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# Neuropharmacologic characterization of strychnine seizure potentiation in the inferior olive lesioned rat

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# NEUROPHARMACOLOGIC CHARACTERIZATION OF STRYCHNINE SEIZURE POTENTIATION IN THE INFERIOR OLIVE LESIONED RAT

by

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

NEUROPHARMACOLOGIC CHARACTERIZATION OF

STRYCHNINE SEIZURE POTENTIATION IN THE

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by Melissa C. Anderson

Adviser: Melvin H. Van Woert, M.D.

Cerebellar stimulation is associated with anticonvulsant activity in several animals models. There are two afferent inputs to cerebellar Purkinje cells: 1) parallel fibers, which relay mossy fiber input, from brainstem, spinal cord, cerebral cortex and cerebellum, and 2) climbing fibers, arising from the inferior olive. Both climbing and parallel fibers release excitatory amino acid neurotransmitters (glutamate and/or aspartate), which stimulate Purkinje cells and cause GABA release in the deep cerebellar nuclei. Climbing fibers also exert tonic

inhibition over Purkinje cell activity by producing an absolute refractory period following stimulation, rendering Purkinje cells unresponsive to parallel fibers.

Climbing fiber deafferentation by bilateral inferior olive lesions produced a specific decrease in threshold for strychnine-seizures in the rat. Inferior olive lesions produced no change in threshold to seizures induced by picrotoxin, bicuculline or pentylenetetrazole. Inferior olive lesions also produced abnormal motor behavior including, myoclonus, backward locomotion and hyperextension, which was significantly aggravated by strychnine, brucine, picrotoxin, bicuculline and pentylenetetrazole.

Inferior olive lesions produced a significant increase in quisqualate sensitive [$^3$H]AMPA ((RS)-alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid) binding to cerebellar membranes. AMPA is a glutamate analog with high affinity for quisqualate sensitive receptors.

Systemic administration of glutamate diethylester, a quisqualate selective glutamate antagonist, caused reversal of inferior olive lesion induced decrease in strychnine-seizure threshold. This suggests that the behavioral effect of inferior olive lesions (i.e.,

decreased threshold for strychnine-seizure) is mediated by an increase in quisqualate sensitive glutamate receptors in cerebellum. Increased receptor binding may be the result of ectopic spine formation on Purkinje cells due to heterotypic reinnervation by parallel fibers.

A similar proconvulsive phenomenon occurs following systemic administration of azaspirodecanedione anxiolytics; buspirone, gepirone and isapirone. Strychnine specific proconvulsive effects of inferior olive lesions and buspirone were additive. This observation indicates that buspirone-induced decrease in strychnine-seizure threshold does not require intact inferior olive - climbing fiber pathways. Although the azaspirodecanediones have high affinity for 5-$HT_{1A}$ sites, the selective 5-$HT_{1A}$ agonist, 8-OH DPAT, had no effect on strychnine seizure threshold.

Decreased threshold for strychnine-seizure produced by bilateral inferior olive lesion and azaspirodecanedione pretreatment may be mediated through increased Purkinje cell activity and resultant decreased cerebellar efferent output.

## Acknowledgments

The completion of this dissertation has seemed like a monumental task for so long that it is hard to even conceive of writing these acknowledgments. There are so many people to thank. Foremost, my parents, for their continual love and support of my efforts. The encouragement received from you has seen me through many valleys, and I hope at the top of some peaks, as well. I also want to give a special thanks to my brother and sister.

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for validation of my fears and aspirations, and for feeding my rats - many, many thanks for your friendship. I would also like to thank Mark Dvorzniak for his ear, encouragement, insight and the occasional joke. To all my friends and colleagues, I thank you, for teaching, guiding and supporting me through my personal and academic crises and triumphs, you have made graduate school (and life) an enriching experience.

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## Dedication

To my parents, William and Suzanne, my brother, Andrew and my sister, Kate.

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List of Abbreviations

| | |
|---|---|
| AMB | abnormal motor behavior |
| AMPA | ((RS)-alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid) |
| 3AP | 3-acetylpyridine |
| AP4 | 2-amino-4-phosphonobutyric acid |
| AP5 | 2-amino-5-phosphonovaleric acid |
| AP7 | 2-amino-7-phosphoheptanoic acid |
| CD50 | dose which causes convulsions in 50% of the test group |
| DDT | 1,1,1-trichloro-2,2-bis (p-chlorophenyl)ethane |
| ETL | electrothermic lesion |
| GABA | gamma-amino-butyric acid |
| GDEE | glutamate diethyl ester |
| gDGG | gamma-D-glutamyl glycine |
| GVG | gamma-vinyl glycine |
| 5-HT | 5-hydroxytryptamine |
| i.p. | intraperitoneal injection |
| KA | kainic acid |
| Kd | dissociation constant |
| LTD | long-term depression |
| LTP | long-term potentiation |
| MK-801 | ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) |
| NAAG | N-acetylaspartyl glutamate |
| NMDA | N-methyl-D-aspartate |
| NMDLA | N-methyl-D,L-aspartate |
| 8-OH DPAT | 8-hydroxy-2-(di-n-propyl-amino)-tetralin |
| PCP | phencyclidine |
| PTZ | pentenyleneterazole |
| QA | quisqualic acid |

# CHAPTER ONE

## Literature Review

## Cerebellar Structure and Seizure Activity

## 1.1 Introduction

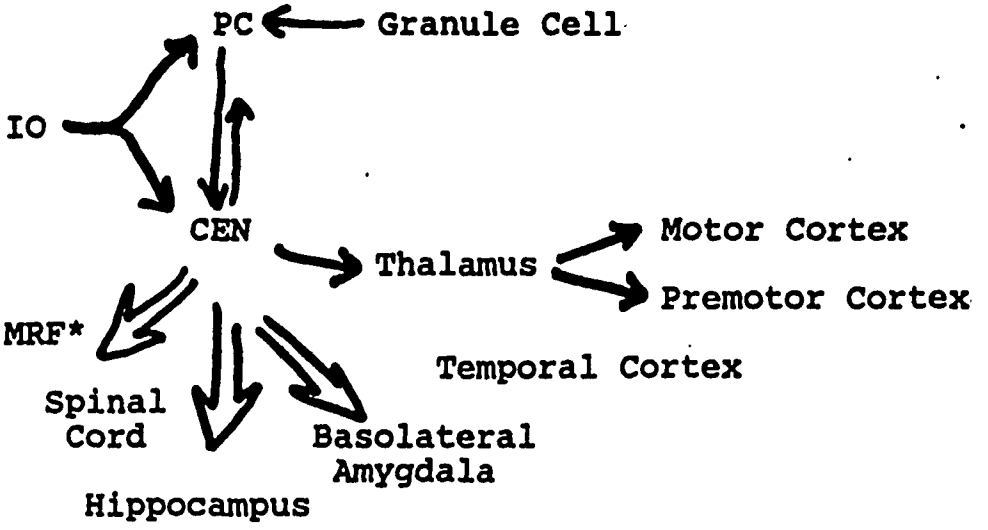
The cerebellum overlies the pons and medulla, with portions hidden under the occipital lobe of the cerebral cortex, and it regulates muscular activity. Referred to by Sherrington as "the head ganglion of the proprioceptive system", our knowledge of the scope of cerebellar influence on cortical function and sensory input has since expanded, as connections between cerebellar structures and virtually every other part of the brain have been discovered. It is now thought that the cerebellum coordinates and controls somatic motor activity, muscle tone and maintenance of equilibrium and this view is expanding to include motor learning (Marr 1969, Albus 1971). Marr's hypothesis of cerebellar function related to motor learning, as later modified by Albus, predicts that parallel fiber - Purkinje cell synapses in the cerebellum are subject to modification by the climbing fiber system. Presumably, climbing fibers influence Purkinje cells in such a way that, over time, a specific pattern of excitation results in a typical and predictable Purkinje cell response. Purkinje cells innervate the deep cerebellar nuclei, which are the fastigial nucleus, the interpositus nucleus and the dentate nucleus, the vestibular nuclei, including Deiters neurons; all of these will be referred to as cerebellar efferent nuclei.

Climbing fibers and mossy fibers are the two major afferent systems to the cerebellum, and the behavioral and biochemical consequences of climbing fiber deafferentation is the topic of this thesis. Background information relating cerebellar function to seizures and excitatory amino acid neurotransmission is presented below.

Previous studies by other investigators on cerebellar influence on seizure propagation and control provided the motivation for this work. In 1953, Snider and Cooke (1953) observed that electrical stimulation of the cerebellar surface can stop an electrically induced cerebral cortical seizure in both cats and monkeys. They further showed that stimulating the inferior olive also stopped this seizure, as did direct stimulation of the cerebellar efferent nuclei and the brachium conjunctivum, a cerebellar efferent system. In doing so, they outlined a three member neuronal circuit connecting the inferior olive, via cerebellar Purkinje cells to the cerebellar efferent nuclei. This pathway and efferent connections pertaining to motor output, are depicted in Figure one. These findings have been corroborated by Dow and coworkers (1962, Dow 1965) and others (Van den Dreissche and Trebaul 1958, Hutton, et al. 1972) who have shown that seizures induced by topical administration of cobalt, penicillin or strychnine to the cerebral cortex are reduced following cerebellar stimulation. Cerebellectomy, on the other

IO-PC-CEN Circuit**

And Cerebellar Efferent Connections




**Inferior olive - Purkinje Cell - Cerebellar Efferent Nuclei

Figure One

hand, increases the duration of cortical epileptiform discharges induced by electrical stimulation of cerebral cortex, or penicillin administration (Van den Dreissche and Trebaul 1958, Rains and Anderson 1976, Gartside 1979). Direct stimulation of the fastigial nucleus arrested seizures induced by metallic cobalt implanted in the hippocampus of both cats (Babb et al. 1974) and

monkeys (Heath 1976); the existence of a fastigio-septal connection has been demonstrated in both species (Heath and Harper 1974), which may be important in relaying inhibitory signals to the seizure focus.

Within the inferior olive - Purkinje cell - cerebellar efferent circuit, there are reciprocal connections between the inferior olive and cerebellar efferent nuclei (Tolbert et al. 1976, 1978), and the cerebellar efferent nuclei and Purkinje cells (Tolbert et al. 1978, Gould 1979). The inferior olive innervates both Purkinje cells and cerebellar efferent nuclei (D'esclin 1974), and efferent Purkinje cell projections are to the cerebellar efferent nuclei (including Deiters neurons of the lateral vestibular nuclei). Within the cerebellar cortex, Purkinje cells also send axon collaterals to cerebellar interneurons, such as basket cells, stellate cells and Golgi cells in the granule cell layer of the cerebellum, and other Purkinje cells (Hamori and Szentagothai 1966). Purkinje cell activity is also influenced by the mossy fiber - granule cell system, which directly innervates the cerebellar Purkinje cell as parallel fibers. As predicted by the Marr-Albus hypothesis, synaptic events and interactions between these systems determine the type of signals that are sent to the cerebellar efferent nuclei and outward, and have implications in motor learning and control (Watanabe 1984, Gellman and Miles 1985, McCormick

et al. 1985) as well as 'seizure arrest' function of the cerebellum (Snider and Cooke 1953).

Surface stimulation of the cerebellum, as employed by Cooke and Snider (1953a) to halt seizure propagation, activates all of the main elements of the above circuit. Purkinje cells are activated directly, and the inferior olive and cerebellar efferent nuclei by antidromic stimulation (Bantli et al. 1976). Direct stimulation of the Purkinje cell causes release of the inhibitory neurotransmitter GABA, which inhibits cerebellar efferent nuclei firing. Antidromic stimulation of the cerebellar efferent nuclei, by the cerebellar efferent nuclear - Purkinje cell collateral, will also occur and may override this inhibition and cause neurons of the cerebellar efferent nuclei to stimulate both Purkinje cells and extra-cerebellar structures (Tolbert et al. 1976). Stimulation of climbing fiber terminals present in the cerebellar cortex will result in antidromic stimulation of the inferior olive, causing tonic release of aspartate, the putative excitatory amino acid neurotransmitter of the climbing fibers (Crepel et al. 1982, Kimura et al. 1985) and production of a complex spike by Purkinje cells. The net result of a complex spike is Purkinje cell excitation followed by temporary suppression of parallel fiber - Purkinje cell impulses. This has been called the 'climbing fiber inactivation response' (Granit and

Phillips 1956). In addition inhibitory interneurons in the cerebellar cortex are stimulated, and they act to inhibit Purkinje cell firing. Thus the net effect of cerebellar surface stimulation may be decreased Purkinje cell activity and increased output by the cerebellar efferent nuclei. To better understand how signals transduced through this circuit, specifically the interactions of climbing fiber and parallel fiber inputs on Purkinje cell firing, affect seizures, a more detailed discussion of cerebellar circuitry is presented.

## 1.2 Cerebellar Circuitry

The cerebellum consists of a cortical shell divided into three layers:

1) The outer molecular layer contains Purkinje cell dendrites, terminals of both major afferent systems (climbing and parallel fibers) and inhibitory intracortical neurons. Intracortical neurons consist of stellate and basket cells, which, when stimulated inhibit Purkinje cell firing by releasing GABA (ten Bruggencate and Engberg 1971).

2) The Purkinje cell layer, which is adjacent to the molecular layer, contains the large Purkinje cell bodies; this portion of the cerebellar cortex also

receives climbing fiber terminals and a minor serotonergic input arising from the nucleus reticularis gigantocellularis of the reticular formation (Bishop and Ho 1985).

3) The granule cell layer, which is innermost, contains many small granule cells. Incoming mossy fibers synapse with the granule cells in rosettes, forming cerebellar glomeruli. The outer portion of these multi-synaptic processing units are contacted by axons of Golgi cells, large intracortical neurons also situated in the granule cell layer.

Mossy fiber input is transmitted to Purkinje cells in the molecular layer via long axonal projections of granule cells, the parallel fibers. The cerebellar efferent nuclei (the dentate, interpositus and fastigial) make up the core of the cerebellum, surrounded by cerebellar cortex, and they receive cerebellar cortical input from Purkinje cells. Purkinje cells also send projections to the vestibular nuclei. The majority of cerebellar output is relayed through efferent projections through the cerebellar nuclei to the brainstem, spinal cord and cortical structures.

Purkinje cells release GABA when stimulated and inhibit cerebellar efferent nuclei. The rate at which

Purkinje cells inhibit cerebellar efferent nuclei and the pattern of inhibition transmitted is determined by both parallel fiber input, in the form of simple spikes and climbing fiber afferents, which produce complex spikes in the Purkinje cell. While both parallel fibers and climbing fibers release excitatory amino acids (glutamate and/or aspartate) when stimulated, the anatomical distributions of synaptic inputs and the electrophysiological consequences of excitation by these two pathways are markedly different. There is evidence suggesting that the post-synaptic receptors subserving these two systems are also different.

### 1.2a Mossy Fiber Input

Mossy fiber input to the cerebellum is diffuse and modified at cerebellar glomeruli. This input is then transmitted to Purkinje cells via granule cell parallel fibers. The mossy fibers arise from spinocerebellar tracts, various regions of the brainstem, particularly the pons and parts of the cerebral cortex. Vestibulo-cerebellar pathways mediate impulses both directly and indirectly from the vestibular apparatus, some of these fibers terminate as mossy fibers, and some project to the dentate nucleus. Mossy fibers account for roughly half of cerebellar afferents. Once inside the cerebellum, mossy

fibers branch repeatedly and send collaterals to several folia. Input from an individual mossy fiber is highly processed and disseminated throughout the cerebellum. Mossy fibers synapse in the granule cell layer as the center of cerebellar glomeruli and these impulses are then relayed to the Purkinje cell via parallel fibers. Parallel fibers contact Purkinje cells at distal dendrites, en passent, in a way that has been described as 'crossing over' (Hamori and Szentagothai 1964). In this way, parallel fibers from a single granule cell contact dendrites of several different Purkinje cells. This type of synaptic distribution acts to further diffuse the effect of a single mossy fiber stimulation. Parallel fibers also contact interneurons, creating a net effect of patterned inhibition and excitation of Purkinje cells in the molecular layer.

Parallel fibers are thought to release glutamate when stimulated and produce a $Na^+$-$K^+$ action potential called a simple spike on the Purkinje cell. The high frequency of simple spike firing may account for Purkinje cell background activity, which ranges from 20-100 Hz. Simple spike production results in the release of GABA to cerebellar efferent nuclei. Parallel fiber stimulation elicits simple spikes from the distal dendrites of Purkinje cells, as does iontophoretic application of glutamate to the same area (Curtis and Johnston 1974,

Crepel et al. 1985). In addition, $K^+$ induced $Ca^{2+}$ dependent release of endogenous glutamate from cerebellar synaptosomes has been demonstrated (Levi and Gallo 1986); this release is reduced in synaptosomes prepared from agranular cerebella (Levi et al. 1982). [$^3$H]-glutamate uptake is also diminished in rats following X-irradiation, a procedure that selectively kills granule cells, the source of parallel fibers (Rohde et al. 1979). Glutamate levels are also reduced in agranular cerebella produced by X-irradiation, and in weaver, reeler and staggerer mutant mice (Rohde et al. 1979). A decrease in kainic acid (KA) receptor sites has been demonstrated in weaver, reeler and staggerer mutant mice (Griesser et al. 1982).

1.2b Climbing Fiber Input

Climbing fibers arise from a single source, the inferior olive and directly innervate Purkinje cells. Climbing fibers give off abundant collaterals and make repeated contact with one, or at most a few Purkinje cells, at the cell body and proximal dendrites, providing a dense one-to-one type of excitation that is distinct from parallel fiber innervation. The spontaneous firingrate of climbing fibers is tonic and low frequency, usually not exceeding 2 Hz. Climbing fiber stimulation results in the complex spike, which is a $Na^+$-$K^+$ spike

superimposed on a slow depolarizing after potential (EPSP) mediated by a $K^+$ - dependent $Ca^{2+}$ current (Crepel et al. 1981, Llinas and Sugimori 1980). Climbing fibers also send collaterals to the cerebellar efferent nuclei (D'esclin 1974) and to cerebellar cortical interneurons, although these are minor compared to the density of synapses made with the Purkinje cell.

Climbing fibers are believed to release aspartate when stimulated. Retrograde labeling studies using the metabolically inactive D-isomer of [$^3$H]-aspartate, show specific labeling of climbing fibers and cell bodies in the inferior olive (Wiklund et al. 1982, 1984). In addition, a $K^+$- induced $Ca^{2+}$- dependent release of endogenous aspartate has been demonstrated <u>in vitro</u> from cerebellar slices and aspartate release decreases significantly following inferior olive lesion (Wiklund et al. 1982). Cerebellar aspartate levels are also reduced following inferior olive lesion (Nadi et al. 1977). Additional evidence that climbing fibers release aspartate comes from pharmacological studies comparing rank order potency of excitatory amino acid antagonists in inhibiting complex spikes produced either by climbing fiber stimulation or iontophoretic application of aspartate.

