the distinct pharmacology of cerebellar NMDA receptor channels and distinguishing cerebellar NMDA receptors from those in forebrain regions, which contain an abundance of NR1, NR2A and NR2B subunits but no NR2C expression (18-20,25).

Cerebellar granule neurons have been used extensively for studying the neurophysiology and neurotoxicology of glutamate receptors. In vitro growth and survival of cerebellar granule neurons requires the presence of high KCl concentrations or NMDA treatment. Prolonged K^+ depolarization specifically upregulates the expression of NR2A mRNA through an increase in resting intracellular calcium concentration (32,33). In addition, these treatments permit developmental changes in NR2B and NR2C mRNA expression that are normally observed during in vivo cerebellar granule neuron development, namely the disappearance of NR2B and increase in NR2C mRNAs (33,34).

The precise NR2 subunit stoichiometry of NMDA receptors in cultured cerebellar neurons is however uncertain and appears to vary with age in culture. Thus, agents acting on NMDA receptors may display a changing pharmacology as a function of age of cerebellar granule neurons in vitro.

In light of these recent findings, it is noteworthy that channel blocking radioligands have not been utilized in a rigorous examination of the pharmacology NMDA receptors in cultured cerebellar granule neurons. Such a characterization may provide further insight into the pharmacologic and functional characteristics of the endogenous cerebellar NMDA receptor phenotype. To address this issue, we have used [^{3}H]MK-801 to investigate the pharmacology of NMDA receptors in membranes derived from cerebellar granule neurons at 12 days in culture. Cerebellar neurons at this age have been shown to maximally express NMDA receptors and to be most sensitive to glutamate-induced toxicity (35). We have also assessed the potency of MK-801 and several other channel blocking agents to exert neuroprotective action against a maximally toxic glutamate challenge in cerebellar granule neurons.

5.3 Materials and Methods

5.3.1 Materials

L-glutamate, glycine, trypsin, cytosine arabinoside and fluorescein diacetate were purchased from Sigma (St. Louis, MO). N-(1- [2-thienyl]cyclohexyl)piperidine (TCP), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), dextrorphan (DX), dextromethorphan (DXM) and (+)-ketamine were purchased from Research Biochemicals Inc. (Natick, MA). Ethidium bromide was obtained from Bio Rad (Hercules, CA), and (+)-[^{3}H]MK-801 was purchased from New England Nuclear

(Boston, MA). Basal Eagle's medium was purchased from Gibco Labs (Grand Island, N.Y.).

5.3.2 Cerebellar Granule Cell Culture

Primary cultures of cerebellar granule neurons were obtained from 8 day old Sprague Dawley rats by a previously described method (36). Briefly, isolated cerebella were stripped of their meninges and minced by mild trituration with a Pasteur pipette. Cerebella were then treated with 2200 U/ml trypsin (Sigma cat. no. T-8918) for 15 min. at 37°C, and the granule cells dissociated by two successive trituration and sedimentation steps. Cells were suspended in Dulbecco's basal Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, 25 mM KCl and 100 μg/ml gentamicin, and plated onto poly-l-lysine (M.W. = 393,000) coated polystyrene culture dishes at a density of 2.5×10^5 cells/cm^2. Care was taken to ensure a uniform neuron distribution by thorough mixing of the suspension after addition to each plate. For excitotoxicity assays, neurons were plated onto 6-well 35 mm culture dishes (Fisher) whereas cells harvested for [^{3}H]MK-801 binding assays were plated onto single 100 mm plates. Cells were incubated at 37°C in a 5% CO_2, 95% humidity atmosphere. Cytosine arabinoside (10 μM) was added 18-24 hrs later to inhibit replication of non-neuronal cells. At 8 days in culture, 50 μL/ml of a 25 mg/ml D-glucose solution was added to replenish energy resources and to compensate for growth media evaporative losses.

5.3.3 Excitotoxicity Assays

Cerebellar granule neurons were exposed to L-glutamate and non-competitive NMDA receptor antagonists at 12 days in culture at a temperature of 22°C. Prior to glutamate exposure, growth medium was removed and the cultures exposed for 5 minutes

to 1 ml Locke's incubation buffer containing (in mM): 154 NaCl; 5.6 KCl; 1 MgCl; 2.3 CaCl; 5.6 glucose; 0.1 glycine; 8.6 HEPES; pH 7.4. The buffer was then replaced with 1 ml of fresh Locke's containing 300 μM L-glutamate with or without NMDA receptor antagonists and the cultures incubated for 2 hrs. Glutamate exposure was terminated by washing the neurons three times in fresh Locke's solution. The neurons were then maintained for a one hour post-exposure incubation in fresh Locke's prior to a five minute staining with the vital dye fluorescein diacetate (5μg/ml). Ethidium bromide (10 μl of a 50 μl/ml solution) was added four minutes after the fluorescein diacetate to stain the nuclei of nonviable neurons. The dye solution was replaced with fresh Locke's and the neurons placed on an inverted microscope (Zeiss model IM35) equipped with fluorescence optics. Soma and neurites of live neurons appear bright green in color whereas dead neurons do not accumulate fluorescein. Nuclei of dead neurons, that are not washed away, appear red due to ethidium bromide staining.