## 1.3 Excitatory Amino Acid Receptors on Cerebellar Purkinje Cells

Glutamate and aspartate are excitatory neurotransmitters in the central nervous system, causing depolarization when applied to neurons. They are released from parallel fibers and climbing fibers following depolarization by $K^+$ or electrical stimulation, in a $Ca^{2+}$- dependent manner. A $Na^+$- dependent uptake system, with high affinity for both glutamate and aspartate has been demonstrated (Wofsey et al. 1971). In addition, multiple signal transduction mechanisms have been linked to excitatory amino acid receptors such as direct activation of ion channels, (Watkins and Evans 1981), stimulation of c-GMP formation (Foster and Roberts 1980) and both inhibition (Baudry et al. 1986) and stimulation (Nicoletti et al. 1986, Sladeczek et al. 1985) of phosphatidylinositol (PI) turnover. The ubiquitous distribution and multiple functions of glutamate and aspartate make it difficult to study and differentiate recognition sites responsible for different physiologic actions mediated by these amino acids. Among these functions are nitrogen storage, protein synthesis and involvement in synthetic pathways, in the nervous system, for example, glutamate is the precursor for GABA. Other biological recognition sites for excitatory amino acids

include active sites of enzymes utilizing these compounds as either substrate or product, uptake systems and transport mechanisms. It is generally recognized in pharmacology that the relative affinities of ligands for specific neurotransmitter receptors is greater than for enzymatic recognition sites and uptake processes, and this criteria is often used to differentiate 'binding' of a specific ligand to a neurotransmitter receptor as opposed to the active site of an enzyme. For excitatory amino acid recognition sites, however glutamate and aspartate are rather poor agonists for the specific excitatory amino acid neurotransmitter receptors. Structural analogs of the excitatory amino acids have much greater affinity and are better probes for studying and categorizing excitatory amino acid receptors. At present, excitatory amino acid analogs, with varying degrees of agonist and antagonist activities are available, however, new compounds with greater selectivity are needed to clarify receptor classifications. Several good reviews on excitatory amino acid receptor classification and function are available (Robinson and Coyle 1987, Watkins and Olverman 1987, Cotman et al. 1987, Foster and Fagg 1984, McLennan 1983).

Excitatory amino acid receptors have been subdivided into three classes based on electrophysiological, biochemical and pharmacologic studies, as presented in Table 1. The most selective compounds for excitatory

amino acid receptors are the glutamate analogs, quisqualic acid (QA), kainic acid (KA) and N-methyl-D-aspartate (NMDA), These receptors are defined by selective electrophysiologic agonist actions, which have been corroborated by radioligand binding studies and activation of several receptor-mediated effector mechanisms. The excitatory amino acid analog, 2-amino-4-phosphonobutyrate (AP4) was proposed to label a putative fourth type of excitatory amino acid receptor, however, it has since been concluded that AP4 acts mainly as an antagonist for QA induced depolarization (Watkins and Olverman 1987) and may label high affinity uptake sites, not post-synaptic receptors (Bridges et al. 1986, Fagg and Lanthorn 1985).

### 1.3a Quisqualic Acid and Kainic Acid Receptors

Both QA and KA generate rapid depolarizations, which result in fast excitatory synaptic transmission. Recent experiments in cultured cerebellar neurons and dissociated hippocampal cells show activation of multiple single channel conductances by QA and KA, as well as by glutamate and NMDA (Cull-Candy and Usowicz 1987, Jahr and Stevens 1987). It has been suggested that the different conductance states observed are mediated by distinct receptors coupled to the same channel (Cull-Candy and Usowicz 1987). Another excitatory amino acid analog,

Table 1. Excitatiory Amino Acid Receptors

Table 1. Excitatory Amino Acid Receptors

| Receptor Subtype | Agonists | Antagonists | Effector Mech |
|---|---|---|---|
| Quisqualate (QA) | AMPA<br>QA<br>L-GLutamate<br>KA | GDEE<br>pBr-piperazine dicarboxylate (pBr-PDA)<br>GAMS<br>gDGG | 1) coupled to triggering mo conductances frequency (a)<br><br>2) stimulates inositol (PI) cerebellar ce hippocampus (1<br><br>3) inhibits a PI hydrolysis |
| Kainic acid (KA) | KA<br>Domoate<br>L-Glutamate<br>QA<br>L-Aspartate | gDGG<br>GAMS<br>2,3-PDA<br>GDEE | 1) coupled to triggering mo conductances frequency (a)<br><br>2) stimulates in the cerebel |
| N-methyl-D-aspartate (NMDA) | NMDA<br>NMLA<br>quinolinic acid<br>ibotenic acid<br>1-amino-1,3-dicarboxy-cyclopentane<br>L-homocysteate<br>L-Glutamate<br>L-Aspartate | Competitive<br>AP5<br>AP7<br>DaAA<br>CPP<br><br>Noncompetitive<br>PCP<br>tyletamine<br>ketamine<br>MK-801<br><br>$Mg^{2+}$ | 1) coupled to triggering div conductances frequency (a) |

(a) Cull-Candy and Usowicz 1987, (b) Croucher et al. 1982, (c) Greenamyre (e) Nicoletti et al. 1986a, (f) Nicoletti et al.1986, (g) Sladeczek et al and Coyle 1987, (j) Foster and Roberts 1980, (k) Simon et al. 1984, (l) M

ceptors

ceptors

| Antagonists | Effector Mechanism | Proposed Actions |
|---|---|---|
| GDEE<br>pBr-piperazine dicarboxylate (pBr-PDA)<br>GAMS<br>gDGG | 1) coupled to cation channel triggering monovalent ion conductances with greatest frequency (a)<br><br>2) stimulates phosphatidyl-inositol (PI) hydrolysis in cerebellar cell culture (e), hippocampus (f) and striatum (g)<br><br>3) inhibits aminergic stimulated PI hydrolysis in hippocampus (h) | Fast synaptic transmission<br>Implicated in seizure (b)<br>ischemic brain damage<br>Alzheimer's disease (c)<br>LTD in cerebellum (d) |
| gDGG<br>GAMS<br>2,3-PDA<br>GDEE | 1) coupled to cation channel triggering monovalent ion conductances with greatest frequency (a)<br><br>2) stimulates cGMP formation in the cerebellum (j) | Fast synaptic transmission<br>?Presynaptic<br>Implicated in seizure,<br>ischemic brain damage<br>Huntington's chorrea (i) |
| <u>Competitive</u><br>AP5<br>AP7<br>DaAA<br>CPP<br><br><u>Noncompetitive</u><br>PCP<br>tyletamine<br>ketamine<br>MK-801<br><br>$Mg^{2+}$ | 1) coupled to cation channel triggering divalent cation conductances with greatest frequency (a) | Associated with slow, regenerative currents<br>Implicated in seizure (b)<br>ischemic brain damage (k)<br>learning and memory (l)<br>Synaptic plasticity (m)<br>Alzheimer's disease (c,m) |

b) Croucher et al. 1982, (c) Greenamyre et al. 1987, (d) Kano and Kato 1987,
coletti et al.1986, (g) Sladeczek et al. 1985, (h) Baudry et al. 1986, (i) Robinson
erts 1980, (k) Simon et al. 1984, (l) Morris et al. 1986, (m) Cotman and Anderson 1987.

Legend for Table 1

Agonists and antagonists are listed in the order of potency (i.e., most potent is listed first).

Abbreviations

| | |
|---|---|
| AMPA | ((RS)-alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid) |
| AP5 | 2-amino-5-phosphonovaleric acid |
| AP7 | 2-amino-7-phosphoheptanoic acid |
| CPP | ((±)2-carboxypiperazin-4-yl)propyl-1-phosphonic acid |
| DaAA | D-alpha-amino adipate |
| GAMS | gamma-glutamyl aminomethylsulphonic acid |
| GDEE | glutamate diethylester |
| gDGG | gamma-D-glutamyl glycine |
| KA | kainic acid |
| LTD | long-term depression |
| MK-801 | ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) |
| NMDA | N-methyl-D-aspartate |
| NMLA | N-methyl-L-aspartate |
| PCP | phenycyclidine |
| pBr-PDA | pBr-piperazine dicarboxylate |
| 2,3-PDA | 2,3-H-piperazine dicarboxylate |
| QA | quisqualate |

alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) is a potent excitant, which also has high affinity for the QA site and appears to be a more specific agonist at QA receptors than QA. QA blocks a $Cl^-$-dependent glutamate sequestration process in neurons (Zaczek et al. 1987) and inhibits a peptidase that cleaves N-acetyl aspartyl glutamate (Robinson et al. 1987), a dipeptide, which is a putative excitatory neurotransmitter (ffrench-Mullen et al. 1985), while AMPA does not affect these processes. In binding studies, [$^3$H]-AMPA has been shown to label a population of sites with high affinity for QA, that are not displaceable by KA or NMDA (Olsen et al. 1987). However, a component of QA sensitive [$^3$H]L-glutamate binding is displaceable by KA (Fagg and Matus 1984). It has been suggested that the QA response is not mediated by a single QA receptor, but may represent co-activation of KA and NMDA receptors (Watkins and Olverman 1987). However this hypothesis is not supported by the following evidence:

1: Co-application of KA and NMDA does not induce a QA-like electrophysiological response in the cerebellum (Crepel et al. 1983).

2: There is a lack of anatomical correlation between KA- and NMDA-sensitive sites with AMPA-sensitive sites, using both [$^3$H]L-glutamate and [$^3$H]AMPA (Cotman et al. 1987, Greenamyre et al.

1985, Rainbow et al. 1984).

3: There are reports of KA-sensitive sites associated with presynaptic terminals, while NMDA- and QA-sensitive sites have been localized to postsynaptic densities (Fagg and Matus 1984).

Glutamate diethyl ester (GDEE) is a glutamate analog with antagonist activity. GDEE is a better inhibitor of QA and AMPA induced depolarizations than it is of KA induced excitation, while it is a weak antagonist at NMDA sites (McLennan 1983). Binding studies show that GDEE preferentially inhibits a high affinity component of [$^3$H]-AMPA binding with the same specificity as either QA or unlabeled AMPA (Olsen et al. 1987), thus making it the most selective QA-sensitive glutamate receptor antagonist available at present. Behaviorally, GDEE has been shown to antagonize QA and homocysteine induced seizures, and increase the latency to strychnine induced seizures (Freed 1985, Schwarz and Freed 1986, Abdul-Ghani et al. 1982). A QA selective antagonist, that is active against any QA-elicited response (electrophysiological, biochemical or behavioral) has yet to be found.

Compounds that appear to show higher affinity for KA excitation and binding sites are the gamma glutamyl derivatives. These include gamma-D-glutamyl glycine (gDGG) and gamma-glutamyl aminomethylsulphonic acid (GAMS)

(Davies and Watkins 1985). GAMS shows preferential antagonism of KA induced seizures and myoclonus (Turski et al. 1985). Hyper-excitation mediated through KA, as well as QA receptors has been implicated in epilepsy and ischemic brain damage (Robinson and Coyle 1987). KA is a potent excitotoxin and is often used to create discrete lesions in the central nervous system.

1.3b N-methyl-D-aspartate Receptors

Iontophoretic application of N-methyl-D-aspartate (NMDA) to neuronal membrane causes a slow depolarization, mediated by a $Ca^{2+}$ current. The $Ca^{2+}$ channel can be activated via receptor coupling, and in a voltage dependent manner (Dingledine 1982). Voltage dependency is mediated by magnesium inhibition of the calcium channel activated by NMDA (Mayer and Westbrook 1984); this inhibitory influence can be removed <u>in vivo</u> when the membrane potential reaches a certain level, for example, by prior synaptic activity (Coan and Collingridge 1985). In the presence of sustained neurotransmitter release, activation of the NMDA receptor leads to rhythmic depolarizations and regular bursts of action potentials, due to the triggering of hyperpolarizing conductances by depolarization and $Ca^{2+}$ entry. This regenerative synaptic process may be involved in tonic activity.

Secondary activation of NMDA-coupled $Ca^{2+}$ channels have been shown to play an important role in long-term potentiation. Long-term potentiation is an increase in synaptic activity that is maintained for a period of hours to days following a single tetanic stimulation that may be involved in long-term information storage, or memory. Blockage of NMDA receptors by local infusion of 2-amino-5-phosphonovaleric acid (AP5), a specific NMDA antagonist, prevents the induction of long-term potentiation and spatial learning in rats, without affecting visual cues (Morris et al. 1986). NMDA receptors may also be important in development since their appearance occurs very early in differentiation (Cotman and Anderson 1987).

The NMDA receptor is the best characterized of all excitatory amino acid receptors. This is by virtue of the fact that NMDA is a potent and highly selective agonist neurophysiologically, and that selective antagonists for this receptor have been identified. These include both 2-amino-5-phosphonic acid (AP5) and 2-amino-7-phosphonic acid (AP7) (Stone 1986), and 3-((±)-2-carboxy-piperazin-4-yl)-propyl-1-phosphonic acid (CPP), a piperazine derivative (Davis et al. 1986). Both amino phosphonic acid derivatives (AP5 and AP7) have high affinity for [$^3$H]L-glutamate sites that are displaced by NMDA. These compounds are also active as anticonvulsants against

seizures induced by NMDA (Monaghan and Cotman 1986) as well as a number of other experimental seizures, such as audiogenic seizures in rodents and photosensitive seizure in baboons (Croucher et al. 1982, Meldrum et al. 1983). CPP has high affinity for [$^3$H]-AP5 labeled sites (Davis et al. 1986), and NMDA displaceable [$^3$H]L-glutamate binding sites (Olverman et al. 1986), and has anticonvulsant activity against NMDA and quinolate induced seizures (Turski et al. 1987).

In addition to competitive antagonists, several noncompetitive NMDA antagonists have been identified, as well as an allosteric enhancer of NMDA activity. Glycine has been shown to augment NMDA induced depolarization by activation of a strychnine-insensitive receptor that may be part of an NMDA receptor - ion channel complex (Johnson and Ascher 1987). Systemic administration of noncompetitive NMDA antagonists results in various behavioral effects, and has prompted much speculation regarding the function of NMDA receptors. The dissociative anesthetics ketamine and phencyclidine (PCP) diminish NMDA induced excitation (Watkins and Olverman 1987), and PCP also has anticonvulsant activity (Hayes and Balster 1985). MK-801, ((+)-5-methyl-10,11-di-hydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine) is a potent anticonvulsant (with the exception of strychnine induced seizures) and may also have anxiolytic activity

(Clineschmidt et al. 1982). MK-801 depresses NMDA induced depolarizations, and binds specifically to synaptic membrane preparations, however no specific [$^3$H] MK-801 binding has been observed in the cerebellum (Wong et al. 1986). This is not surprising, since there is very little [$^3$H]-NMDA binding (Monaghan and Cotman 1986) or NMDA displacement of [$^3$H]L-glutamate binding in the cerebellum (Greenamyre et al. 1985, Monaghan and Cotman 1985). Electrophysiologic activity produced by NMDA in the cerebellum is somewhat ambiguous. In the presence of $Mg^{2+}$, NMDA and NMDLA, the mixture of L- and D- isomers, failed to elicit agonist action, but instead were active as antagonists of complex spikes induced by stimulation of climbing fibers and iontophoresis of both glutamate and aspartate on mature cerebellar Purkinje cells (Kimura et al. 1985). In the absence of $Mg^{2+}$, however, NMDA has recently been shown to induce depolarizations and spike formation in the proximal dendrites of cerebellar Purkinje cells (Sekiguchi et al. 1987). Antagonism by NMDA of glutamate and excitatory amino acid agonist induced depolarizations, as well as those induced by release of the endogenous neurotransmitter, was also observed on the photoreceptor of horizontal cells from carp, which has been described as an 'atypical glutamate receptor' (Ariel et al.).

NMDA receptors have recently been implicated in a

number of neurologic disorders. Experimental ischemic brain damage has been slowed by prior treatment of animal models with CPP and AP5, the NMDA antagonists (Rothman and Olney 1987, Simon et al. 1984). As mentioned previously, NMDA receptor activation has been inferred in a number of animals models for epilepsy (Croucher et al. 1982, Meldrum et al. 1983). The NMDA antagonist, AP7 delays onset, and in some doses prevents experimental induction of high pressure neurologic syndrome (Wardley-Smith et al. 1984). Loss of NMDA receptors in cortex, has recently been implicated in Alzheimer's disease (Greenamyre et al. 1987, Cotman and Anderson 1987)

### 1.3c Parallel Fiber and Climbing Fiber Systems

There is considerable evidence to suggest that both parallel fibers and climbing fibers release excitatory amino acids, however whether there are distinct excitatory amino acid receptors on Purkinje cells mediating the characteristic response (simple spike or complex spike) elicited by stimulation of either afferent is not yet clear. Both electrophysiological and receptor binding experiments indicate the presence of multiple excitatory amino acid receptor subtypes in the cerebellar cortex. A summary of the available data on excitatory amino acid receptors localized to the molecular layer of the

cerebellum is presented in Table 2. While absolute numbers of receptors measured vary with the binding protocol used, the data indicate that there is a high density of QA-sensitive sites and a low density of NMDA-sensitive sites.

Both aspartate and glutamate, when iontophoretically applied to cerebellar slice preparations will produce a complex spike similar to that produced by climbing fiber stimulation in intact animals. However, the rank order potencies of excitatory amino acid antagonists for inhibition of complex spikes produced by climbing fiber stimulation, aspartate or glutamate are different (Table 3). The complex spike induced by climbing fiber stimulation is inhibited most potently by AP5 and gDGG, followed by N-methyl-D,L-aspartate (NMDLA, the racemic mixture of NMDA) and finally, GDEE (Kimura et al. 1985). The rank order potency of excitatory amino acid antagonists for inhibition of the aspartate induced complex spike is nearly identical to that for the climbing fiber elicited response, although the inhibition is not as complete. For example, maximum inhibition by AP5 was 70% for the aspartate induced response as compared to 99% for the complex spike induced by climbing fiber stimulation (Kimura et al. 1985). The fact that the rank order potencies for inhibition of both the climbing fiber stimulated complex spike and the complex spike induced by

Table 2. Distribution of excitatory amino acid subtypes in molecular layer of cerebellar cortex as determined by autoradiography

| LABELED LIGAND [ ]nM | DISPLACER (conc.) | INCUBATION CONDITIONS | SPECIFIC BINDING (fm/mg) |
|---|---|---|---|
| [$^3$H]-L-Glutamate | | | |
| 300 nM | 1 mM glutamate | 1 hour, 25 C | 165 ± 19 (a) |
| 200 nM | 1 mM glutamate | 45 min., 2 C | 3300 ± 200 (b) |
| 100 nM | 100 uM NMDA | 10 min., 0 C | 78 ± 28 (c) |
| 100 nM | 100 uM NMDA | 45 min., 2 C | |
| "displaces 20% specific glutamate binding, corresponding to low affinity to QA sites" (d) | | | |
| 100 nM | 2.5 uM QA | 45 min., 2 C | |
| "displaces 80% specific glutamate binding, corresponding to high affinity to QA sites" (d) | | | |
| [$^3$H]AMPA | | | |
| 80 nM | 1 mM glutamate | 1 hour, 25 C | 161 ± 47 (e) |
| 50 nM | 100 uM AMPA | 30 min., 30 C | 28 ± 3 (f) |
| [$^3$H] KA | 100 uM KA | 30 min., 0 C | "moderate levels, compared to high levels in granule cell layer"(g) |

(a) Halpain et al. 1984, (b) Greenamyre et al. 1984, (c) Monaghan and Cotman 1985 (d) Greenamyre et al. 1985 (e) Rainbow et al. 1984 (f) Monaghan et al. 1984 (g) Monaghan and Cotman 1984

iontophoretic application of aspartate are almost identical suggests that the complex spike may be produced by an aspartate preferring receptor. This receptor, however, does not conform to the current classification scheme for excitatory amino acid receptors.

As mentioned before, complex spikes are made of two distinct components, an action potential superimposed on a slow degenerating EPSP. Complex spikes can be recorded from either somatic or dendritic climbing fiber - Purkinje cell synapses and both complex spikes have the same components and shape, but their time courses vary. The action potential in the somatic complex spike is very rapid (1 ms) and of low threshold, since it begins near the bottom of the EPSP rise. Dendritic complex spikes, on the other hand, have a longer action potential (2-6 ms) of higher threshold which starts during the rise of the EPSP or near its' peak (Crepel et al. 1981). The different time courses with respect to action potential formation suggest that this component of the complex spike may be generated in the Purkinje cell body, while the EPSP may originate in either the dendritic region or the cell body. The dendritic spike may be initiated locally (at the dendrite), by a process that is voltage dependent, requiring a long-lasting prior depolarization. The sequence of events might then be 1) generation of the EPSP by activation of one type of receptor (for example, the

NMDA receptor), followed by 2) depolarization and a change in membrane potential which would cause activation of voltage dependent channels in the dendrite and 3) production of the dendritic spike (Crepel et al. 1981).

Classification of the excitatory amino acid receptor or receptors mediating the complex spike-response has been difficult because there is so much apparently conflicting data. Information available thus far is:

1) QA elicits a complex spike-like response when applied to both proximal and distal dendrites of the Purkinje cell (Sekiguchi et al. 1987, Crepel et al. 1982)

2) the presumed QA-selective antagonist, GDEE is a poor inhibitor of the complex spike induced by both climbing fiber-stimulation and aspartate (Kimura et al. 1985)

3) NMDA and NMDLA, if applied at endogenous concentrations of $Mg^{2+}$ (1-4 mM) do not act as agonists, but instead antagonize complex spikes induced by both climbing fiber-stimulation and aspartate (Kimura et al. 1985)

4) in a $Mg^{2+}$- depleted environment (< 10 uM), NMDA does induce complex spike-like depolarizations when applied to proximal Purkinje cell dendrites (Sekiguchi et al. 1987)

5) AP5 the 'selective NMDA antagonist' is the most potent antagonist of the climbing fiber-induced complex spike (Kimura et al. 1985).

6) QA also elicits a simple spike-like response that is well inhibited by GDEE and kynurenate and gamma-D glutamyl glycine (Kano et al. 1988, Crepel et al. 1982)

One interpretation of these data is that complex spike production occurs by either simultaneous or sequential activation of different excitatory amino acid receptor subtypes, possibly NMDA and QA receptors (Sekiguchi et al. 1987).

The recent report of functionally active NMDA receptors, localized to proximal dendrites on Purkinje cells from adult guinea-pig cerebella (Sekiguchi et al. 1987) is of extreme importance in eventual identification of the receptor or receptors mediating the complex spike response. Prior to this report, the lack of agonist response to NMDA suggested an absence of NMDA receptors on cerebellar Purkinje cells, although in fetal and neonate rats NMDA induces a depolarization (Crepel et al. 1983). Quantitative autoradiography of NMDA-sensitive [$^3$H]-glutamate binding in mature rats, shows a low density of sites in the molecular layer of the cerebellar cortex (Monaghan and Cotman 1985).

There is a high concentration of [$^3$H]AMPA binding

sites in the cerebellum, as determined by quantitative autoradiography. These values are similar to those seen for [$^3$H]L-glutamate binding (~160 fm/mg) (Halpain et al. 1984, Rainbow et al. 1984). Lower levels were seen when [$^3$H]AMPA was displaced by lower concentrations of unlabeled AMPA (Monaghan and Cotman 1984a). The highest density of radiolabelled binding was observed with [$^3$H]L-glutamate (Halpain et al. 1984, Greenamyre et al. 1984).

Curiously, binding to the putative dipeptide neurotransmitter, N-acetylaspartyl glutamate, occurs with high affinity in cerebellar synaptosomal preparations, and QA has a high affinity for these sites (Koller and Coyle 1984, 1985). In addition, N-acetylaspartyl glutamate produces a complex spike - like depolarization in cultured cerebellar neurons, and is reported to be a glutamate agonist at Purkinje cells in guinea-pig cerebellar slices (Mori-Okamato et al. 1987, Sekiguchi et al. 1988). Whether this binding corresponds to a unique receptor is not yet clear; immunocytochemical localization of N-acetylaspartyl glutamate in the hippocampus suggests that while binding is high, presence of the endogenous ligand is relatively low (Anderson et al. 1986). N-acetylaspartyl glutamate has been proposed as the endogenous neurotransmitter for both the lateral olfactory tract and the retinogeniculate pathway (ffrench-Mullen et

al. 1985, Molinar-Rode and Pasik 1987).

It has been fairly well established that parallel fibers release glutamate to induce simple spikes in Purkinje cells, however, iontophoretically applied glutamate can also produce a complex spike in Purkinje cells (Kimura et al. 1985). The rank order potency for antagonism of glutamate induced complex spikes is the reverse of that seen for complex spikes induced by either climbing fiber stimulation or aspartate application as shown in Table 3. The most striking difference was that

Table 3. Rank Order Potency For Antagonism of Complex Spike (CS) Production In Guinea-Pig Cerebellum (a)

| CS Induced By | Rank Order Potency Antagonists |
|---|---|
| Climbing Fiber Stimulation | AP5 > gDGG > NMDLA > GDEE |
| Aspartate | gDGG > AP5 > NMDLA > GDEE |
| Glutamate | GDEE > NMDLA > gDGG > AP5 |

(a) Kimura et al., 1985.

GDEE, which is a poor antagonist of both the climbing fiber and aspartate induced complex spike, was the most potent antagonist for glutamate induced complex spikes (63% inhibition) (Kimura et al. 1985); NMDLA, gDGG and AP5 were all considerably less effective (inhibition ranged

from 22% to 14%). The fact that glutamate also induces complex spikes further suggests that two populations of excitatory amino acid receptors on Purkinje cells may be capable of generating this response.

Patch-clamp studies of cerebellar Purkinje cells grown in culture indicate that different excitatory amino acid analogs (glutamate, aspartate, QA, KA and NMDA) activate different sub-conductance levels of the same type of ion channel (Cull-Candy and Usowicz 1987). Rigid excitatory amino acid analogs represent different structural conformations that glutamate and/or aspartate can have in vivo, depending on the local ionic environment (Esposito et al. 1984, Coyle 1980). In primary cultures of hippocampal neurons, glutamate itself has been shown to activate sub-conductance levels of the same monovalent cation channel (Jahr and Stevens 1987). Whether individual channels are linked to multiple receptor types, or if each subtype of receptor interacts with only one channel remains to be seen. However, the data indicate that glutamate and/or aspartate can initiate different types of responses in the same cell (Cull-Candy and Usowicz 1987, Jahr and Stevens 1987). Along this same line, QA elicits a complex spike - like response (Sekiguchi et al. 1987, Crepel et al. 1982) and a simple spike (Kano et al. 1988, Crepel et al. 1982). In addition, QA can substitute for parallel fiber stimulation in a

paired climbing fiber - parallel fiber stimulation paradigm which results in a long lasting suppression (upward of 1 hour) of Purkinje cell simple spike activity. This phenomenon, known as long-term depression, is produced by sequential stimulation of climbing fibers and parallel fibers (Ito and Kano 1982). The induction of long-term depression by QA occurred only when QA was applied following climbing fiber stimulation, application of QA alone did not affect Purkinje cell response to parallel fiber stimulation (Kano and Kato 1987). This experiment suggests that a QA receptor may mediate the glutamate effect produced by parallel fibers. Since extracellular $K^+$ and $Ca^{2+}$ levels fluctuate as a function of climbing fiber stimulation (increase and decrease, respectively), configurational changes in extracellular glutamate might be expected under these conditions (Stockle and ten Bruggencate 1980).

In summary, under normal physiologic conditions, cerebellar Purkinje cells receive excitatory input from parallel fibers and climbing fibers, via excitatory amino acid neurotransmitters. The effect of parallel fiber stimulation is simple spike production by Purkinje cells culminating in the release of GABA in the cerebellar efferent nuclei. Simple spike production may proceed via activation of QA-sensitive glutamate receptors. Climbing

fiber stimulation causes production of a complex spike by Purkinje cells, which also results in GABA release in cerebellar efferent nuclei, and is always followed by temporary suppression of parallel fiber-induced simple spikes. Purkinje cells become unresponsive to simple spikes as a result of the slow EPSP component of the complex spike. Climbing fibers fire in a rhythmic fashion, thus initially producing Purkinje cell GABA release followed by a quiet period due to interference with simple spike production by parallel fiber input. The net effect of climbing fiber and parallel fiber activity is a pulsatile release of GABA from the Purkinje cells.

## 1.4 Climbing Fiber Deafferentation

### 1.4a 3-Acetylpyridine Induced Inferior Olive Lesion

The neurotoxin 3-acetylpyridine (3AP) causes complete destruction of the inferior olive (D'esclin and Escubi 1974, Llinas et al. 1975, Anderson and Flumerfelt 1980, Balaban 1985) and climbing fibers (D'esclin 1974, Balaban 1985), resulting in ataxia and transient tremor which are behavioral signs of cerebellar dysfunction. As with all neurotoxins, other areas of the central nervous system are affected; substantive portions of the hypoglossal nucleus, dorsal motor nucleus of X and the motor nucleus of the

facial nerve have been reported to be damaged by 3AP, as well. In addition, varying degrees of cell injury have been observed in the nucleus ambiguus (D'esclin and Escubi 1974, Balaban 1985). Injury to the hippocampus, is the subject of some controversy. D'esclin and Escubi (1974), in a study using male Sprague-Dawley rats, specifically looked for damage in this area and therefore did not confirm previous reports of hippocampal degeneration due to 3AP toxicity (Coggeshall and McLean 1958). More recently, however, Balaban (1985) has reported consistent, albeit incomplete lesions of dentate granule cells and subsequent destruction of their short axon mossy fibers to CA3 fields in hippocampus. Hippocampal damage may be dose dependent, since D'esclin and Escubi (1974) used a dose of 65 mg/kg 3AP, i.p., while Balaban (1985) used 75-80 mg/kg. It does not seem probable that ataxia associated with 3AP lesion is due to hippocampal damage. Furthermore, ataxia occurs following either dose (65 or 75-80 mg/kg, i.p.) of 3AP (Balaban 1985, Anderson and Flumerfelt 1980, D'esclin and Escubi 1974, Herken 1968, Coggeshall and McLean 1958), while only higher doses of 3AP produced hippocampal degeneration (Balaban 1985, Coggeshall and McLean 1958).

### 1.4b Biochemical and Electrophysiological Studies in 3-Acetylpyridine - Inferior Olive Lesioned Rats

3AP lesioned rats have been used as an experimental model that resembles genetic torsion dystonia (Oltmans et al. 1985). Biochemical studies show that both genetically dystonic rats and those with 3AP inferior olive lesions have an increase in glutamic acid decarboxylase activity, in cerebellar cortex and cerebellar efferent nuclei (Oltmans et al. 1986, 1984). Glutamic acid decarboxylase is the synthetic enzyme for GABA, the inhibitory transmitter released by Purkinje cells. These observations suggest that Purkinje cells are more active following inferior olive lesion. No change in $GABA_A$ receptors has been detected in the cerebellar efferent nuclei, however binding to $GABA_B$ sites in the cerebellum was decreased following 3AP lesion (Kato and Fukuda 1985). $GABA_B$ binding was measured in synaptosomes prepared from whole cerebellum, therefore the decrease in binding sites may mean either that $GABA_B$ receptors are present on climbing fibers (Kato and Fukuda 1985), or that 'pre-synaptic, GABA autoreceptors' on Purkinje cell terminals have been down regulated in response to excessive GABA release caused by hyper-excitation of Purkinje cells.

Increased electrophysiological activity in cerebellar Purkinje cells as a function of inferior olive degeneration or reversible inactivation has been reported by many investigators. As discussed in previous sections,

Purkinje cells have a characteristic electrophysiologic response to both climbing fiber and parallel fiber input. Parallel fibers produce simple spikes which cause release of GABA at cerebellar efferent nuclei, while climbing fibers produce complex spikes, which, in addition to causing release of GABA at cerebellar efferent nuclei, appear to regulate Purkinje cell simple spike production. A temporary suppression of simple spike activity has been observed in both cats and rats, secondary to climbing fiber stimulation (Rawson and Tiloskulchai 1982, Colin et al. 1980). Complete inhibition of the simple spike response is seen at a climbing fiber firing frequency of 2 Hz, a rate that can be attained in vivo, without pharmacologic intervention. When climbing fibers are driven at a higher frequency (8-10 Hz), by administration of the tremor inducing agent harmaline, for example, a prolonged inhibition of simple spikes occurs and Purkinje cells register only a complex spike. Following harmaline administration, the frequency of complex spikes is the same as the frequency of tremor (Headley et al. 1976). The rate of climbing fiber activity under normal physiological conditions, in vivo, is much slower than 8-10 Hz; however climbing fibers do fire tonically, thus mediating a pulsatile release of neurotransmitter to Purkinje cells. The pattern of GABA release by Purkinje cells is in response to simple spike and complex spike

production, which results in GABA release as well as suppression of simple spike activity. This pattern of inhibition is then registered at the cerebellar efferent nuclei. A climbing fiber collateral from the inferior olive also innervates the cerebellar efferent nuclei. Ergo, climbing fibers from the inferior olive provide

1) a pulsatile release of GABA to the cerebellar efferent nuclei
2) tonic regulation of the Purkinje cell with respect to simple spike production, and 'unchecked' GABA release at the cerebellar efferent nuclei
3) excitation of the cerebellar efferent nuclei via the climbing fiber collateral.

The effect of climbing fiber destruction then is quite complex. Inferior olive lesions remove climbing fiber input to both Purkinje cells and cerebellar efferent nuclei. Acute inferior olive lesion, as well as temporary inactivation of inferior olive neurons results in a rise in simple spike activity (Benedetti et al. 1983, Montarolo et al. 1982, Colin et al. 1980), and diminished output by the cerebellar efferent nuclei and Deiters neurons of the lateral vestibular nucleus (Benedetti et al. 1983, Bardin et al. 1983). These observations have been extended by Batini and Billard (1985), who have reported a simultaneous increase in Purkinje cell activity and a decrease in cerebellar efferent nuclear firing following

acute inferior olive lesion. Although a decrease in the elevated simple spike levels occurs after chronic lesion, the system by no means returns to normal; Purkinje cells no longer produce complex spikes or display tonicity in releasing GABA to the cerebellar efferent nuclei (Batini et al. 1985). Ito, et al. (1978) reported that cerebellar stimulation failed to elicit an inhibitory response in Deiters neurons, following chronic inferior olive lesion, suggesting diminished response capacity by the inferior olive - Purkinje cell - cerebellar efferent nuclei system.

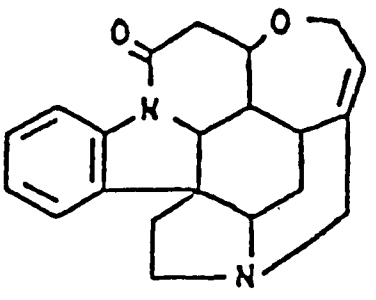
In corroboration of electrophysiological studies, an increase in metabolic activity in the cerebellar cortex has been demonstrated following inferior olive inactivation and suggests increased synaptic activity of Purkinje cells (Batini and Billard 1985). These observations are also consistent with the increase in glutamic acid decarboxylase activity in both Purkinje cells and cerebellar efferent nuclei already discussed (Oltmans et al. 1985). The net effect of climbing fiber removal appears to be increased simple spike production by Purkinje cells, in response to parallel fiber activity, and deregulation of cerebellar efferent nuclei.

## 1.5 Strychnine Seizures

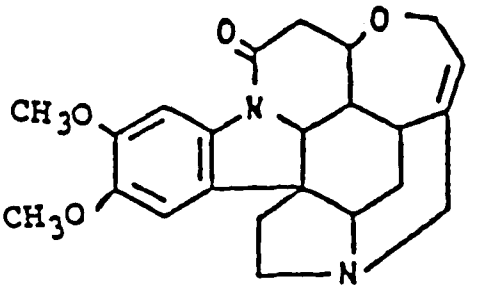
Strychnine is a natural alkaloid, found in nux vomica,

the seeds of the tree Strychnis nux vomica. When iontophoretically applied to spinal motoneurons, strychnine reversibly inhibits glycine induced inhibitory post synaptic potentials (IPSPs), while having no effect on IPSPs produced by GABA (Curtis et al. 1968). In fact, strychnine is one of the most selective antagonists available for glycine receptors. Glycine induced inhibitions in the spinal cord (Curtis et al. 1968, 1968a, 1971) and supraspinal structures, including the medullary reticular formation (Tebecis and DiMaria 1972), ventromedial hypothalamus (Dreifuss and Matthews 1972), Deiters neurons of the lateral vestibular nucleus (ten Bruggencate et al. 1972) and the cuneate nucleus (Hill et al. 1976) are all selectively antagonized by strychnine.

When administered systemically to rodents, strychnine and to a lesser extent, its' structural congener, brucine, act as convulsants (Figure 2). Subconvulsant doses of




Strychnine




Brucine

Figure Two

strychnine do not effect GABA IPSPs or endogenous Renshaw cell discharge, but the same doses reduce glycine-induced potential changes and those resulting from stimulation of inhibitory reflex loops in the spinal cord (Curtis et al. 1968a). Similarly, in the cuneate nucleus, strychnine antagonized glycine-induced IPSPs, but only rarely blocked GABA induced hyperpolarizations. This is in marked contrast to bicuculline, picrotoxin and penicillin, other dis-inhibitory convulsants, which act primarily at GABA sites, but also cause substantial inhibition of glycine-induced IPSPs (Hill et al. 1976). By specifically antagonizing inhibitory glycinergic receptors in the central nervous system, strychnine reduces inhibition. This includes the reciprocal inhibition between antagonistic muscles. The pattern of strychnine seizure, then is determined by the most powerful muscle acting at a joint, which happen to be the extensor muscles. There is no clonic component to a strychnine seizure, because there is no direct activation of a muscle. A typical strychnine seizure is characterized by tonic extension of the body and all limbs, preceded and followed by phasic symmetrical extensor thrusts (Woodbury 1980).