5.3.4 Assessment of Neuronal Viability

Five to six randomly selected fields from each plate were photographed at 80X magnification. Live neurons were counted from the photographic slides and the results averaged for each concentration of noncompetitive NMDA receptor antagonist. Results were referenced to the average control culture populations processed in parallel to experimental plates.

5.3.5 Preparation of Cerebellar Granule Cell Membranes

Membranes were prepared from neurons at 12 days in culture. Growth media was replaced with 2 ml of cold (4°C) 5 mM HEPES buffer (pH 7.4) containing 10 mM EDTA, the neurons scraped and collected in a 40 ml Dounce, homogenized, and then

centrifuged 10 minutes at 18000 rpm. The supernatant was discarded and the pellet resuspended with 20 ml HEPES/EDTA and recentrifuged. This last step was repeated in 5 mM HEPES without EDTA and the pellet was frozen for at least 30 min at -70° C. Membrane pellets could be stored frozen for up to one month with no loss in binding sites. Pellets were then thawed for 30 min, resuspended in 20 ml HEPES without EDTA, and centrifuged. This final pellet was resuspended in an appropriate volume of 5 mM HEPES for use in radioligand binding assays. Membrane protein content was determined by the method of Lowry (37).

5.3.6 Radioligand Binding

[^{3}H]MK-801 binding was determined in a reaction mixture containing 75-100 µg of cerebellar granule cell membrane protein, 10 µM glutamate, 10 µM glycine, H_2O or competitor and 10 µM EDTA in 5 mM HEPES pH 7.4 at 22°C, for a final volume of 1 ml. Non-specific binding is defined as that occurring in the presence of 10 µM (+)-MK-801. Under these conditions at 22°C, the (+)-[^{3}H]MK-801 association reaction progresses to >97% completion by 5 hr. After an incubation of 5 hr at 22°C, the reaction was terminated by rapid filtration over Whatman GF/B glass fiber fliters in a Brandel M48-R cell harvester. Filter strips were presoaked in 0.5% polyetheleneimine for a minimum of 2 hr to reduce binding of ligand to the filters. Filter discs were allowed to elute for at least 9 hr in Cytoscint liquid scintillation cocktail and then counted on a Beckman LS 6000SC scintillation counter.

5.3.7 Data Analysis

Nonlinear regression analysis was performed with Graphpad Inplot software (San Diego, CA). EC_{50} values for neuroprotection against exposure to glutamate and [^{3}H]MK-

801 competition binding to membranes were determined by nonlinear least squares fitting of a logistic equation to concentration response data. These data were analyzed according to the following equation:

$$Y = A + \frac{(B - A)}{1 + (C/[X])^D}$$

where Y equals the response, C is the ED_{50}, [X] the antagonist concentration, D the slope factor and A and B the minimum and maximum plateaus of the concentration-response curve. Saturation isotherms were similarly parameterized through non-linear regression analysis-based fitting of a hyperbolic equation to the data.

The parameters of [^{3}H]MK-801 association and dissociation kinetics were determined by fitting the following exponential equations:

$$RB_t = \sum_{i=1}^{n} [RB_{eqi}] \cdot \left(1 - e^{-k_{obs} \cdot t}\right)$$

$$RB_t = \sum_{i=1}^{n} [RB_o] \cdot e^{-k_{-1} \cdot t}$$

where, for site i of n total sites, RB_t is the amount of bound radioligand at time t, RB_{eq} is the amount of radioligand bound at equilibrium, k_{obs} is the apparent association rate constant, RB_o is the amount of bound radioligand at $t = 0$, and k_{-1} is the dissociation rate constant.

The criterion for assigning a multiple site model to equilibrium and kinetic ligand binding analyses is the F statistic:

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{(SS_2/df_2)}$$

where SS_1 and SS_2 are the sum of squares of the residuals for the one-and two-site fits, respectively, and df_1 and df_2 are the degrees of freedom for one- and two-site fits.