The glycine receptor is coupled to a chloride channel, and activation causes an increase in cell membrane permeability to chloride. There is evidence that strychnine interacts directly with the chloride channel,

and therefore is not a competitive glycine antagonist. This is also suggested by binding experiments where it was found that strychnine displaced [$^3$H]strychnine 300 times as effectively as glycine, in synaptic membranes from spinal cord (Young and Snyder 1973). Localization of [$^3$H]strychnine binding also paralleled endogenous distribution of glycine in the central nervous system (Young and Snyder 1973).

Using the method of receptor autoradiography, [$^3$H]strychnine binding displaced by either unlabeled strychnine or glycine has been localized in the central nervous system. [$^3$H]strychnine binding displaced by unlabeled strychnine, shows a high density of sites in the spinal cord and medulla, specifically the dorsal horn, cranial nerve nuclei, the dorsal column nuclei and medullary reticular formation (Frostholm and Rotter 1985). This distribution parallels glycine-sensitive [$^3$H]strychnine binding (Zarbin et al. 1981). Comparison of glycine displaceable [$^3$H]glycine binding sites to glycine displaceable [$^3$H]strychnine binding sites show a different distribution. [$^3$H]glycine labeled sites have a more widespread distribution within the central nervous system, suggesting the presence of strychnine-insensitive glycine receptors (Bristow et al. 1986). Glycine enhancement of NMDA depolarization, which is strychnine insensitive, has already been discussed (Johnson and

Ascher 1987).

Conversely, non-glycinergic events are also mediated by strychnine. At high doses, strychnine completely inhibits both acetylcholinesterase and butryl-cholinesterase, which might account for excitatory effects induced by topical strychnine (Alid et al. 1974). Strychnine inhibition of GABA effects on the cortex has also been proposed to explain strychnine induced tetany (Terzuolo 1954). Strychnine has also been shown to block $Na^+$ and $K^+$ conductances in non-mammalian systems (Calahan and Almeus 1979, Freeman 1973), inhibit presynaptic acetylcholine release (McKinstry and Koelle 1967), facilitate dopamine release (Morgan and Kamp 1983) and inhibit norepinephrine induced hyperpolarization (Biscoe and Curtis 1966). Most of these actions, however, require much higher doses of strychnine than the dose required for antagonism of glycine-induced hyperpolarization.

# CHAPTER TWO

## Effect of Inferior Olive Lesion on Seizure Threshold in the Rat*

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## 2.1 Summary

Cerebellar stimulation has been associated with anticonvulsant activity in several experimental seizure models. We examined the effect of destruction of cerebellar climbing fibers, by systemic administration of 3-acetylpyridine (3AP) or electrothermic lesion of the inferior olive, on seizures produced by various chemical convulsants in rats. We found that inferior olive lesioned rats had lower threshold to seizures induced by strychnine and brucine, both glycine antagonists. The dose response curve for strychnine seizure was shifted 2.5 times to the left in 3AP lesioned rats. No difference in seizure threshold was seen when picrotoxin, bicuculline or pentylenetetrazole (PTZ) were used to produce seizures. Abnormal motor behavior (AMB) including myoclonus, backward movement and hyperextension, produced by all of the convulsants tested, was significantly aggravated in 3AP pretreated rats. The inferior olive-climbing fiber projection to the cerebellum appears to modulate seizures induced by inhibition of glycinergic neurotransmission.

## 2.2 Introduction

Cerebellectomy increases the duration of cortical epileptiform discharges (Gartside 1978, Halpern and Julien 1972, van den Driessch and Trebaul 1958) while direct electrical stimulation of the cerebellum can suppress seizure activity produced by local application of cobalt, penicillin or strychnine to the cerebral cortex (Hutton et al. 1972, Dow 1965, Dow et al. 1962, Moruzzi 1941). Seizure patterns in the cat cerebral cortex induced by strong hippocampal stimulation are also inhibited by stimulation of the cerebellar cortex (Iwata and Snider 1959). On the other hand, stimulation of the cerebellar hemisphere has produced only minimal or no effect on generalized seizures produced by systemic injections of penicillin, enflurane, PTZ or chloralose (Myers et al. 1959). Cerebellectomy markedly reduced the anticonvulsant effect of phenytoin but did not affect the ability of diazepam to antagonize seizures (Raines and Anderson 1976). These observations suggest that inhibitory cerebellar efferent pathway(s) may exert an arresting effect on seizure spread from certain but certainly not all epileptogenic foci.

Afferent connections to the cerebellum emanate from two major pathways: the mossy and the climbing fibers. Mossy fibers arise from diffuse regions of the brainstem,

particularly the pons, and terminate in the granule layer of the cerebellum (Brodal 1981). Neurons of the inferior olive are the source of climbing fibers which project to the Purkinje cells in the cerebellar cortex (D'esclin 1974). Cooke and Snider (1955) observed that electrically induced cerebral cortex seizures could be blocked by electrical stimulation of the inferior olive, brachium pontis and brachium restiform as well as the cerebellar cortex. Greater voltage was required to prevent seizures by pontine as compared to inferior olive or restiform body stimulation. Since climbing fibers constitute a major portion of the restiform body, the anticonvulsant effect observed by electrical stimulation of this pathway may be due to its climbing fiber components.

In the present study, we report the effect of inferior olive-climbing fiber destruction on seizure activity in the rat. The inferior olive was destroyed by both 3-acetylpyridine (3AP) a neurotoxin which produces selective degeneration of the inferior olive (D'esclin and Escubi 1974) and electrothermic lesions of the same nucleus to remove climbing fiber projections to the cerebellum. Strychnine, brucine, bicuculline, picrotoxin or PTZ were administered systemically to induce seizures.

## 2.3 Materials and Methods

## 2.3a Materials

Male Sprague-Dawley rats, weighing 150-200 g. were obtained from Harlan, Sprague Dawley Inc., Walkersville, MD. Chemicals were obtained from Sigma Chemical Company, St. Louis, MO, except for bicuculline which was from Regis Chemical Company, Morton Grove, IL.

## 2.3b Lesions

Rats were housed 6/cage, kept on a 12:12 hour light-dark schedule and provided food and water ad libidum. Chemical lesions of the inferior olive were made by intraperitoneal (i.p.) injections of 80 mg/kg 3AP. Recovery from acute toxicity (stridulous breathing, weight loss, diarrhea and tremor) occurred within 2-4 days. Seizure studies were carried out after a 2-3 week recovery period. The only persistent neurological sign was ataxia. Animals that did not recover from acute toxicity were discarded.

Electrothermic lesions of the inferior olive were made using coordinates from lambda, A-P: -4.0 mm, L: $\pm$ 0.7 mm, V: -10.2 mm, with bite bar at -2.5 cm. A Grass LM-4 lesion maker (Grass Medical Instruments, Quincey, MA) was used to apply a 120 volt, 4 mA current for 10 seconds. Convulsants were administered one week after lesioning.

Ataxia was present in these animals, but to a lesser extent than in 3AP lesioned rats. Location of lesions was confirmed histologically.

2.3c Convulsive Behavior

Seizure experiments were performed between 1300 and 1900 hours. Lesioned and naive rats were given i.p. injections of convulsants (strychnine, brucine, picrotoxin bicuculline or PTZ) and observed individually in 16 1/2" x 8 1/2" x 7" lucite cages for 45 minutes to 2 hours, depending on the convulsant (Table 6). Naive rats were weight matched to lesioned animals. A typical strychnine induced convulsion was initiated by tonic extension of the entire body and all limbs and occurred while the animal was upright or supine; this was often followed by phasic symmetrical extensor thrusts during a period of postictal depression. Brucine convulsions followed the same general pattern of motor behavior, but were less intense than those induced by strychnine. Both bicuculline and picrotoxin produced clonic and uncoordinated convulsions, which on occasion progressed to full tonic-clonic seizures, and were accompanied by excess salivation. PTZ produced the most characteristic convulsion which was always initiated by forelimb and jaw clonus when the animal was in an upright position; this often progressed

into a tonic-clonic seizure similar to that produced by bicuculline and picrotoxin. Parameters monitored were incidence of and latency to seizure, number and duration of seizures and presence and type of other convulsant induced abnormal motor behavior (AMB). AMB occurred with all convulsive agents tested and included whole body and individual limb myoclonus, forepaw tapping, tremor, backward movements and hyperextension of head, back and limbs; rats were considered to have AMB when any one or more of these behaviors were present.

### 2.3d Statistics

The Fisher exact method of chi-square analysis (Goldstein 1964) was used to determine significance of seizure incidence. The non-parametric Mann-Whitney test was used to assess differences in convulsion number and AMB scores.

## 2.4 Results

The effects of 3AP and bilateral electrothermic lesions of the inferior olive on seizures induced by i.p. injections of 1.5 mg/kg strychnine and 40 mg/kg brucine are shown in Table 4. Both types of inferior olive lesions significantly increased the incidence as well as

Table 4. Effect of IO Lesion (3AP and ETL) on Seizures Induced by Strychnine and Brucine

| Drug | Group | Seizure Incidence | Latency To Seizure(c) (minutes) | Mean Number (per group) of Seizures(c) |
|---|---|---|---|---|
| Strychnine 1.5 mg/kg | Control | 1/16 | 7:25 (a) | .06 (a) |
| | 3AP | 12/16 ** | 5:15 ± 0:40 | 10.7 ± 2.2 + |
| | Control | 3/12 | 5:27 ± 1:21 | 0.4 ± 0.3 |
| | ETL (b) | 7/9 * | 8:38 ± 1:46 | 2.3 ± 0.9 + |
| Brucine 40.0 mg/kg | Control | 0/7 | - | - |
| | 3AP | 5/7 * | 6:33 ± 2:08 | 8.1 ± 2.3 + |

(a)ETL: bilateral electrothermic lesion of IO,
(b)only one value; (c)values are means ± SEM
*p< .05,**p < .001, Fisher Exact chi-square; + p < .05 Wilcoxin Rank Sum Test

the mean number of seizures produced. In the few control rats where 1.5 mg/kg strychnine induced seizures, the latency to seizure was not different from that of inferior olive lesioned rats. Brucine (40 mg/kg) did not produce seizures in control rats, but 5 out of 7 3AP-lesioned animals had seizures at the same dose. In 3AP-lesioned rats, and to a lesser extent in animals with electrothermic lesions, the intensity of the strychnine induced seizure was greater and associated with more violent movements which lifted the animal off the cage floor.

Figure 3 shows a composite drawing of the electro-

thermic inferior olive lesions. The area of overlap of all lesions is the inferior olive.

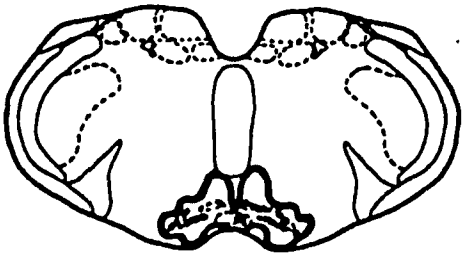


Figure Three

Coronal section (11.0 mm posterior to bregma) through rat medulla from Pellegrino et al. (1967) showing composite drawing of inferior olive electrothermic lesions.

The dose response curves for the incidence of strychnine induced seizures in control and 3AP lesioned rats are shown in Figure 4. The dose response curve of 3AP lesioned rats is shifted to the left indicating an increased sensitivity to strychnine. The dose of strychnine producing convulsions in 50% of the animals (CD50) is 0.92 mg/kg in the 3AP group and 2.32 mg/kg in weight matched control rats.

Unlike the results with the glycine antagonists strychnine and brucine, 3AP lesioning did not

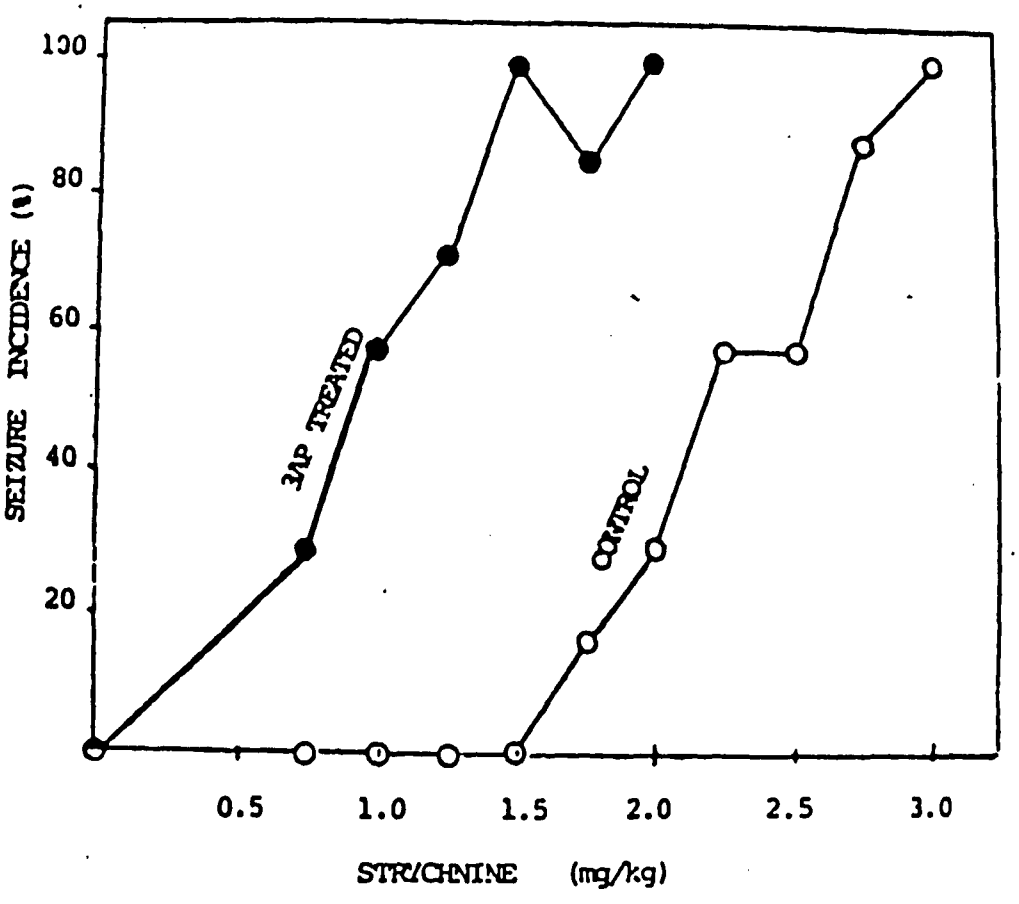



Figure Four

Strychnine dose response curve for strychnine in control (O) and 3AP lesioned (●) rats. Each point represents the incidence of seizure in 5-7 male Sprague-Dawley rats.

significantly aggravate the seizures produced by bicuculline, picrotoxin or PTZ (Table 5).

In addition to seizures, all the convulsants tested in this study induced abnormal motor behavior (AMB) consisting of myoclonus, tremor, forepaw tapping, backward movements and hyperextension of head, back and

Table 5. Effect of 3AP Lesion on Seizures Induced by Other Convulsants

| Drug | Group | Seizure Incidence | Latency To Seizure(c) | Mean Number Of Seizure(c) |
|---|---|---|---|---|
| Bicuculline 2.5 mg/kg | Control | 7/10 | 1:12 ± 0:19 | 1.0 ± .3 |
| | 3AP | 6/9 | 1:18 ± 0:18 | .9 ± .3 |
| Picrotoxin 2.0 mg/kg | Control | 1/7 | 9:10 (b) | .14 (b) |
| | 3AP | 0/7 | - | - |
| PTZ (a) 40.0 mg/kg | Control | 9/10 | 3:03 ± 1:32 | .9 ± .1 |
| | 3AP | 8/10 | 1:49 ± 0:19 | 1.0 ± .3 |

(a)PTZ: pentylenetetrazole; (b)only one value obtained
(c) values are mean ± SEM

extremities. As seen in Table 6, the percentages of the observation periods that the rats had AMB were significantly greater in the 3AP -lesioned groups compared observation periods that the rats had AMB were significantly greater in the 3AP -lesioned groups compared to controls for all convulsants tested. For example, at a dose of 40 mg/kg PTZ, the 3AP lesioned rats spent 52.5% of the observation period exhibiting AMB compared to 8.2% in non-lesioned controls. Electrothermic lesions of the inferior olive, however did not significantly alter AMB produced by 1.5 mg/kg strychnine.

## 2.5 Discussion

Table 6. Effects of IO Lesion on Convulsant Induced Abnormal Motor Behavior (AMB)

| Drug | Dose (mg/kg) | Obs. Period (min.) | Control (% of time in AMB) | IO Lesion (% of time in AMB) |
|---|---|---|---|---|
| Strychnine | 1.5 | 60 | 3.0 ± 0.2 | 11.7 + 0.3 * |
| Brucine | 40.0 | 60 | 0.4 ± 0.1 | 2.8 ± 0.1 * |
| Bicuculline | 2.5 | 50 | 6.6 ± 0.2 | 22.6 ± 0.4 ** |
| Picrotoxin | 2.0 | 45 | 4.5 ± 3.1 | 11.4 ± 0.2 * |
| PTZ (a) | 40.0 | 120 | 8.2 ± 0.3 | 52.5 + 0.7 *** |
| Strychnine(b) | 1.5 | 60 | 1.0 ± 0.1 | 1.5 ± 0.6 |

(a)PTZ: pentylenetetrazole
(b)Electrothermic lesioned rats, all others are 3AP lesioned.
*p< 0.02, **p<0.001 as compared to respective controls by Mann-Whitney test.

Each value is the mean ± SEM of 6-10 rats

Our results suggest that interruption of the olivocerebellar pathway (climbing fibers from inferior olive to cerebellar cortex) by either 3AP or electrothermic lesions of the inferior olive, lowers the threshold for seizures induced by strychnine and brucine. Brucine is the 2,3-dimethoxy derivative of strychnine. Both strychnine and brucine specifically antagonize the effects of the inhibitory neurotransmitter glycine (Woodbury 1980). Brucine is less potent than strychnine as a convulsant (Sandberg and Kristianson 1970), however the convulsions follow the same general pattern of motor

behavior. We report a 2.5 times shift to the left in the dose response curve for strychnine in 3AP lesioned rats (CD50 = 0.92 mg/kg), as compared to naive controls (CD50 = 2.32 mg/kg).

Electrothermic lesions did not completely obliterate the inferior olive in all animals and sometimes included adjacent areas of the medulla; however the only consistent area of overlap was the inferior olive. Furthermore, areas adjacent to the inferior olive which were damaged by electrothermic lesions are known not to be affected by 3AP. In another set of experiments, the effects of strychnine were assessed in rats that had lesions dorsal or lateral to, but not involving the inferior olive, and the CD50 for strychnine was 2.53 mg/kg which is not significantly different from the control group (CD50 = 2.32 mg/kg). Therefore, we conclude that destruction of the inferior olive by both 3AP and electrothermic lesions produces the same strychnine specific proconvulsive state.

A direct consequence of inferior olive destruction is degeneration of climbing fibers, and the climbing fiber-Purkinje cell synapse. The neurotransmitter released by climbing fibers is believed to be the excitatory amino acid, aspartate (Kimura et al. 1985, Wiklund et al. 1984). Purkinje cells also indirectly receive excitatory input from the mossy fibers, via the granule cell's parallel fibers which release glutamate. In

the intact system, excitation of climbing fibers causes a complex spike in the Purkinje cells (Llinas and Sugimori 1980, 1980a). Parallel fiber stimulation generates simple spikes. The complex spike is characterized by a typical Na+-K+ action potential prolonged by a calcium dependent afterpotential. During this afterpotential, simple spikes are inhibited and a silent period ensues. The silent period has been shown to be directly related to the duration of the stimulating volley (Rawson and Tilokskulchai 1981) of the climbing fibers. Thus, the net effect of climbing fiber stimulation may be inhibition of Purkinje cell activity (Montarolo et al. 1982, Colin et al. 1980).

Destruction of the inferior olive results in loss of complex spikes in the Purkinje cell (Savio and Tempia 1985, Benedetti et al. 1982) and a net increase in simple spikes, resulting in increased GABA release from Purkinje cell nerve terminals in the deep cerebellar nuclei. Batini and Billard (1985) have shown that after acute 3AP lesioning, there is a sharp rise in simple spikes concomitant with almost complete inhibition of firing of the cerebellar nuclei. Similarly, Benedetti and coworkers (1983, 1982), have shown that both reversible and irreversible inactivation of the inferior olive inhibit firing of intracerebellar and lateral vestibular nuclei. Biochemically, 3AP lesions of the inferior olive have been

shown to increase the activity of the GABA synthetic enzyme, glutamic acid decarboxylase in Purkinje cell nerve terminals in cerebellar nuclei (Oltmans et al. 1985). The sum of these observations support the hypothesis that climbing fiber destruction increases simple spike activity in the Purkinje cell and increases release of GABA by Purkinje cell nerve terminals in cerebellar nuclei. Climbing fibers also send excitatory collaterals to these nuclei (fastigial, interpositus and dentate) and to Deiters neurons in the lateral vestibular nucleus (Eccles 1967). Hence an inferior olive lesion would remove the stimulatory climbing fiber collateral input to, as well as enhancing Purkinje cell inhibition of these nuclei. Since the deep nuclei are the only source of cerebellar output, inhibition of these nuclei is analogous to cerebellectomy which is also known to create a proconvulsive state (Gartside 1978, Halpern and Julien 1972, van den Driessch and Trebaul 1958).

There are several cerebellar efferent pathways which could modify strychnine seizure activity. The dentate nucleus projects to the ventrolateral thalamus, whose efferents terminate predominantly in the sensorimotor cortex. Fastigial nuclear projections, which mainly influence the reticular and vestibular nuclei, could modify reciprocal cortical-brain stem interactions. The fastigial nucleus also has a small ascending pathway that

terminates in various thalamic nuclei. One of the more likely pathways which could be involved in modulation of the threshold for strychnine seizures is the projection from fastigial nucleus to the medullary reticular formation, an area which has dense 3H-strychnine binding (Frostholm and Rotter 1985).

We have previously reported that unilateral stereotaxic infusions of DDT, strychnine and brucine into rat medullary reticular formation induced generalized myoclonus identical to that produced by intragastric DDT (Chung and Van Woert 1984). We also found that 3AP lesioning of the inferior olive shortened the latency period and increased the intensity of myoclonus in rats administered DDT (Hwang et al. 1981). This type of myoclonus appears to be due to hyper-excitable medullary reticular formation neurons which are under glycinergic inhibitory control. Localized disinhibition of medullary reticular formation neurons by stereotaxic injection of strychnine produces myoclonus, while widespread interference with glycinergic inhibition throughout medullary reticular formation by systemically administered strychnine could produce a sufficient neuronal hyperexcitability which then spreads rostrally causing seizures. Fastigial nucleus neurons projecting to medullary reticular formation are known to have an excitatory effect (Ito et al. 1970) and could synapse with

glycine interneurons. Reduction of fastigial nucleus activity by inferior olive lesions could lower glycine inhibition thus making medullary reticular formation neurons more susceptible to antagonism of glycinergic neurotransmission by strychnine.

It is of interest to note that bicuculline and picrotoxin induced seizures were not affected by 3AP lesioning and these two GABA antagonists did not produce myoclonus when injected into the medullary reticular formation (Chung and Van Woert 1984). Inferior olive lesioning appears to selectively facilitate medullary neuronal hyperexcitability induced by interference with glycinergic neurotransmission.

# CHAPTER THREE

## Effect of Inferior Olive Lesions on [$^3$H]Strychnine and [$^3$H]GABA Binding

## 3.1 Introduction

Since the behavioral effect we observed following inferior olive destruction was a decreased threshold specific for strychnine-induced seizures, specific [$^3$H]strychnine binding was measured in inferior olive lesioned rats. The hypothesis was that the decreased threshold for seizure could be due to either more strychnine receptors; or strychnine receptors with higher affinity for strychnine. We looked at [$^3$H]strychnine binding displaced by unlabeled strychnine or glycine in synaptosomal preparations from medulla and spinal cord in inferior olive lesioned and control rats.

We also measured [$^3$H]GABA binding to the cerebellar nuclei of inferior olive lesioned and control rats. An increase in simple spike production by Purkinje cells following inferior olive lesion has been observed, concomitant with diminished cerebellar efferent nuclear output (Batini and Billard 1985, Batini et al. 1985). This suggests an increase in GABA release by Purkinje cells. Biochemical studies show an increase in activity of the synthetic enzyme for GABA, glutamic acid decarboxylase in cerebellar cortex and cerebellar efferent nuclei from rats with inferior olive lesions (Oltmans et al. 1986, 1984). In the case of chronic, excess GABA release, we might expect to see a decrease in the number

of $GABA_A$ receptors, postsynaptic to Purkinje cells. A decrease in $GABA_B$ sites in the cerebellum from inferior olive lesioned rats was reported (Kato and Fukuda 1985), and may reflect down-regulation of 'pre-synaptic, GABA autoreceptors' on Purkinje cells, in response to excessive GABA release by Purkinje cells.

## 3.2 Materials and Methods

### 3.2a Lesions

Male Sprague-Dawley rats (150-175 g) were housed 6 per cage, kept on a 12:12 hour light:dark schedule and provided with food and water ad libidum. Animals were pretreated with a single injection of 80 mg/kg 3AP. Recovery from acute toxicity, stridulous breathing, diarrhea and weight loss occurred 3-5 days following 3AP administration; rats which retained a chronic, rolling ataxia as the predominant symptom of cerebellar dysfunction, were considered lesioned. Animals were used for binding studies 2-3 weeks post lesion.

### 3.2b [$^3$H]Strychnine Binding

[$^3$H]Strychnine binding was measured using the method of Young and Snyder (1974). Determinations were made in

triplicate for individual rats, n=9. Briefly, synaptic membranes were prepared from medulla or spinal cord from 3AP-inferior olive lesioned and control rats. Tissue was homogenized in 50 volumes of 50 mM Tris buffer, pH 7.7 for three 15 sec intervals using a polytron at speed 5, then washed twice in 100 volumes of 50 mM Tris buffer, pH 7.5, and resuspended at a final concentration of 10 mg/ml. Specific [$^3$H]strychnine binding has been shown to be enriched in this fraction (Young and Snyder 1974). Protein was determined by the method of Lowry et al. (1951).

The Kd for strychnine in synaptic membranes prepared from spinal cord is 3 nM, as compared to 10 uM for glycine (Young and Snyder 1974). For the binding assay, 1 ml aliquots of tissue, were incubated with 1 nM [$^3$H]strychnine (29 Ci/mmol, Amersham, Arlington Heights, IL.) for 10 min at 0$^o$ C. Non-specific binding was determined by the inclusion of 100 uM unlabeled strychnine or glycine. Free and bound ligand were separated by filtration through GF/B Whatman glass fiber filters. Filters were washed twice with 5 ml ice cold 50 mM Tris buffer, pH 7.5. Dried filters were counted in 8 ml liquid scintillation fluid. Specific binding was determined by subtracting nonspecific counts from total counts.

### 3.2c [$^3$H]GABA Binding

[$^3$H]GABA binding was measured in pooled samples of cerebellar nuclei. The cerebellum was removed from the whole brain immediately following decapitation, and dissected into three pieces by making an anterior cut along the primary fissure, and another cut about halfway through the corpus cerebelli, along the horizontal fissure. From the ventral side then, the deep nuclei are readily visible, as they are white matter and stand out from the gray, cerebellar cortex. The deep nuclei were then dissected out, and pooled cerebellar efferent nuclei from each individual rat was used for binding studies.

The Kd for GABA at $GABA_B$ sites measured in Triton-X 100 treated membranes from whole brain is 34 nM (Bowery et al. 1983), compared to 3.4 nM for $GABA_A$ sites (Heaulme et al. 1986). [$^3$H]GABA binding was done using the method outlined by Bennet (1978). Briefly, synaptosomes were prepared from cerebellar nuclear tissue from control and inferior olive lesioned rats, n=9, as described in section 3.2b, with the addition of 10 uM amino-oxyacetate to the buffer. Amino-oxyacetate is a transaminase inhibitor and is used to inhibit GABA-transaminase, a GABA synthetic enzyme. Synaptosomes were partially solubilized by incubation with 0.05% Triton X-100 at 37$^o$ C, for 35 min in 100 volumes Tris Buffer, pH 7.5 + amino-oxyacetate, collected by centrifugation and washed with 50 mM Tris buffer, pH 7.5. Tissue was resuspended at a final

concentration of 10 mg/ml. Protein was determined by the method of Lowry et al. (1951). For the binding assay, 1 ml aliquots of tissue were incubated, in triplicate, with 1 nM [$^3$H]GABA (70 Ci/mmol, Amersham Corp., Arlington Heights, IL.) for 30 min at 0$^o$ C. Non-specific binding was determined by the inclusion of 100 uM unlabeled GABA. Free and bound ligand were separated by centrifugation for 20 min at 12,000g (SS34 rotor, Sorval centrifuge). The pellet was rinsed twice, and radioactivity extracted with 1 ml 95% ethanol for 1 hour, and counted in 6 ml liquid scintillation fluid. Specific binding was determined by subtracting nonspecific counts from total counts.

## 3.3 Results

The results of [$^3$H]strychnine binding are presented in Table 7. There was no difference in specific binding displaced by either unlabeled strychnine or glycine in synaptosomes from either spinal cord or medulla, in control and inferior olive lesioned rats. Glycine displacable [$^3$H]strychnine binding was lower than strychnine displaced [$^3$H]strychnine binding in both spinal cord and medulla.

In Table 8, the results of [$^3$H]GABA binding to synaptosomal preparations from cerebellar nuclei are presented . There is no difference between control and

inferior olive lesioned rats.

Table 7. Effect of Inferior Olive (IO) Lesions on [3H]Strychnine Binding in Medulla and Spinal Cord (fm/mg protein).

| Tissue | Displaced by | |
|---|---|---|
| | 100uM Strychnine | 100uM Glycine |
| Spinal Cord | | |
| Control | 180.3 ± 32.9 | 141.4 ± 16.2 |
| IO Lesion | 195.7 ± 13.2 | 139.6 ± 15.9 |
| Medulla | | |
| Control | 72.3 ± 4.7 | 41.8 ± 6.1 |
| IO Lesion | 77.8 ± 10.5 | 42.9 ± 14.3 |

Each value is the mean ± SE of 9 rats.

## 3.4 Discussion

Inferior olive lesion had no effect on [$^3$H]strychnine or [$^3$H]GABA binding. Specific [$^3$H]strychnine binding was measured using both unlabeled strychnine and glycine as displacers. Since strychnine has a much higher affinity for the [$^3$H]strychnine labeled site than glycine, it has been suggested that there is an allosteric relationship between sites displaced by glycine and strychnine, however, the

Table 8. [3H]GABA Binding (fm/mg) in Control and Inferior Olive (IO) Lesioned Rats.

| Tissue | Displaced by |
|---|---|
| Cerebellar Nuclei | 100uM GABA |
| Control | 74.0 ± 3.5 |
| IO Lesion | 81.8 ± 7.1 |

Each value is the mean ± SE of 9 rats.

differences in affinity may be due to the fact that strychnnine is an antagonist and glycine is an agonist. Since no difference in binding was observed, the increased sensitivity of inferior olive lesioned rats to strychnine-induced seizure cannot be accounted for by a direct increase in binding sites.

There was no difference in specific [$^3$H]GABA binding to high affinity $GABA_A$ receptors in cerebellar nuclei in control and inferior olive lesioned rats. A decrease in $GABA_B$ receptors to cerebellar tissue from inferior olive lesioned rats as compared to controls has been shown (Kato and Fukuda 1985), and may reflect presynaptic GABA receptor down regulation. The absence of postsynaptic receptor down regulation might be due to prior compensation by the presynaptic 'autoreceptors', thus preventing postsynaptic changes.

The negative findings led us to investigate the changes in excitatory amino acid receptor binding in the cerebellum which are presented in the next two chapters.

# CHAPTER FOUR

## [$^3$H]L-Glutamate Binding to Postsynaptic Densities

## 4.1 Introduction

In this chapter, I report the results of [$^3$H]L-glutamate binding to postsynaptic densities prepared from cerebella of inferior olive lesioned and control rats. As discussed in the literature review, three distinct excitatory amino acid receptors have been identified and classified on the basis of selective interaction with the glutamate analogs NMDA, QA and KA. These compounds elicit a characteristic excitatory electrophysiologic response when applied iontophoretically to brain slices or neuronal cell cultures, and have high affinity for sites labeled with [$^3$H]L-glutamate. A putative fourth site was proposed following the observed potentiation of [$^3$H]L-glutamate binding by $CaCl_2$ (Nadler et al. 1985, Mena et al. 1982, Fagg et al. 1982). In the synaptic plasma membrane preparation, which is a mixture of both pre- and post- synaptic membranes, the glutamate analog 2-amino-4-phosphonobutyrate (AP4) inhibited the $CaCl_2$-stimulated [$^3$H]L-glutamate binding (Wu et al. 1986, Fagg et al. 1982), and [$^3$H]-AP4 was subsequently shown to preferentially label this site (Fagg and Foster, 1984). When applied directly to a neuronal membrane however, AP4 antagonized depolarizations induced by glutamate, aspartate, QA and N-acetylaspartyl glutamate, but does not induce a direct response. It was

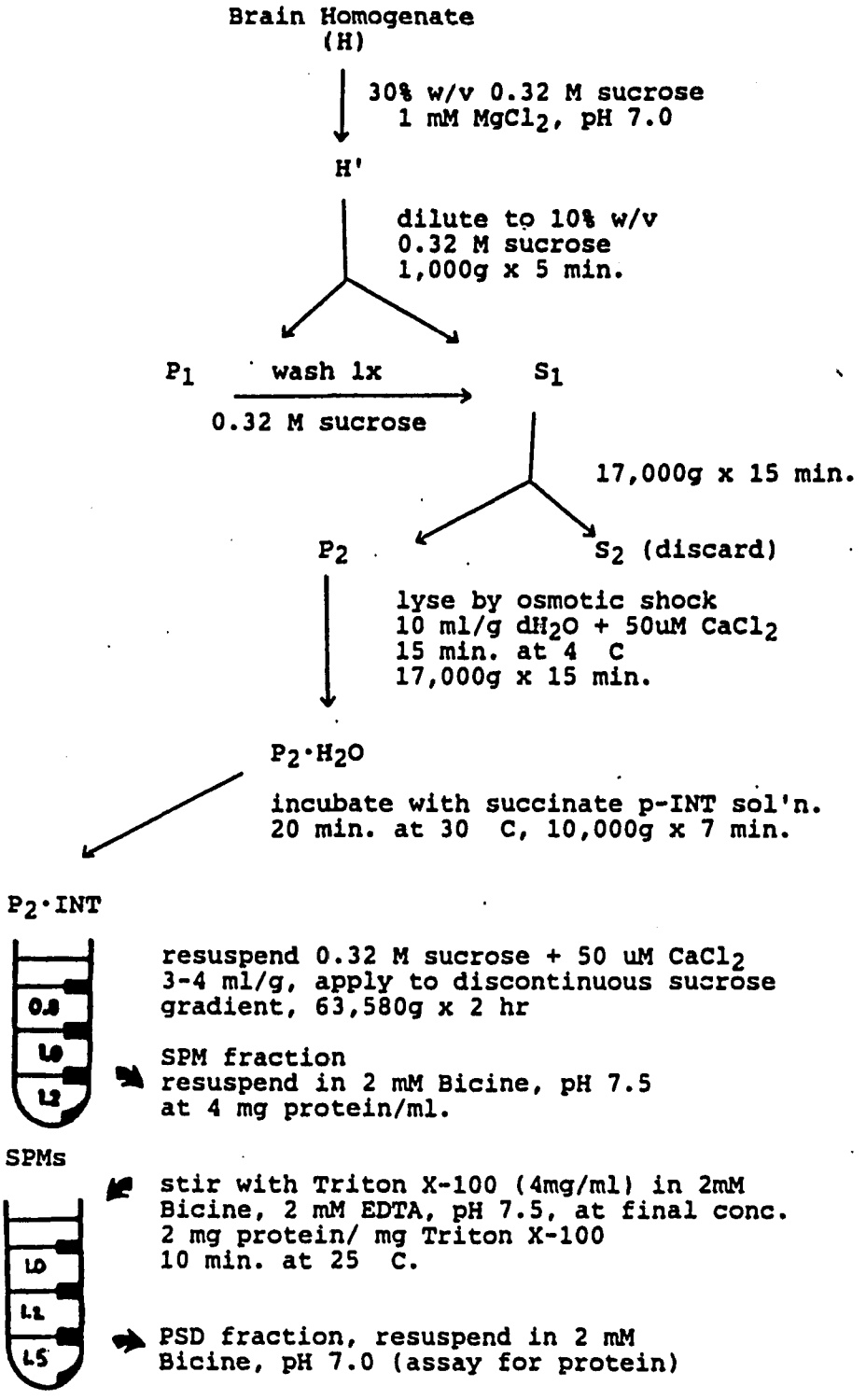
thought for some time that AP4 sites represented a class of neurophysiologically active excitatory amino acid receptors coupled to a chloride channel (Fagg et al. 1982), or a desensitized form of glutamate receptor with divalent cations promoting transition from a low affinity to a high affinity state (Fagg et al. 1983). It appears now, that these sites represent a non-physiologic vesicular uptake system that results from sonication and partial solubilization of the membranes which occurs during preparation of both synaptosomes and synaptic plasma membranes for in vitro binding studies (Fagg and Lanthorn 1985, Pin et al. 1984). In essence, [$^3$H]L-glutamate is captured in these vesicles and sequestered in a dose dependent fashion. This process is stimulated by both divalent and monovalent cations, but disappears as a function of osmolarity. While much of the work done characterizing the phenomenon of vesicular sequestration has focused on the anomalous binding of [$^3$H]AP4, or AP4 displaceable [$^3$H]L-glutamate binding, sequestration occurs to a certain extent as a function of the membrane preparation, whether QA, KA or NMDA are used as displacing ligands. A similar stimulation of artifactual uptake by sodium ions has been observed for D-aspartate, and has been confused for binding sites (Danbolt and Storm-Matehisen, 1986). However, in preparations that do not contain vesicles, such as

postsynaptic densities, there is no ionic stimulation of [$^3$H]L-glutamate binding (Zaczek et al. 1987, Wu et al. 1986, Kessler et al. 1986, Pin et al. 1984) and no binding of [$^3$H]-AP4, the putative antagonist of this 'binding site' (Kessler et al. 1986, Pin et al. 1984). It is not entirely clear if all the 'sites' labeled by [$^3$H]-AP4 are due to the artifactual vesicular sequestering described above. For example, Fagg and Lanthorn (1985) have demonstrated that there is no correlation between $Cl^-/Ca^{2+}$-dependent AP4 displaceable [$^3$H]L-glutamate binding and AP4 mediated electrophysiologic inhibition of glutamate induced depolarization. To avoid the contribution of [$^3$H]L-glutamate sequestration by artifactual vesicles, I measured [$^3$H]-glutamate binding to postsynaptic densities.