5.4 Results

Non-competitive NMDA receptor antagonist binding is known to be selectively stimulated by the NMDA receptor coagonists L-glutamate and glycine in extensively washed, divalent cation depleted rat brain membranes under low ionic strength conditions (30,38). Initial experiments revealed that concentrations of 100μM glutamate and glycine were required to maximally stimulate [^{3}H]MK-801 binding. When such conditions were utilized in equilibrium binding studies using membrane preparations from 12 DIC cerebellar granule neurons, [^{3}H]MK-801 labeled high affinity sites in a saturable manner

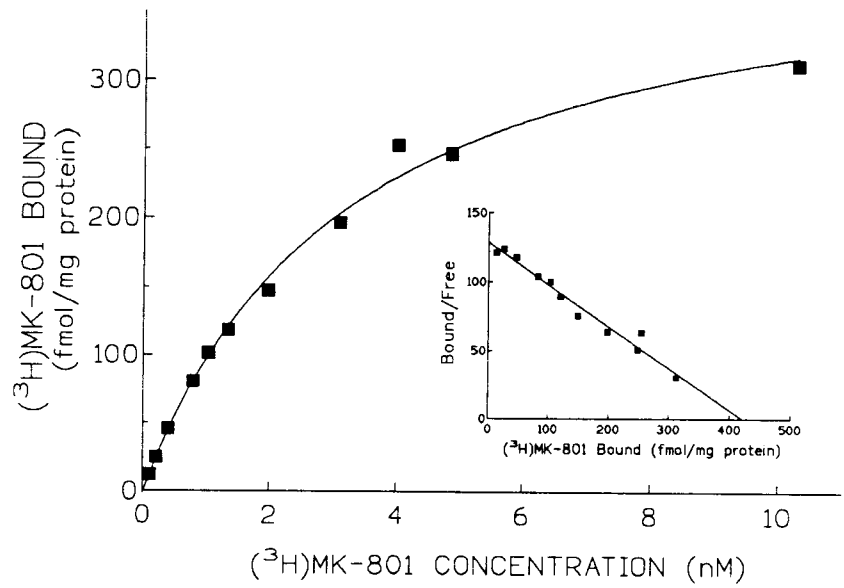

Fig. 5.1 Saturation analysis of 0.1-10 nM (^{3}H)MK-801 binding to extensively washed 12 DIC cerebellar granule neuron membranes. Membranes were incubated for 5 hrs at 22° in the presence of 100μM L-glutamate and glycine. Scatchard plot (inset) of the data yielded a K_d of 3.2 ± 0.3 nM and a B_{max} of 416 ± 18 fmol/mg protein in this preparation. Data is from one of three similar experiments.

(Fig. 5.1). Using 10μM unlabeled MK-801 to define non-specific binding, specific binding accounted for > 60% of the total binding at a [^{3}H]MK-801 concentration of 1.5 nM. Nonlinear regression analysis of the saturation data averaged from three experiments

indicated the presence of a uniform population of sites with apparent affinity (K_d) and site density (B_{max}) values of 3.2 ± 0.3 nM and 416 ± 18 fmol/mg protein, respectively (Fig. 5.1). The inset depicts the linear Scatchard replot of these data.

The association of [^{3}H]MK-801 with its recognition site on 12 DIC cerebellar granule cell membranes, in the presence of 100μM L-glutamate and glycine at 22° and at a radioligand concentration of 1.5 nM, progressed with a $t_{0.5}$ of 41.5 minutes (Fig. 5.2). Equilibrium was not attained under these conditions until after 300 min., at which time binding was > 97% complete. Non-linear regression analysis of the kinetics of association of [^{3}H]MK-801 indicated, however, that a simple bimolecular reaction model could not adequately account for the observed binding data. The fit was significantly improved by