The postsynaptic density is the protein attachment to the postsynaptic membrane which is rich in neurotransmitter receptor binding sites. A postsynaptic density fraction can be isolated from crude synaptosomes by lysis followed by a series of discontinuous sucrose gradients and detergent solubilization (Jones and Matus 1974, Cotman and Taylor 1972). A detailed protocol for postsynaptic density isolation from rat cerebellum is presented in the methods section of this chapter and outlined schematically in Figure 5. Postsynaptic densities prepared using several slightly different

Figure Five

POSTSYNAPTIC DENSITY PREPARATION*




*Adapted from Cotman and Taylor, J.Cell Biol. 55, 696-711, 1972.

protocols yield a fraction that is primarily protein with no nucleic acid and very little phospholipid (Jones and Matus 1974, Davis and Bloom 1973, Cotman and Taylor 1972). Detailed electron microscopic studies by Cohen et al. (1977) and Cotman's group (Cotman et al. 1971) showed structures approximately 400 nm x 40 nm composed of 13-28 nm particles. Isolation of postsynaptic densities from synaptic plasma membranes by sonication produces a vesicular preparation with residual ATPase activity (Jones and Matus 1974), whereas detergent treatment of synaptic plasma membranes, a crucial step in the postsynaptic density isolation procedure used to measure specific glutamate binding (Wu et al. 1986, Fagg and Matus 1984), yields a vesicular free sample with no ATPase or GTPase activity (Cotman and Taylor 1972, Cohen et al. 1977). Specific binding of radiolabelled ligands to synaptic plasma membranes and postsynaptic densities provides information about subcellular localization (i.e., presynaptic or postsynaptic) of receptors. Both NMDA and QA sensitive glutamate binding sites have been observed in postsynaptic density preparations (Fagg and Matus 1984), while KA sensitive [$^3$H]L-glutamate binding has been shown to occur in synaptic plasma membranes, suggesting a localization of this receptor in presynaptic, as well as postsynaptic sites (Foster et al. 1981).

Neurotransmitter receptors for excitatory amino acids

are particularly difficult to measure due to the ubiquitous distribution of both glutamate and aspartate in cells and the multiple functions they subserve. To measure glutamate neurotransmitter receptor sites, [$^3$H]L-glutamate binding should be performed using postsynaptic densities, not only to avoid artifactual 'sequestration' described above, but also to avoid binding to other high affinity excitatory amino acid recognition sites. Synthetic and degradative enzymes for both glutamate and aspartate, and transport mechanisms for these amino acids are present in all cells. Mitochondrial membranes, for example, contain a receptor mediated exchange system, the malate-aspartate shuttle, which acts to maintain charge distribution for oxidative - phosphorylation. This system contains a specific, high affinity carrier for aspartate. Aspartic amino transaminase is present in the cytosol near the mitochondrial membrane, while glutamic-oxaloacetic transaminase is in the mitochondria, both of these enzymes have a high affinity for aspartate and glutamate (from Stryker, 1981). While it is generally accepted that affinity coefficients for recognition sites of enzymes are less than those required for neurotransmitter receptor-ligand interactions, Kds for glutamate neurotransmitter receptors have been reported in the micromolar range (Wu et al. 1986, Fagg and Matus, 1984).

Nerve terminals contain a high concentration of mitochondria, and to avoid potential contamination by binding to 'specific' sites not relevant to neurotransmission, we measured [$^3$H]L-glutamate binding to postsynaptic densities.

## 4.2 Materials and Methods

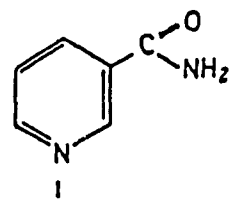
### 4.2a Lesions

Male Sprague-Dawley rats (150-175 g) (Harlan Sprague Dawley, Walkersville, MD) were used. The inferior olive was lesioned by systemic administration of 3AP. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO., unless otherwise indicated), using a modification of the procedure described by Llinas et al. (1975). 65 mg/kg 3AP was given, followed 4.5 hours later by 300 mg/kg niacinamide. Drugs were dissolved in 0.9% saline. The mortality rate using this procedure was zero. Lesioned animals displayed a persistent ataxic gait.

Unlike non-specific neurotoxins, such as kainic acid, 3AP does not exert effects directly, but apparently through a metabolite, as neuronal hypertrophy is not visible until 7 hours after i.p. injection (D'esclin and

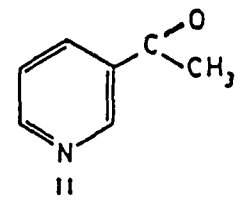
Escubi 1974). Since 3AP is toxic in general, a major drawback to its use is the high mortality rate: the $LD_{50}$ for 3AP is very near the dose which produces inferior olive lesions and cerebellar dysfunction (65 mg/kg). Llinas et al. (1975), reported that administration of harmaline, following 3AP injection produced a discrete lesion, localized to the inferior olive, presumably due to specific activation of olivo-cerebellar fibers by harmaline (de Montigny and Lamarre 1973, Llinas and Volkind 1973). Subsequent treatment with nicotinamide was thought to protect against severe niacin depletion, which occurs secondary to systemic flooding by 3AP.

A multi-drug protocol for 3AP lesioning was proposed, whereby 3AP administration was followed by harmaline (to increase inferior olive metabolism) and then nicotinamide




I

Nicotinamide




II

3-acetylpyridine

Figure Six

to prevent death and minimize the general toxic effects. 3AP is a nicotinamide analog (Figure 6). Nicotinamide functions metabolically as a component of two important coenzymes; nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). The mechanism of 3AP neurotoxicity is believed to be due to the synthesis of abnormal coenzymes in which nicotinamide is replaced by 3AP.

A comparative study by Balaban (1985), however, showed that there was no consistent difference in lesion specificity between the protocol just described and a single i.p. injection of 75-80 mg/kg 3AP. Initially, we had given a single dose of 80 mg/kg 3AP i.p. which resulted in a mortality rate ranging from 35-70%. When 300 mg/kg nicotinamide was given 4.5 hours after 3AP injection, rats still displayed some signs of acute toxicity (diarrhea, partial aphagia, severe ataxia and transient tremor), but these were not as pronounced as those seen following the injection of 3AP alone. Severe respiratory depression was absent and weight loss was significantly less, if at all. With this protocol, the mortality rate was zero. A long-lasting, broad based ataxic gait was present in rats lesioned by both methods.

### 4.2b [$^3$H]L-Glutamate Binding

## Membrane Preparation

Postsynaptic densities were isolated from rat cerebella by the method of Cotman and Taylor (1972). As depicted in figure 5, a crude mitochondrial fraction ($P_2$) was prepared, lysed using 50 uM $CaCl_2$ and treated with 1 mM p-iodonitro-tetrazolium violet (p-INT) in 60 mM succinate/40 mM $KH_2PO_4$ buffer, containing 50 uM $CaCl_2$, pH 7.5, to facilitate mitochondrial separation. Isolation of synaptic plasma membrane was performed by discontinuous sucrose density centrifugation using a gradient made up with equal volumes 0.8 M, 1.0 M and 1.2 M sucrose, containing 50 uM $CaCl_2$, pH 7.0 and centrifuged for 2 hrs at 63,500g (Beckman Model L5-50, Ultra-Centrifuge). Synaptic plasma membranes were collected from the 1.0 M/1.2 M sucrose interface, washed in 50 uM $CaCl_2$ and resuspended in 20 mM Bicine, 50 uM $CaCl_2$, pH 7.4. Synaptic plasma membranes were treated with 1 ml Triton X-100 (4 mg/ml) per mg protein. Postsynaptic densities were then isolated via discontinuous sucrose density centrifugation using a gradient consisting of equal volumes of 1.0 M, 1.2 M and 1.5 M sucrose, containing 50 uM $CaCl_2$, pH 7.0. Postsynaptic densities were collected from the 1.2 M/1.5 M sucrose interface, washed in 50 uM $CaCl_2$ and resuspended in 20 mM Bicine, 50 uM $CaCl_2$, pH 7.4. Protein was

determined using the method of Lowry et al. (1951). Postsynaptic densities were stored on ice at 0-4 C, and glutamate binding performed within 12 hours of the final preparation step.

## Binding Assay

[$^3$H]L-glutamate binding was carried out using a modification of the microfuge assay described by Foster et al. (1981). 40-50 ug protein of Postsynaptic densities were incubated in a final volume of 200 ul 20 mM Bicine/KOH buffer, pH 7.4, containing 2.5 mM $CaCl_2$ and 45 nM [$^3$H]L-glutamate (56 Ci/mmole, Amersham, Arlington Heights, ILL.) for 20 minutes at 30$^{o}$C. Non-specific binding was determined by the inclusion of 0.5 mM unlabeled L-glutamate, KA, NMDA, QA, AP4 or glutamate diethylester (GDEE). Bound ligand was separated from free by centrifugation (Fisher Micro-centrifuge Model 235A) for 3.5 minutes and the pellet superficially rinsed with distilled $H_2O$. The pellet was then solubilized in 200 ul 0.2 N NaOH for 12 hrs, suspended in 8 mls liquid scintillation fluid (Liquiscint, National Diagonstics, Manville, NJ.) kept in the dark for 12 hours and counted for 5 min, using a Beckman LS-9000 Scintillation Counter.

Equilibrium saturation binding was carried out using [$^3$H]L-glutamate ranging in concentrations from 45 to

1440 nM. Non-specific binding was determined in the presence of either 0.5 mM unlabeled glutamate or quisqualic acid.

### 4.2c Statistics

Differences in binding, between 3AP lesioned and naive groups were assessed using Student's t-test. Scatchard analysis was performed using EBDA (Equilibrium Binding Data Analysis program, adapted by G.A. MacPherson, 1984). The numbers obtained for Bmax using the EBDA analysis are extrapolated values, to insure a valid statistical analysis, the slopes of the scatchard analysis curves for both the inferior olive lesioned and control groups were compared using Student's t-test (Zar 1974, p.228).

## 4.3 Results

Purity of the postsynaptic density preparation was examined by electron microscopy, with the help of Dr. Gay Holstein, Departament of Neurology, Mt. Sinai Medical Center, and no vesicles were observed. The results of three separate experiments on [$^3$H]L-glutamate binding to cerebellar postsynaptic densities from both inferior olive lesioned and control groups, are presented in Table 9. For each experiment, postsynaptic densities were prepared

using cerebellum pooled from 6-7 rats for both groups. The values presented are means of 6, 6 and 9 determinations, for experiments 1, 2 and 3, respectively. In experiment 3, [$^3$H]L-glutamate binding displaced by 0.5 mM unlabeled glutamate and 0.5 mM QA increased significantly in the inferior olive lesioned group compared to the control ($p< 0.05$, Student's t-test). The increase in QA-sensitive binding was also seen in experiment 2. NMDA displaceable [$^3$H]L-glutamate binding is increased in the inferior olive lesioned group in experiment 3.

A decrease in [$^3$H]L-glutamate binding displaceable by 0.5 mM KA in inferior olive lesioned rats was observed in experiment 1. KA-sensitive binding in postsynaptic densities consisted of only 6-12 % of specific binding. There were no differences in [$^3$H]L-glutamate binding displaced by 0.5 mM AP4 in control or inferior olive lesioned groups. GDEE displaceable [$^3$H]L-glutamate binding was measured in experiments 1 and 2 and was observed to increase significantly ($p< 0.05$, $p< 0.01$) in the inferior olive lesioned groups compared to the control groups. GDEE displaceable specific binding ranged from 2-11 %.

Binding parameters for both glutamate and quisqualate displaceable [$^3$H]L-glutamate binding in cerebellar post-synaptic densities were estimated from scatchard analysis.

Table 9. Effect of inferior olive lesions on [3H]L-Glutamate binding to cerebellar postsynaptic densities.

| Displacer (0.5mM) | EXP. | Control | Inferior Olive Lesioned |
|---|---|---|---|
| Glutamate | 1 | 1497 ± 155 | 1329 ± 118 |
| | 2 | 2442 ± 278 | 3000 ± 487 |
| | 3 | 3008 ± 169 | 3642 ± 349* |
| QA | 1 | 1395 ± 194 | 1380 ± 147 |
| | 2 | 467 ± 159 | 1232 ± 501** |
| | 3 | 2464 ± 169 | 3207 ± 349* |
| NMDA | 1 | 546 ± 155 | 632 ± 138 |
| | 2 | 337 ± 150 | 690 ± 374 |
| | 3 | 247 ± 94 | 759 ± 290* |
| AP4 | 1 | 546 ± 150 | 355 ± 103 |
| | 2 | --- | 741 ± 392 |
| | 3 | 486 ± 128 | 949 ± 307 |
| KA | 1 | 601 ± 155 | 140 ± 79* |
| | 2 | 109 ± 94 | 191 ± 191 |
| | 3 | 173 ± 78 | 440 ± 236 |
| GDEE | 1 | 221 ± 126 | 624 ± 118* |
| | 2 | 62 ± 62 | 1058 ± 461** |

Each value is the mean ± SEM (fm/mg) of 6-9 determinations from pooled samples.
*p< 0.05, **p< 0.01, compared to the respective control Student's t-test.

This data is summarized in Table 10. [$^3$H]L-glutamate concentrations ranged from 45-1440 nM, and non-specific binding was determined by the inclusion of either 0.5 mM unlabeled glutamate or QA, each point was determined in triplicate. In the control group, the Kd for glutamate displaceable radioligand binding was 402.2 nM and the Bmax

Table 10. Scatchard analysis of equilibrium [$^3$H]L-glutamate binding (pmole/mg protein) to cerebellar Postsynaptic densities from control and inferior olive lesioned rats.

| Group | Kd (nM) | Bmax ± SEM (pmole/mg) | r* | Hill Coefficient |
|---|---|---|---|---|
| Displacer: 0.5 mM unlabeled glutamate | | | | |
| Control | 402.2 | 55.7 ± 7.3 | -0.979 | 0.987 ± 0.049 |
| IO Lesion | 423.3 | 42.9 ± 8.9 | -0.908 | 0.988 ± 0.045 |
| Displacer: 0.5 mM quisqualate | | | | |
| Control | 325.8 | 30.9 ± 9.0 | -0.890 | 1.046 ± 0.203 |
| IO Lesion | 214.5 | 17.8 ± 0.8 | -0.996 | 1.008 ± 0.021 |

* correlation coefficient

55.7 ± 7.3 pmole/mg protein. The Kd estimated for glutamate binding in inferior olive lesioned rats was 423.3 nM, with a Bmax of 42.9 ± 8.9 pmole/mg protein. Student's t-test (t=-0.066) shows that there is no difference in the binding parameters between groups. For QA displaceable [$^3$H]L-glutamate binding, the Kds estimated for control and lesioned groups were again similar (325.8 and 214.5 nM, respectively), the Bmax for the control group was 30.9 ± 9.0 pmole/mg, while in the inferior olive lesioned group, the Bmax was estimated at 17.8 ± 0.8 pmole/mg protein, again there is no significant difference between these groups (t=-0.234). Hill coefficients were near unity for all binding isotherms,

indicating that [$^3$H]L-glutamate was interacting with a single population of sites.

## 4.4 Discussion

In this chapter we demonstrate high specific binding of [$^3$H]L-glutamate, displaceable by a number of glutamate analogs, to postsynaptic densities isolated from rat cerebellum. The signal was robust, with specific binding generally greater than 80%, indicating specific recognition site interaction between [$^3$H]L-glutamate and the displacing ligands; however limitations inherent in the binding assay may have prohibited detection of differences in receptor populations between the inferior olive lesioned and control groups. The results presented in this chapter are inconsistent. In Table 9, [$^3$H]L-glutamate binding displaced by glutamate is either unchanged in inferior olive lesioned rats, or, as in experiment 3 it is increased. [$^3$H]L-glutamate binding displaced by QA in inferior olive lesioned rats appears to be increased in both experiments 2 and 3; however, QA-displaceable [$^3$H]L-glutamate binding in control rats in experiment 2 is much lower than experiments 1 or 3, making interpretation of these data difficult. In fact, because of the high degree of variability in the data, no clear conclusions can be drawn. Before any interpretation

of the data are presented, it is important to explain some of the shortcomings in the experimental protocol. As discussed in the introductory section, several factors unique to glutamate and aspartate interfere with the determination of meaningful radioligand binding data from crude synaptosomal preparations; foremost are 1) artifactual vesicular sequestration of [$^3$H]L-glutamate, and 2) contamination by other biological high affinity recognition sites. To avoid these complications, [$^3$H]L-glutamate binding was measured in postsynaptic densities, the are a highly specialized protein attachment to neuronal membranes. Postsynaptic densities constitute only a small fraction of brain tissue. A typical preparation yielded a postsynaptic density fraction of 0.2% wet weight, with values ranging from 0.04% to 0.5%, therefore necessitating the use of a pooled sample from 6-7 rats for each experiment.

Once isolated, postsynaptic densities are unstable and cannot be stored frozen because disruption of the residual membrane and denaturation of the integral proteins abolish [$^3$H]L-glutamate binding. Postsynaptic densities can be stored refrigerated, on ice, for a short period of time (Fagg et al. 1981). In practice, I found that binding activity virtually disappeared by 24 hours after preparation, in spite of all precautions, due to denaturation and microbial contamination. The choice of

storage buffer is crucial as it can accelerate or retard these processes considerably. We found, as have others (Wu et al. 1986, Fagg et al. 1981) that while [$^3$H]L-glutamate binding is high in HEPES/KOH buffer, it interferes with protein determination, and accelerates loss of binding. Bicine/KOH, on the other hand lends stability to the postsynaptic density preparation and does not interfere with protein determination. Unfortunately, [$^3$H]L-glutamate binding is less in Bicine/KOH buffer, thus making it difficult to see small differences in binding between the control and lesioned groups.

Additional problems in determining [$^3$H]L-glutamate binding were inherent to the binding assay itself. Separation of bound from free ligand had to be accomplished by centrifugation, because the filtration technique is suitable only for binding processes with Kds of $10^{-8}$ M or less. The Kds for [$^3$H]L-glutamate binding are in the micromolar range, as shown in this study and by others. In cerebellar membranes, the Kd for [$^3$H]L-glutamate binding to cerebellar membranes was calculated as 0.82 uM (Honore et al. 1981) and in whole brain postsynaptic densities, Kds of 0.33 uM and 1.8 uM were observed (Fagg and Matus 1984, Head et al. 1979). Centrifugation posed a problem because the postsynaptic densities did not pellet uniformly. Samples that were

grossly aberrant were discarded, however, it appears that inevitably variable amounts of bound [$^3$H]L-glutamate were measured. In addition, pellets were often lost during the washing procedure, further diminishing the number of samples. In spite of these difficulties, a number of determinations were made, however, no clear trends emerged.

If interpretation of the data were limited only to experiment 3, in Table 9, one might conclude that either QA-sensitive glutamate binding sites are increased in the inferior olive lesioned group, or that receptors from these animals have a higher affinity. The increase in GDEE-sensitive [$^3$H]L-glutamate binding, from experiments 1 and 2, also tempt one to suspect a change in QA-receptors, however GDEE is not a very good displacer of [$^3$H]L-glutamate binding (Olsen et al. 1987). But, when scatchard analysis was performed for both glutamate- and QA-displaceable [$^3$H]L-glutamate binding, there was no difference in either receptor population in control and inferior olive lesioned rats using the microfuge assay for postsynaptic density binding. Therefore it is not possible to draw any definite conclusions about [$^3$H]L-glutamate receptors in cerebellum as a function of climbing fiber denervation on the basis of the data presented in this chapter.

As discussed in the literature review, there is

evidence to suggest that both climbing and parallel fibers release excitatory amino acids that interact with a QA-sensitive receptor (Kimura et al. 1985, Kano and Kato 1987). An increase in QA-sensitive binding could be due to supersensitivity following climbing fiber denervation or compensatory innervation by the parallel fiber system. However, to draw any conclusions, further experiments with a more selective binding ligand needed to be done. These experiments are reported in the next chapter.

The variability in postsynaptic density binding data also presented the possibility that there might be a change in NMDA-sensitive binding sites in inferior olive lesioned rats. This is particularly interesting, in light of the recent report demonstrating the presence of functionally active NMDA receptors on distal dendrites of cerebellar Purkinje cells in the adult guinea-pig (Sekiguchi et al. 1987). Prior to this report, cerebellar NMDA receptors were found only in cultured neurons from fetal and neonatal tissue (Cull-Candy and Usowicz 1987). If NMDA receptors on Purkinje cells are postsynaptic to climbing fibers, an increase in NMDA-sensitive [$^3$H]L-glutamate binding sites could also represent denervation supersensitivity. This is an interesting speculation, and an area that should be explored further.

# CHAPTER FIVE

## Increased [$^3$H]AMPA Binding in the Cerebellum May Mediate Decreased Strychnine Seizure Threshold in Inferior Olive Lesioned Rats

Submitted for publication to Brain Research, June, 1988.

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## 5.1 Summary

Bilateral inferior olive lesions produce a proconvulsant state specific for strychnine-induced seizures and myoclonus. We have proposed that these phenomena may be mediated by increased excitation of glutamate receptors on cerebellar Purkinje cells in response to climbing fiber deafferentation (Anderson et al. 1987). We found that inferior olive lesions produced a selective increase in [$^3$H]AMPA binding sites displaceable by QA. There was no difference in the number of [$^3$H]AMPA binding sites displaceable by glutamate, KA or GDEE.

We next examined the effects of excitatory amino acid antagonists on strychnine seizures in control and inferior olive lesioned rats. Administration of either GDEE, a QA selective antagonist or MK-801, an NMDA antagonist to control rats, did not affect strychnine-induced seizures or myoclonus. However, both GDEE and MK-801 did counteract the inferior olive lesion-induced leftward shift in the strychnine seizure dose-response curve. In inferior olive lesioned rats, GDEE also inhibited strychnine induced myoclonus while MK-801 had no effect on strychnine myoclonus. The increase in QA sensitive [$^3$H]AMPA binding sites in the cerebellum may mediate the inferior olive lesion induced proconvulsant state specific

for strychnine induced seizures and myoclonus and GDEE pretreatment restores the threshold for strychnine induced seizure in inferior olive lesioned rats by blocking QA receptors.

## 5.2 Introduction

We have previously reported that bilateral inferior olive lesions produce a decreased threshold specific for strychnine-induced seizures. Furthermore, inferior olive lesions aggravate myoclonus induced by administration of a number of disinhibitory convulsants, such as picrotoxin, bicuculline and pentylenetetrazole (Anderson et al. 1987). Inferior olive lesioned rats also display a decreased latency to DDT-induced myoclonus (Hwang et al. 1981)

Climbing fibers, originating from the inferior olive project to cerebellar Purkinje cells where they release the neurotransmitter aspartate (Wiklund et al. 1982). Stimulation of climbing fibers produces a characteristic electrophysiologic signal called the complex spike. The presence of complex spikes reduces the responsiveness of Purkinje cells to glutamatergic excitatory input from the parallel fibers, which produce simple spikes (Rawson and Toliksulchai 1982). Inferior olive lesions remove climbing fiber-induced complex spikes which result in

Purkinje cell hyperexcitation due to unimpeded simple spike production by parallel fibers (Colin et al. 1980).

In the mammalian central nervous system, glutamate and aspartate are excitatory neurotransmitters that interact with specific receptors which have been classified on the basis of their preference for the glutamate analogs quisqualate (QA), kainic acid (KA) and N-methyl-D-aspartate (NMDA) (Watkins and Evans 1981, Curtis and Johnston 1974). The molecular layer of the cerebellar cortex, which contains Purkinje cell dendrites, is rich in QA-receptors, as demonstrated by binding studies with [$^3$H]AMPA ((RS)-alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid), a ligand with high affinity for the QA preferring site (Olsen et al. 1987, Honore et al. 1982). Glutamate diethylester (GDEE) is a QA-receptor antagonist that prevents QA, homocysteine and alcohol withdrawal seizures in mice (Schwarz and Freed 1986, Freed 1985) and delays the onset of strychnine seizures in rats (Abdul-Ghani et al. 1982). MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine), an NMDA receptor antagonist, is also a potent anticonvulsant in some animal models, but has been reported to have no effect on strychnine-induced seizures (Clineschmidt et al. 1982). NMDA displaceable [$^3$H]L-glutamate binding is not present in the cerebellum (Monaghan and Cotman 1985).

In the present study, we examined the effects of

bilateral inferior olive lesions on [$^3$H]AMPA binding in the cerebellum. In addition, we investigated whether the excitatory amino acid antagonists, GDEE and MK-801 would have any effect on inferior olive lesion-induced alterations in threshold for strychnine seizure and myoclonus.

## 5.3 Materials and Methods

Male Sprague-Dawley rats, 125-150 g, were used (Harlan Sprague Dawley, Walkersville, MD.). Animals were provided with food and water ad libidium and kept on a 12:12 hour light:dark schedule. The inferior olive was lesioned by the neurotoxin 3-acetylpyridine (3AP), using a modification of the procedure described by Llinas et al (1975). 65 mg/kg 3AP was administered intraperitoneally (i.p.), followed 4.5 hours later by 300 mg/kg niacinamide. i.p.. The mortality rate was zero. Lesioned animals displayed a persistent ataxic gait. All drugs were dissolved in 0.9% saline. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO., unless otherwise specified.

Cerebellar membranes for [$^3$H]AMPA binding were prepared by a modification of the method of Honore and Neilsen (1985), using fresh tissue. All steps were carried out at 0$^o$ C unless indicated otherwise, the

cerebellum was removed immediately following decapitation and homogenized in a glass-teflon homogenizer for 90 sec in 2 x 5 ml 30 mM TRIS-HCl, 2.5 mM $CaCl_2$, pH 7.1 (Buffer A) and centrifuged at 30,000g x 15 min. The pellet was washed three times with 10 ml of Buffer A, resuspended in 10 ml Buffer A and incubated at 37° C for 30 min then collected by centrifugation at 30,000g x 10 min at 25°C. The pellet was washed again by centrifugation at 30,000g x 10 min at 0° C and frozen in acetone-dry ice for 20 min, thawed and centrifuged at 48,000g x 10 min, washed two more times and the final pellet resuspended by homogenization in Buffer A + 0.1 M KSCN (50 vol. per gram wet weight). Membranes were stored as a frozen pellet after first washing once in 10 ml of Buffer A without KSCN. For the binding assay, 0.5 ml aliquots of membranes were incubated with 5 nM [$^3$H]AMPA (27.6 Ci/mmole, New England Nuclear, Boston, MA) for 1 hr at 0 C°. Non-specific binding was determined by the inclusion of 10 uM QA or 0.5 mM glutamate, KA or GDEE. Free and bound ligand were separated by filtration through GF/C Whatman glass fiber filters and washed three times with 5 ml of ice cold Buffer A + 0.1 M KSCN, pH 7.1. Dried filters were counted in 8 ml liquid scintillation fluid.

Behavioral experiments were initiated 3 weeks post lesion and performed between 1300 and 1900 hours. GDEE was administered i.p. 20 min prior to strychnine

administration (Schwarz and Freed 1986, Abdul-Ghani et al. 1982). MK-801 (Merck, Sharp & Dohme Research Laboratories, West Point, PA.) was administered orally, 1 hour prior to strychnine treatment (Clineschmidt et al. 1982). Animals were observed individually in 16 1/2" x 8 1/2" x 7" lucite cages at 5 minute intervals for 1 hour after strychnine administration. A typical strychnine seizure began by tonic extension of the entire body and all limbs and occured while the animal was upright or supine; this was often followed by phasic symmetrical extensor thrusts during a period of postictal depression. Incidence of seizures in groups of 7-9 rats were recorded. In addition, percentage of the observation period during which rats displayed strychnine induced myoclonus was also recorded.

## 5.4 Results

### 5.4a [$^3$H]AMPA Binding in the Cerebellum

The effect of inferior olive lesions on specific binding of [$^3$H]AMPA to washed cerebellar membranes, displaced by excess glutamate, QA, KA and GDEE are shown in Table 11. There was no statistically significant difference in [$^3$H]-AMPA binding displaced by 0.5 mM glutamate between control and inferior olive lesioned

groups. However, a significant increase in [$^3$H]-AMPA binding sites displaced by 10 uM QA was observed in the

Table 11. Effect of 3AP-induced inferior olive lesions on 5 nM [$^3$H]AMPA binding in cerebellar membranes.

| Displacer | Conc. uM | Control | (%GLU) | IO Lesion | (%GLU) |
|---|---|---|---|---|---|
| L-GLU | 500 | 202.4 ± 8.6 | | 248.3 ± 34.4 | |
| QA | 10 | 55.3 ± 3.1 | (27%) | 71.7 ± 4.1* | (29%) |
| KA | 500 | 84.3 ± 9.5 | (41%) | 101.4 ± 17.4 | (41%) |
| GDEE | 500 | 22.8 ± 5.2 | (11%) | 26.8 ± 6.1 | (11%) |

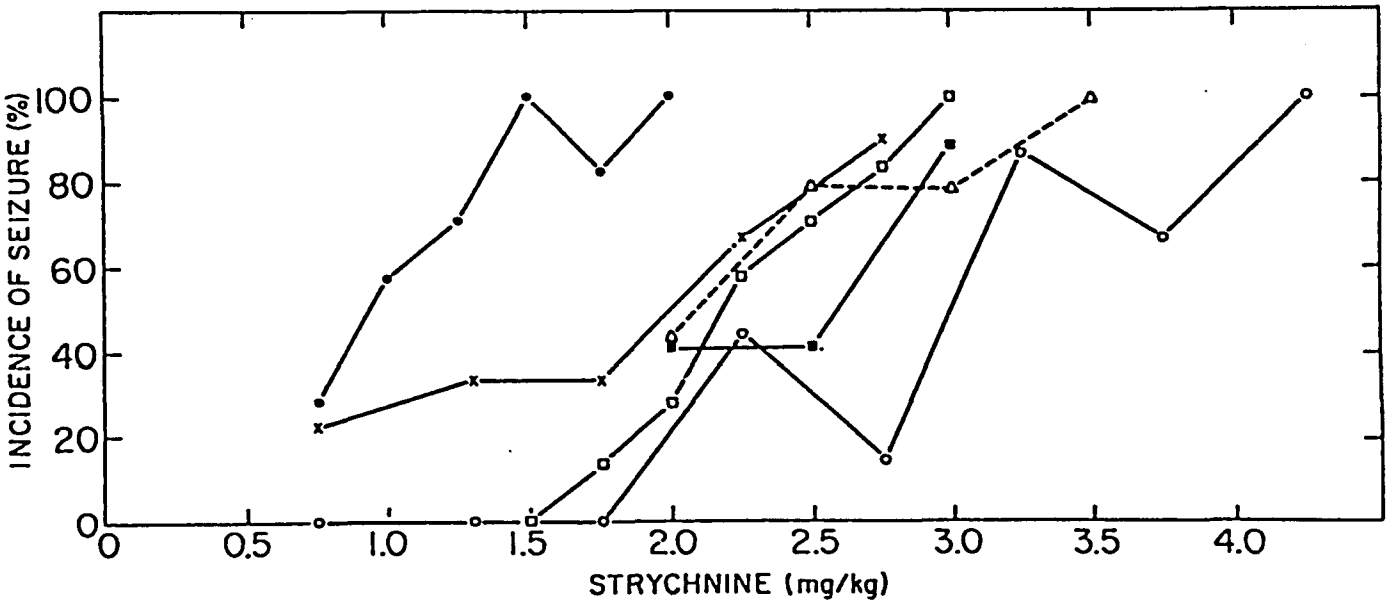
Each value is the mean ± SEM (fm/mg) of 6 rats in triplicate per determination.

inferior olive lesioned rats compared to control ($p<0.005$, Student's t-test). The $IC_{50}$ for QA is 60 nM in whole brain membranes (Olsen et al. 1987), therefore 10 uM QA should displace >90% of [$^3$H]AMPA binding. [$^3$H]-AMPA sites displaced by 10 uM QA constituted approximately 1/4 to 1/3 of the [$^3$H]-AMPA sites displaced by 0.5 mM glutamate. Kainic acid, at 0.5 mM, a concentration 50 times higher than QA, displaced approximately the same number of sites as QA, but there was no difference in KA displacable [$^3$H]-AMPA sites between inferior olive lesioned and control groups. Although GDEE is a potent antagonist for both QA and AMPA induced depolarizations (Segal 1976, Wheal and Miller 1980), GDEE is a poor displacer of [$^3$H]AMPA binding (Honore et al. 1982). In cerebellar membranes, GDEE displaced 11% of [$^3$H]AMPA

sites displaced by glutamate, with no difference between 3AP-induced inferior olive lesioned rats and controls.

## 5.4b Strychnine Seizure and Myoclonus

Figure 7 shows the dose response curves for strychnine seizures in control and inferior olive lesioned rats following pretreatment with either 750 mg/kg GDEE, i.p. or 0.1 mg/kg MK-801, p.o.. For comparison, strychnine seizure dose response curves in inferior olive lesioned and control rats with no drug treatment are also included in this figure (Anderson et al. 1987). An inferior olive lesion alone shifts the dose response curve for strychnine seizures to the left: the dose which produced convulsions in 50% of the test group ($CD_{50}$) for control rats is 2.18 mg/kg compared to 0.92 mg/kg following inferior olive lesion. GDEE pretreatment has no significant effect on the dose response curve in control animals ($CD_{50}$=2.6 mg/kg), however, GDEE clearly shifts the dose response curve for strychnine seizures in inferior olive lesioned rats back to the right ($CD_{50}$=3.0 mg.kg). MK-801 appears to have an effect similar to that of GDEE on strychnine seizure threshold in inferior olive lesioned rats ($CD_{50}$=2.0 mg/kg), although it is difficult to interpret this data in light of the fact that the seizure-type induced by strychnine in inferior olive lesioned rats




Dose-response curves for strychnine-induced seizures in IO lesioned rats (●), IO-lesioned rats pretreated with MK-801 (X), IO lesioned rats pretreated with GDEE, control rats (□), control rats pretreated with MK-801 (Δ) and control rats pretreated with GDEE (■). Each point is the incidence of seizure in groups of 7-17 rats.

**Figure Seven**

pretreated with MK-801 is changed. In these rats, seizures differed from typical strychnine seizures, which are characterized by tonic extension. Instead, convulsions in inferior olive lesioned rats pretreated with MK-801 were preceded specifically by forelimb clonus in an upright position and rearing, in a manner reminiscent of seizures induced by pentylenetetrazole (Browning et al. 1981, Woodbury 1980). MK-801 pretreatment had no effect on the strychnine seizure threshold in control rats ($CD_{50}$=2.1 mg/kg).

Strychnine administration to rats also produced arrhythmic myoclonus which occured either alone or preceding a seizure. Duration of myoclonus increased with increasing doses of strychnine in both control and inferior olive lesioned rats (Table 12). As in the case with strychnine seizure, inferior olive lesioned rats had a lowered threshold for strychnine-induced myoclonus. As shown in Table 12, control rats displayed myoclonus during 12.4% of the observation period with 2.0 mg/kg strychnine, however inferior olive lesioned rats displayed myoclonus during 13.8% of the observation period with only 0.75 mg/kg strychnine.