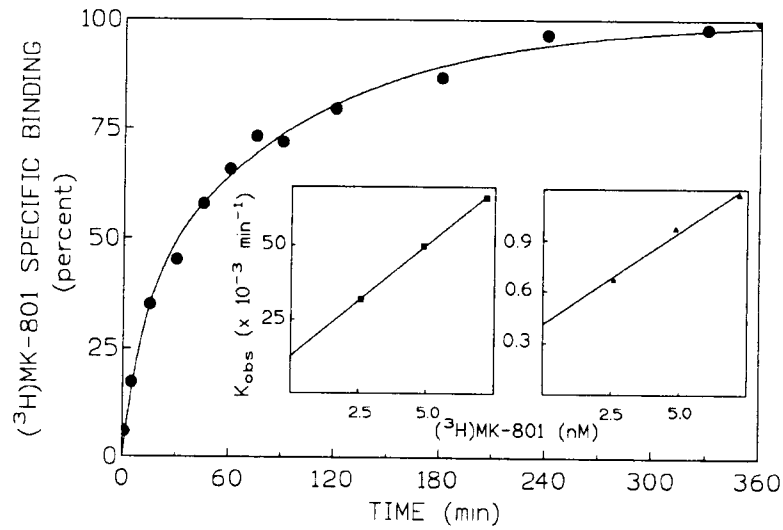

Fig. 5.2 Association kinetics of (^{3}H)MK-801 binding to extensively washed 12 DIC cerebellar granule cell membranes. Binding was initiated at 22° by addition of 1.5 nM (^{3}H)MK-801 to membrane preparations at specific time intervals in the presence of 100 μ M L-glutamate and glycine. Termination of the binding reaction was achieved by simultaneous filtration using a Brandel 48-well cell harvester as described in Materials and Methods. Normalized binding values represent the average of three experiments. Error bars were removed for clarity, but were no greater than 10% SEM. **Inset**: K_{obs} vs (^{3}H)MK-801 concentration for the fast and slow components of radioligand binding. Plots are from a single experiment using (^{3}H)MK-801 concentrations of 2.5, 5.0 and 7.5 nM.

acceptance of a more complex biexponential binding model ($F = 70.6$, $p < 0.0001$) which resolved [^{3}H]MK-801 binding into two kinetic components ($k_{obs1} = 88.2 \pm 12 \times 10^{-3}$ min^{-1} and $k_{obs2} = 10.1 \pm 0.44 \times 10^{-3}$ min^{-1}), with 35% of the total in the fast phase ($t_{1/2} = 8$ min) and 65% in the slow ($t_{1/2} = 69$ min). The kinetics of [^{3}H]MK-801 dissociation (Fig. 5.3), initiated by addition of 10μM MK-801, was similarly described by a more complex biexponential binding model ($F = 97.5$, $p < 0.0001$), while the $t_{0.5}$ for the overall

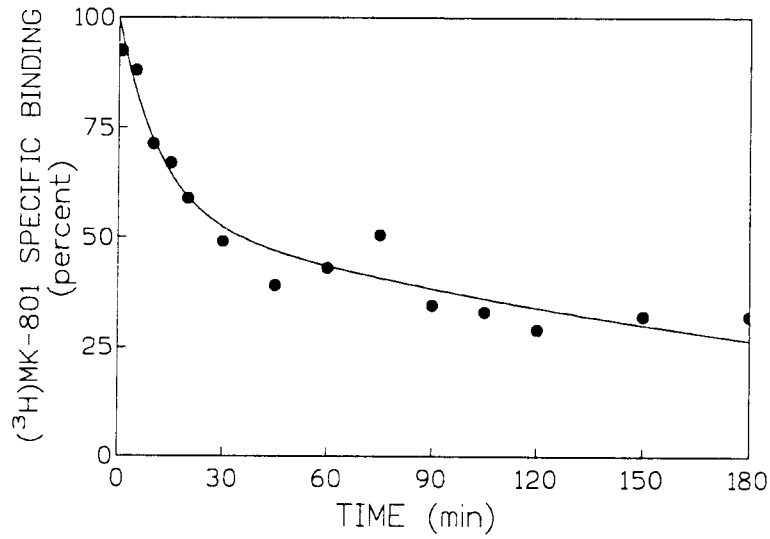

Fig. 5.3 Dissociation kinetics of (^{3}H)MK-801 binding to 12 DIC cerebellar granule cell membranes. After incubation of 1.5 nM (^{3}H)MK-801 with membranes for 5-6 hrs at 22° and in the presence of 100 μM L-glutamate and glycine, 10 μM MK-801 was added at specific time intervals and the binding terminated by rapid filtration. Normalized binding values represent the average of three experiments. SEM for each time point was no greater than 10%.

dissociation process was 54.2 min. The dissociation rate constants for the two components derived from non-linear regression analysis were: $k_{-1_1} = 83.3 \pm 13 \times 10^{-3}$ min^{-1} and $k_{-1_2} = 4.25 \pm 0.48 \times 10^{-3}$ min^{-1}, with 45% of the total in the fast phase ($t_{1/2} = 8$ min) and 55% in the slow ($t_{1/2} = 172$ min).

Association (k_{+1}) constants for the fast and slow components of [^{3}H]MK-801 binding were ascertained by determining the k_{obs} values for the fast and slow components of binding as a function of [^{3}H]MK-801 concentration. The kinetics of both components of [^{3}H]MK-801 association followed a pseudo-first order pattern as demonstrated by the k_{obs} values varying linearly as a function of ligand concentration (Fig. 5.2 insets). The rate constants (k_{+1}) derived from this kinetic analysis were respectively $7.53 \pm 0.25 \times 10^6$ M^{-1} min^{-1} and $0.108 \pm 0.013 \times 10^6$ M^{-1} min^{-1} for the fast and slow components. The ratios of dissociation and association rate constants, k_{-1}/k_{+1}, were used to calculate dissociation constants (K_D) for high and low affinity binding sites. The resultant kinetically derived K_D values for [^{3}H]MK-801 were 0.56 and 771 nM.

NMDA receptor pharmacology was examined further by ascertaining the affinity of MK-801 and other channel blocking compounds as displacers of [^{3}H]MK-801 from 12 DIC cerebellar granule cell membranes. For all compounds except ketamine, competition

TABLE 5.1

Neuroprotective potencies in live neurons and competition potencies for (^{3}H)MK-801 binding in membranes prepared from 12 DIC cerebellar granule cells. In vitro neuroprotection and (^{3}H)MK-801 binding assays were performed as described in Materials and Methods. Data were fit to a logistic equation by nonlinear regression analysis using Graphpad Inplot software as described in Materials and Methods.

Compound	**Neuroprotective EC50** (nM)		**Competition Binding IC50** High affinity (nM)	%	Low affinity (μM)	%	**95% CI** (high)
MK-801	8.28	(8.0-9.0)	3.3	73	4.18	27	2-5
TCP	165	(115-236)	14.9	73	38.3	27	10-22
Dextrorphan	1427	(1260-1620)	147	71	313	29	111-194
Ketamine	7022	(4400-11000)	1074	100			820-1400
Dextrometh-orphan	14820	(13000-16900)	402	69	26.8	31	241-672

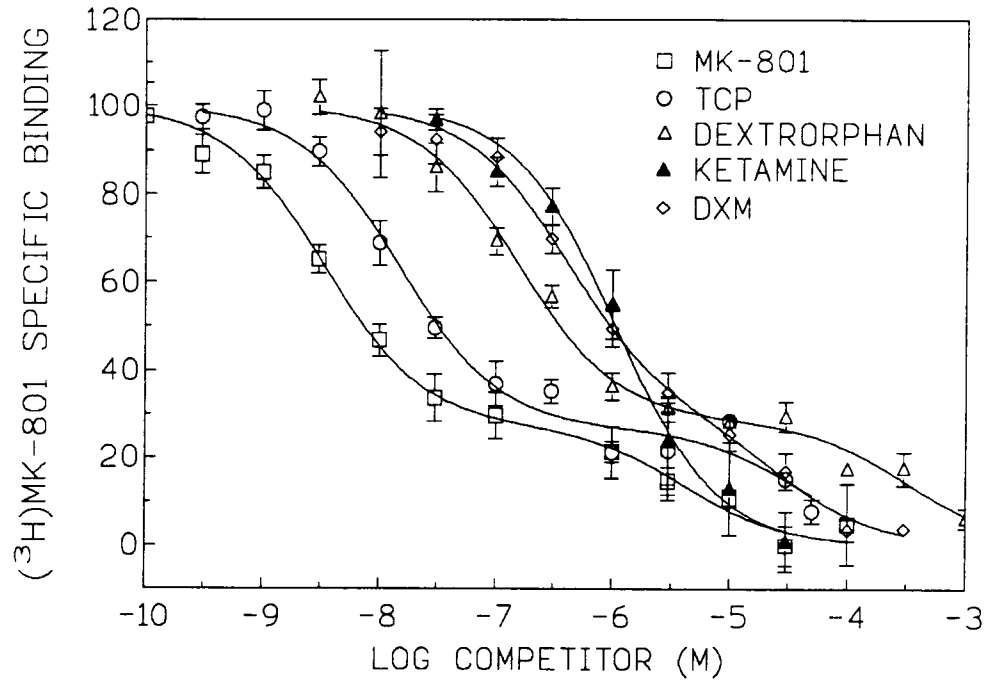

Fig. 5.4 (^{3}H)MK-801 competition binding in 12 DIC cerebellar granule cell membranes. Assay was performed as described in Materials and Methods, using washed membranes in the presence of 100 μM glycine and L-glutamate. Values for each point are the means ± SEM of data from at least three experiments. Non-specific binding was defined by 10 μ M MK-801.

curves were best described by a two site model ($p < 0.0001$), with 69-73% of the total being high affinity sites (Fig. 5.4 and Table 5.1). The rank order potency profile of competitor binding at the high affinity site was (+)-MK-801 (IC_{50H} = 3.3 nM) > TCP (IC_{50H} = 14.9 nM) > dextrorphan (IC_{50H} = 147 nM) > dextromethorphan (IC_{50H} = 402 nM) > (+)-ketamine (IC_{50} = 1074 nM). For the low affinity site, the rank order of potency was (+)-MK-801 (IC_{50L} = 4.18 μM) > dextromethorphanrphan (IC_{50L} = 26.8 μ M) > TCP (IC_{50L} = 38.3 μM) > dextrorphan (IC_{50L} = 313 μM).

In order to determine the relative potencies of noncompetitive antagonists as neuroprotectants, these compounds were tested for their ability to block glutamate-induced neurotoxicity in cerebellar granule cells. In the absence of non-competitive

antagonists, viable neurons were reduced by 65% when exposed for 2 hrs to 300μM L-glutamate in physiological buffer at 22° C (data not shown). As shown in figure 5.5, NMDA receptor noncompetitive antagonists potently and completely protected against glutamate-induced toxicity with a rank order potency profile of (+)-MK-801 (EC_{50} = 8.28 nM) > TCP (EC_{50} = 165 nM) > dextrorphan (EC_{50} = 1.43 μM) > (+)-ketamine (EC_{50} = 7.02 μM) > dextromethorphan (EC_{50} = 14.8 μM). This rank order potency profile was significantly correlated with the affinities of these compounds for the high affinity [^{3}H]MK-801 binding sites (Fig. 5.6; r = 0.96, p = 0.008).

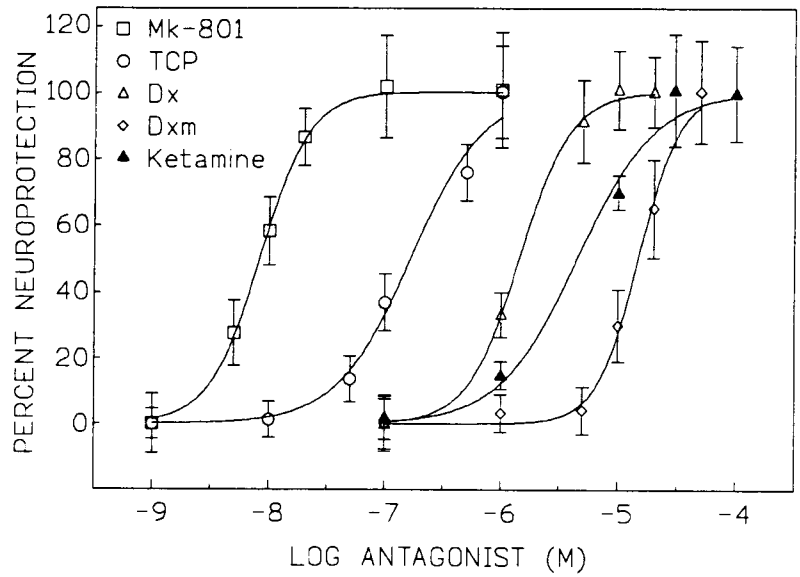

Fig. 5.5 Concentration response curves for non-competitive NMDA receptor antagonist neuroprotection of cerebellar granule neurons at 12 days in culture. Neuron cultures were exposed to 300 μM L-glutamate for 2 hr at 22° in the presence of 100 μM glycine. Quantitation of the neurotoxic response was performed as described in Materials and Methods. Results represent the average ± SEM of at least three separate experiments.

5.5 Discussion

A requirement for the growth and survival of cerebellar granule neurons in vitro is the presence of depolarizing conditions, which are thought to mimic the influence of tonic physiological activation in immature neurons during cerebellar development (39,40). An advantage of the use of primary cultures of neurons is the presence of native receptor

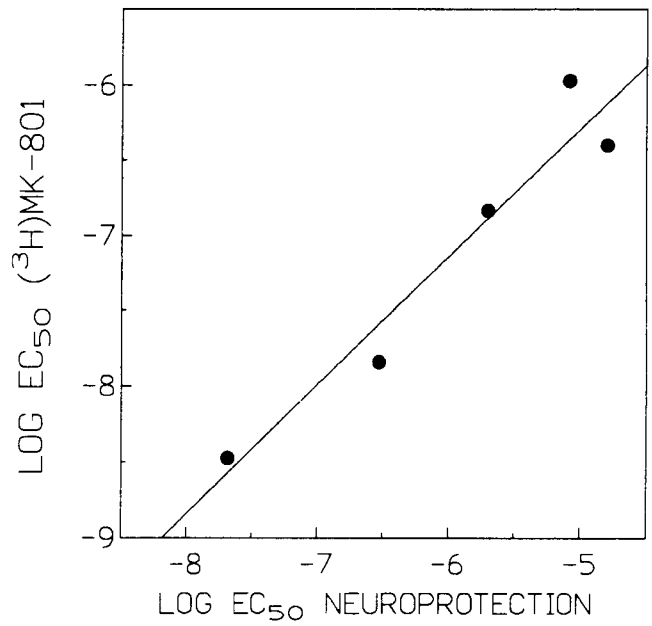

Fig. 5.6 Correlation between the potencies of compounds to compete for high affinity (^{3}H)MK-801 binding in 12 DIC cerebellar granule neuron membranes and their neuroprotective potencies against a 300 μM L-glutamate challenge ($r = 0.96$, $p = 0.008$). The significance of the correlation was evaluated by two-tailed t test of the probability that $r = 0$.

ensembles and signal transduction elements. These preparations afford the opportunity to explore the pharmacologic properties of cells expressing distinct heteromeric forms of NMDA receptors. It has been shown that depolarizing conditions induce the up-regulation of NR2A subunit mRNA and down-regulate NR2B mRNA followed by a gradual upregulation of NR2C subunit mRNA in cerebellar granule cells (32-34). These

changes in NMDA receptor subunit mRNA expression in cultured cerebellar granule cells parallels in vivo granule cell maturation, which is associated with down-regulation of NR2B and upregulation of NR2A and NR2C expression (41-43). In cerebellar granule cell cultures at 12-14 days in vitro the levels of NR2A and NR2C are comparable, whereas NR2B is undetectable (34). One maturational trend which is not preserved in culture is the developmental increase in the ratio of the NR1b splice variant compared with NR1a, which lacks an N-terminal insertion encoded by exon 5. Thus, in cerebellar granule cell cultures grown in depolarizing conditions the NR1a subunit mRNA predominates, whereas in vivo the adult cerebellum displays a ratio of NR1b/NR1a of approximately 5:1 (34). In the present report we investigated the pharmacologic and toxicologic significance of the NMDA receptor subunit composition of 12 day in culture cerebellar granule neurons.

The affinities of NMDA receptor ligands for heterologously expressed recombinant homomeric and heteromeric NMDA receptors have been measured and compared to those found in situ in rodent brain. Heteromeric NMDA receptors consisting of NR1-NR2A or NR1-NR2B have been shown to have [^{3}H]MK-801 affinities that are consonant with those found in native forebrain membranes (24,44-46). The [^{3}H]MK-801 binding affinity observed in the present study (K_d = 3.2 nM) suggests that NMDA receptors labelled by this radioligand in membranes derived from 12 DIC cerebellar granule neurons resemble that of assemblies of NR1 and either NR2A or NR2B subunits. Thus, the reported absence of NR2B mRNA in 12 DIC cerebellar granule cells suggests that the observed high-affinity binding of [^{3}H]MK-801 represents the labeling of NR1-NR2A complexes. This K_D value for [^{3}H]MK-801 is consonant with the values (4.0-7.3 nM) reported for the labeling of NMDA receptors in membranes prepared from 10 day in culture murine cerebellar granule cells (35). The affinity of [^{3}H]MK-801 for heterologously expressed heteromeric NR1-NR2C receptors has been demonstrated to be

substantially lower than that measured in adult murine cerebellum, which expresses high levels of NR2C subunit mRNA (24,44).

Recent studies using heterologously expressed NMDA receptor subunits suggest that NMDA receptor assemblies may be composed of three rather than two subunit types (44,45). Thus, Chazot, et. al. (1994) found that transfection of HEK 293 cells with varying ratios of NR1, NR2A and NR2C DNA resulted in expressed receptors that displayed an affinity for [^{3}H]MK-801 which was identical to that measured in membranes from mouse cerebellum, but four-fold lower than that in mouse forebrain. Interestingly, when the level of transfected NR2A DNA was increased relative to NR2C, the resultant affinity for [^{3}H]MK-801 tended to increase (lower K_D values) as well. Thus, the characteristic affinity of [^{3}H]MK-801 for mature granule cell NMDA receptors most closely parallels that of recombinantly expressed NMDA receptors containing NR1 and NR2A (and NR2C) subunits (44). It may therefore be inferred that the observed [^{3}H]MK-801 binding affinity on 12 DIC cerebellar granule cell membranes is due to an interaction with NMDA receptors containing primarily NR1-NR2A subunits with lesser amounts of NR2C. The use of a maximal [^{3}H]MK-801 concentration of 10 nM in these equilibrium saturation studies precluded labeling of the lower affinity [^{3}H]MK-801 binding sites associated with either NR1-NR2A-NR2C or NR1-NR2C assemblies.

Membranes from 12 DIC cerebellar granule neurons were further distinguished by the apparent biexponential kinetics of [^{3}H]MK-801 association and dissociation. Biexponential association and dissociation kinetics have similarly been reported for [^{3}H] MK-801 (47), [^{3}H]dextrorphan (16) and [^{3}H]TCP (48,49) binding to membranes derived from cerebral cortex and hippocampus. Although speculative, the most parsimonious interpretation of the biexponential kinetics of [^{3}H]MK-801 binding is that this represents binding to distinct NMDA receptor complexes with differing affinities. Consonant with this interpretation, the ratios of dissociation and association rate constants yielded kinetically derived K_D values of 0.56 and 771 nM which are in reasonable agreement

with the affinities of [^{3}H]MK-801 for recombinant NMDA receptors composed of NR1-NR2A and NR1-NR2C, respectively (44).

The notion that a heterogenous population of NMDA receptor channels exists on developing cerebellar granule neurons is further supported by the electrophysiologic studies of Farrant, et al. (50), who recorded single channel currents on cerebellar neurons during the period of migration from the external germinal layer to the inner granular layer of the cerebellum in rats from postnatal days 7 through 14. NMDA receptors on premigratory and early postmigratory neurons were shown to possess high conductance channels whereas low conductance channels appear at later postnatal stages and eventually predominate with age. These low conductance channels are characterized by much shorter (< 1ms) mean open times and burst lengths than the higher conductance channels, which is a characteristic shared by recombinant NMDA receptor channels consisting of NR1-NR2C heteromers (51). Moreover, the electrophysiologic characteristics of the high conductance channels measured in premigratory cerebellar granule neurons (50) are similar to those reported for cloned NMDA receptors consisting of NR1-NR2A or NR1-NR2B subunits (51). These findings have been extended by Ebralidze, et al. (52), who compared NMDA receptor channel conductances in cerebellar granule neurons from wild type mice and from mutant mice carrying a deletion of the gene encoding the NR2C subunit. In the mutants, spontaneous channel openings in whole-cell recordings and NMDA-activated single-channel currents from outside-out patches revealed the presence of an exclusively large conductance channel. In contrast, wild type mice possesed NMDA receptors with a wide range of single-channel conductances, which strikingly implies that individual channels in vivo are made up of different combinations of all three subunits. Thus, rather than two distinct populations of channels made up of only two subunit types (eg NR1-NR2A or NR1-NR2C), NMDA receptors in mature granule cells appear to exist as multimeric complexes with varying stoichiomeric ratios of NR2A and NR2C subunits in combination with NR1 (52). The

existence of NMDA receptor channels in cerebellar granule cells with differing proportions of NR2A and NR2C is consistent with the observed complexities of [^{3}H]MK-801 binding in the present study.

In equilibrium competition experiments, [^{3}H]MK-801 binding was displaced by a variety of non-competitive NMDA receptor antagonists. The rank order potency profile of these competitors was in general agreement with descriptions of the competitive displacement of [^{3}H]MK-801 in human (26) and rat (15,27,28,53) cerebral cortex and cerebellum. Moreover, these affinities for [^{3}H]MK-801 binding sites are well correlated with anticonvulsant potencies measured in rat (54) and mouse (55) experimental seizure models, and with the in vitro neuroprotection assay described in this report. The equlibrium competition binding data was best described by an interaction with both high and low affinity sites. The inability to adequately describe competition data with a one-site model has similarly been described for [^{3}H]MK-801 binding sites in human frontal cortex (56), [^{3}H]TCP sites in rat brain homogenates (38), and for [^{3}H]dextrorphan sites in rat forebrain and cerebellar membranes (16). All three radioligands label NMDA receptor channels, and the heterogeniety of the [^{3}H]MK-801 binding sites in membranes derived from 12 DIC cerebellar granule cells is again consistent with the existence of NMDA receptor channels with distinct assemblies of NR2 subunit forms.

Regarding the relationship between multiple [^{3}H]MK-801 affinity sites and neuroprotection, the non-competitive NMDA receptor antagonist rank order potency profile for neuroprotection reported here was highly correlated with the rank order profile for competition at high affinity [^{3}H]MK-801 binding sites. These high affinity sites comprised approximately 70% of total displaceable [^{3}H]MK-801 binding. These data suggest that the preponderance of NMDA receptor channels in 12 DIC cerebellar granule cells are composed of NR1-NR2A complexes and these channels mediate the neuroprotective effects of noncompetitive antagonists. The NR1-NR2A NMDA receptor channels in cerebellar granule cells are responsible for the large-amplitude conductances

and longer open time (40-52) properties and appear to mediate the neurotoxic effects of glutamate in these neurons. In contrast to NR1-NR2A channels, NMDA receptor channels composed of NR1-NR2C subunits display smaller-conductance levels and shorter open time (51). These channels are also less sensitive to Mg^{2+} and more sensitive to glycine than NR2A-containing complexes and therefore may be more likely to pass current in a given physiological situation (52).

In conclusion, the observed characteristics of [^{3}H]MK-801 binding provide pharmacological evidence that NMDA receptors on 12 DIC cerebellar granule neuron membranes are composed of at least two populations of channels with distinct subunit composition. One population of native NMDA receptor channels appears to be composed of NR1-NR2A subunits which constitute the high affinity binding site for [^{3}H]MK-801 and the target for the neuroprotective actions of MK-801 and related non-competitive antagonists of the NMDA receptor. These data also suggest that glutamate induced excitotoxicity in 12 DIC cerebellar granule cells is related to the activation of NR1-NR2A heteromeric NMDA receptor channels.

5.6 References

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