In the control rats, pretreatments with either GDEE or MK-801 did not significantly affect the duration of strychnine-induced myoclonus when compared to the untreated groups. However, GDEE pretreatment in the

Table 12. Effect of GDEE or MK-801 on strychnine induced myoclonus in Inferior Olive lesioned rats.

| Strychnine (mg/kg) | No Treatment | GDEE | MK-801 |
|---|---|---|---|
| | (% time in myoclonus) | | |
| CONTROL | | | |
| 2.0 | 12.4 ± 6.6 | 18.2 ± 6.2[2] | 2.2 ± 0.4 |
| 2.5 | 18.6 ± 4.7 | 15.8 ± 5.5 | 15.1 ± 7.2 |
| 3.0 | 67.9 ± 3.6 | 52.1 ± 9.2 | 40.5 ± 8.0 |
| 3AP-INFERIOR OLIVE LESIONED | | | |
| 0.75 | 13.8 ± 6.5 | 4.0 ± 2.7 | 7.8 ± 3.3 |
| 1.25 | 33.3 ± 9.9 | 8.7 ± 4.9[1,2] | 41.1 ± 8.4 |
| 1.75 | 60.2 ± 12.4 | 26.4 ± 8.6[1] | 43.5 ± 5.6 |

1: $p < 0.05$, treatment vs. no treatment group
2: $p < 0.05$, GDEE treatment vs. MK-801 treatment group

Each value is the mean ± SEM of 6-9 rats per group.

inferior olive lesioned rats significantly reduced duration of strychnine-induced myoclonus. Therefore the effect of GDEE on strychnine myoclonus is similar to the effect of GDEE on strychnine seizure. On the other hand, pretreatment with MK-801 in the inferior olive lesioned rats did not affect the duration of strychnine myoclonus. Hence it appears that MK-801 has a differential effect on strychnine induced myoclonus versus seizure.

## 5.5 Discussion

In the present study we demonstrate that inferior olive lesions are accompanied by an increase in QA displaceable [$^3$H]AMPA binding in the cerebellum. There are several lines of evidence to suggest that QA-sensitive glutamate receptors are present on cerebellar Purkinje cells. Firstly, iontophoretic application of QA to cerebellar slices elicits a fast, all-or-none action potential, which is readily antagonized by L-GDEE (Crepel et al. 1982), kynurenate and gamma-D-glutamyl glycine (Kano et al. 1988). In addition, QA can substitute for parallel fiber stimulation to produce an electrophysiologic phenomenon called long-term depression (Kano and Kato 1987). Long-term depression is an overall decrease in Purkinje cell firing in response to parallel fiber stimulation. Long-term depression is produced by sequential stimulation of climbing fibers and parallel fibers in a specific pattern( Kano and Kato 1987, Ito and Kano 1982).

In the absence of climbing fibers, constant stimulation of Purkinje cells by parallel fibers has been demonstrated, as evidenced by increased simple spike production by Purkinje cells (Batini and Billard 1985). The electrophysiologic activity may be accompanied by morphological changes mediating the increased excitation.

In fact the formation of ectopic spines has been observed in climbing fiber deafferented Purkinje cells (Anderson and Flumerfelt 1987). Increased cerebellar [$^3$H]AMPA binding in inferior olive lesioned rats may represent a heterotypic reinnervation of the Purkinje cell by parallel fibers.

Administration of the QA antagonist, GDEE, reversed the decreased threshold for strychnine seizure in inferior olive lesioned rats, without affecting the threshold for strychnine seizures in control animals. Similarly, GDEE pretreatment significantly reduced strychnine induced myoclonus in inferior olive lesioned rats without altering strychnine induced myoclonus in the control group. The selective 'anti-convulsant' activity of GDEE in the inferior olive lesioned group accompanied by the observed increase in [$^3$H]AMPA binding supports the hypothesis that the decreased threshold for strychnine seizure, is mediated, in part, by increased Purkinje cell excitation occurring through QA-receptors. Antagonism of these receptors by GDEE would only occur in the inferior olive lesioned rat, accounting for the lack of 'anti-convulsant' activity by GDEE in control rats.

A different effect was observed following administration of MK-801, the non-competitive NMDA antagonist. A change in the type of seizure observed following strychnine administration, and thus a change in

the shape of the dose response curve in inferior olive lesioned rats was seen. Since the type of seizure observed was different, it is not possible to compare the strychnine-seizure dose response curve obtained following the other treatments (for example, either MK-801 administration to non-lesioned rats, or 3AP treatment alone). The duration of strychnine-induced myoclonus, in inferior olive lesioned rats pretreated with MK-801, however, was unchanged when compared to the untreated group. It has been reported that MK-801 is a potent anticonvulsant against seizures induced by GABA antagonists, bicuculline and picrotoxin, and pentylenetetrazole; however it is not normally effective against strychnine-induced seizures in rats (Clineschmidt et al. 1982). A change in MK-801 binding sites, or affinity, may occur at some site other than the cerebellum as a result of the 3AP induced inferior olive lesion. It is not likely that MK-801 is acting in the cerebellum, since [$^3$H]MK-801 binding is absent from the cerebellum (Wong et al. 1986).

In summary, we propose that the increase in [$^3$H]AMPA binding sites reflect an increase in QA-sensitive glutamate receptors on Purkinje cells, which can be antagonized by GDEE, thus accounting for the selective anticonvulsant activity of GDEE in 3AP-induced inferior olive lesioned rats. MK-801, a non-competitive NMDA

antagonist, also suppressed seizure activity in inferior olive lesioned rats, but the site of MK-801 action is probably not in the cerebellum, since there are no [$^3$H]MK-801 binding sites in the cerebellum, and few NMDA sensitive [$^3$H]L-glutamate binding sites in cerebellar cortex.

# CHAPTER SIX

## Strychnine Seizure Potentiation by Azaspirodecanedione Anxiolytics in Rats

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## 6.1 Abstract

Buspirone, gepirone and ipsaperone administered intraperitoneally (40 mg/kg) to naive rats were found to be proconvulsive for strychnine-induced seizures. The dose of strychnine required to induce seizures in 50% of test animals ($CD_{50}$) was 2.18 mg/kg in naive rats, while $CD_{50}$s for rats treated with the azaspirodecanediones ipsaperone, gepirone and buspirone were 1.65, 0.97 and 0.70 mg/kg respectively. Azaspirodecanediones have high affinity for the 5-$HT_{1A}$ serotonergic receptor, however the specific 5-$HT_{1A}$ agonist, 8-hydroxy-2-(di-n-propyl-amino)-tetralin (8-OH DPAT) had no effect on strychnine seizure in naive rats ($CD_{50}$=2.0 mg/kg). The strychnine specific proconvulsive effects of IO lesions and buspirone were additive, resulting in a $CD_{50}$ of 0.1 mg/kg. This observation indicates that the buspirone induced decrease in strychnine seizure threshold does not require intact inferior olive-climbing fiber pathways. Cerebellar sites for possible azaspirodecanedione action are discussed.

buspirone, gepirone, ipsaperone, inferior olive, strychnine seizure, 5-$HT_{1A}$

## 6.2 Introduction

Seizures induced by picrotoxin and bicuculline may result from interactions with the GABA-benzodiazepine-chloride receptor complex, causing a disinhibition by blocking inhibitory GABAergic neurotransmission. Benzodiazepines, such as diazepam act at this type of receptor to confer anxiolytic as well as anticonvulsant activity (Olsen 1982) by facilitating GABAergic neurotransmission. A class of compounds that are structurally dissimilar to the benzodiazepines, the azaspirodecanediones (buspirone, gepirone and ipsaperone) have recently been introduced as anxiolytic agents (buspirone; Goldberg and Finnerty 1979) or potential anxiolytics (gepirone, ipsaperone; McMillen and Mattiace 1983, Traber et al. 1984).

Neither azaspirodecanediones, nor their metabolites are active at the GABA-benzodiazepine-chloride receptor complex (Taylor et al. 1984; Hirsch et al. 1982). It has been postulated that the anxiolytic activities of azaspirodecanediones are mediated by interaction with serotonin ($5HT_{1A}$) receptors, since a high affinity for $5HT_{1A}$ recognition sites has been demonstrated for buspirone and ipsaperone (Peroutka 1985, Traber et al. 1984). Furthermore, buspirone, gepirone, ipsaperone and 8-OH DPAT (8-hydroxy-2-(di-n-propylamino)-tetralin) have

agonist activity at the 5-$HT_{1A}$ site which is negatively coupled to adenylate cyclase (Bockaert et al. 1987, Yocca and Maayani 1986). Buspirone, gepirone and ipsaperone have been reported to both induce and antagonize certain components of the serotonin syndrome (Eison et al. 1986, Smith and Peroutka 1986, Traber et al. 1986).

8-OH DPAT, a specific 5-$HT_{1A}$ agonist, has been found to potentiate seizures induced by pentylenetetrazole (PTZ) and maximal electroshock in mice but not in rats (Loscher and Czuczwar 1985). Buspirone has been reported to potentiate picrotoxin induced seizures in both mice and rats (Eison and Eison 1984). These observations suggest an effect of azasprirodecanedione anxiolytics on seizure control mechanisms, which is different from the anxiolytic/anticonvulsant benzodiazepines. We recently found that bilateral inferior olive lesions reduced the threshold for seizures induced by strychnine, a glycine antagonist, in rats. Because azaspirodecanedione anxiolytics have a proconvulsant effect in certain seizure models, we decided to examine the effect of buspirone, gepirone, ipsaperone and 8-OH DPAT on strychnine-induced seizures in naive rats.

## 6.3 Materials and methods

### 6.3a Animals

Male Sprague-Dawley rats (150-200 g) (Harlan, Walkersville, MD) were used. Rats were housed 6/cage, kept on a 12:12 hour light-dark schedule and provided food and water ad libidum. Chemical lesions of the inferior olive were made by i.p. injections of 80 mg/kg of the neurotoxin 3-acetylpyridine (Hwang et al. 1981). 3-acetylpyridine is a nicotinamide analog which produces complete destruction of the inferior olive (D'esclin and Escubi 1974), possibly through a metabolite or by competitive antagonism of NAD or NADP (Herken 1968), but is ineffective when administered directly into brain tissue. Although minor pathological changes in other areas of the brain may occur after 3-acetylpyridine administration, the proconvulsive effect of inferior olive degeneration was confirmed by bilateral electrothermic inferior olive lesions (Anderson et al. 1987). Recovery from acute toxicity (stridulous breathing, diarrhea, weight loss and tremor) occurred within 2-4 days. Seizure studies were carried out after a 4 week recovery period. The only persistent neurological sign was ataxia. On rare occasions, animals that did not recover from acute toxicity were excluded.

6.3b Drugs

All drugs were administered i.p. in 0.9% saline, with

the exception of ipsaperone which was injected as a suspension in 0.5% CM-cellulose. 8-OH DPAT was purchased from Research Biochemicals Inc., Wayland, MA. Buspirone and gepirone (MJ 13805) were gifts from Bristol-Myers Company, Evansville, IN, and ipsaperone (TVX Q 7821) was generously donated by Dr. J. Traber, Troponwerke, Koln, FRG. All other chemicals were obtained from Sigma Chemical Company, St. Louis, MO.

6.3c Seizures

Experiments were performed between 1300 and 1900 h. All drugs were given 1 h prior to strychnine administration, and animals were observed individually in 16 1/2" x 8 1/2" x 7" lucite cages at 5 min intervals for 2 h. A typical strychnine seizure began by tonic extension of the entire body and all limbs and occurred while the animal was upright or supine; this was often followed by phasic symmetrical extensor thrusts during a period of postictal depression. Incidence of seizures in groups of 6-8 rats were recorded.

6.3d 5-HT Syndrome

Animals were observed at 5 min intervals for presence of behavioral elements of the 5-HT syndrome (Jacobs

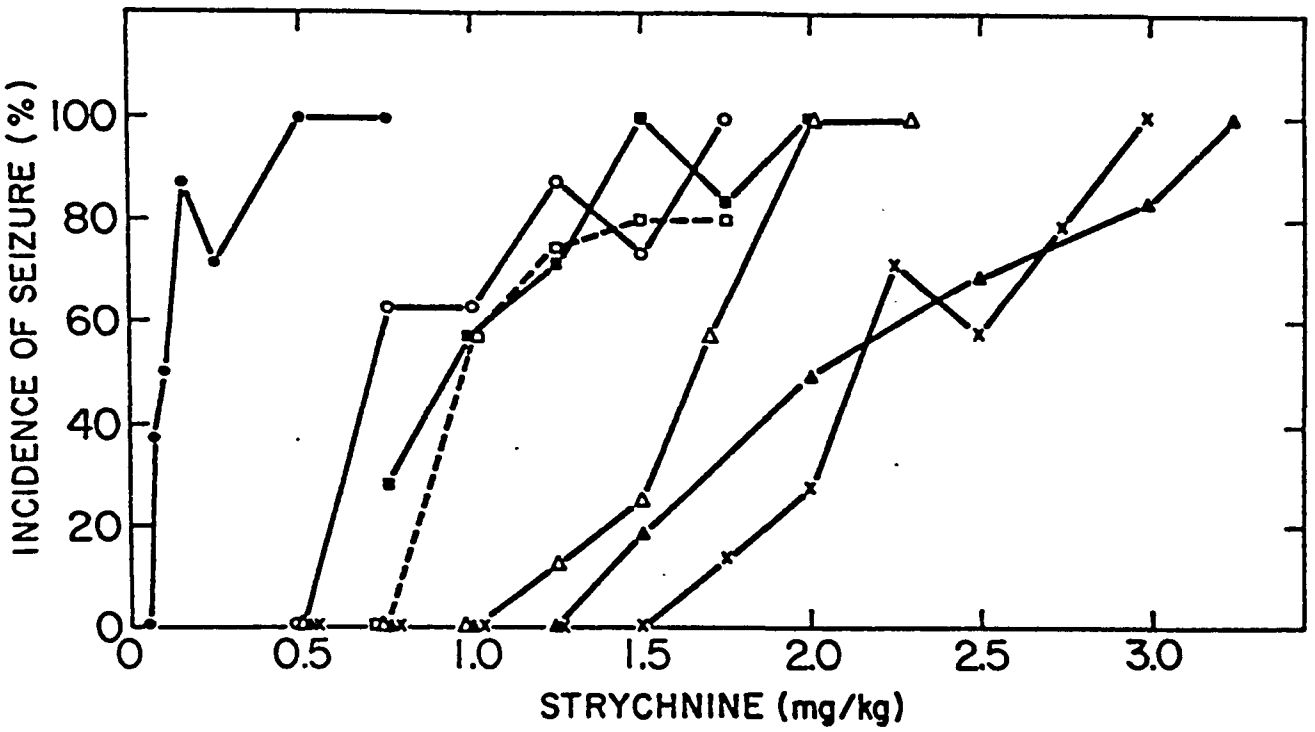
1976). 5-HT receptor activation produces flattened body posture, tremor, forepaw treading, hindlimb abduction and lateral headweaving.

## 6.4 Results

### 6.4a Strychnine induced seizure in naive rats

Figure 8 shows the dose response curves for strychnine induced convulsions in naive rats and those pretreated with buspirone, gepirone, ipsaperone or 8-OH DPAT. A dose of 40 mg/kg, i.p. for azaspirodecanediones was chosen since preliminary results indicated that this dose produced a maximal effect in facilitating picrotoxin-induced seizures. A dose of 0.5 mg/kg, 8-OH DPAT has been reported to induce elements of the 5-HT syndrome (Tricklebank et al. 1984) and facilitate maximal electroshock and PTZ seizures in mice (Losher and Czuczwar 1985); therefore this dose was used in our experiments.

Parallel leftward shifts in the strychnine dose response curves for the groups pretreated with azaspirodecanediones indicate an increased susceptibility to strychnine seizure. Buspirone was most potent, with a $CD_{50}$ (convulsive dose of strychnine in 50% of rats) of 0.7 mg/kg, followed by gepirone ($CD_{50}$ = 0.097 mg/kg) and ipsaperone ($CD_{50}$ = 1.65 mg/kg). 8-OH DPAT, on the other




Strychnine seizure dose response curve in naive rats (X) and following 1 hr. pretreatment with 40 mg/kg each of buspirone (○), gepirone (□), isapirone (△) or 0.5 mg/kg 8-OH DPAT (▲). Rats with bilateral IO lesion produced by systemic administration of 3AP (■) and IO lesioned rats treated with 40 mg/kg buspirone (●). Seizure incidence was determined in groups of 5-8 rats.

Figure Eight

hand did not alter the dose response curve ($CD_{50}$ = 2.0 mg/kg) from that of naive rats ($CD_{50}$=2.18 mg/kg).

### 6.4b Strychnine induced seizure in inferior olive lesioned rats

The dose response curves for strychnine induced seizures in 3-acetylpyridine inferior olive lesioned rats and combined treatment of inferior olive lesion and buspirone are also presented in Figure 8. The $CD_{50}$ for strychnine induced seizures in 3-acetylpyridine lesioned rats is 0.92 mg/kg, and that for rats pretreated with buspirone is 0.7 mg/kg. The combined treatment of 40 mg/kg buspirone and 3-acetylpyridine induced inferior olive lesion yielded a $CD_{50}$ of 0.1 mg/kg. A similar effect was seen in inferior olive lesioned rats pretreated with 40 mg/kg gepirone: 0.75 mg/kg strychnine was subconvulsant in naive and gepirone treated groups, while in inferior olive lesioned rats this dose produced a 29% seizure incidence. The combined treatments of inferior olive lesion and gepirone resulted in 85% seizure incidence ($p < 0.01$, Fisher Exact chi-square).

### 6.4c 5-HT Syndrome

Elements of the 5-HT syndrome (Jacobs 1976) were

observed following azaspirodecanediones or 8-OH DPAT treatment and the results are summarized in Table 13.

Table 13. Percent of rats showing elements of 5-HT syndrome induced by 40 mg/kg azaspirodecanediones (buspirone, gepirone and isapirone) or 0.5 mg/kg 8-OH DPAT, i.p., N = number of rats tested, observations made at 5 minute intervals for 1 hour following drug treatment.

| SYMPTOM | BUSPIRONE (N=8) | ISAPIRONE (N=8) | GEPIRONE (N=16) | 8-OH DPAT (N=32) |
|---|---|---|---|---|
| Flattened Body Posture | 100 | 87.5 | 50 | 96.7 |
| Forepaw Treading | 87.5 | 18.75 | 0 | 84.4 |
| Hindlimb Abduction | 50 | 68.75 | 0 | 9.4 |
| Lateral Headweaving | 37.5 | 56.25 | 50 | 0 |
| Tremor | 100 | 18.75 | 0 | 46.9 |

These include flattened body posture, forepaw treading, hindlimb abduction, lateral head weaving and tremor. Animals treated with buspirone and ipsaperone displayed all of the behaviors but, tremor and forepaw treading were minimal in ipsaperone treated animals. Gepirone treated animals did not have forepaw treading or tremor, while 8-OH DPAT treated rats did not have lateral headweaving.

## 6.5 Discussion

The azaspirodecanedione anxiolytic buspirone, and putative anxiolytics gepirone and ipsaperone all showed proconvulsive activity for strychnine induced seizure in naive rats. Strychnine seizure potentiation induced by buspirone, gepirone and ipsaperone were similar phenomenologically to that previously described in rats with bilateral inferior olive lesions (Anderson et al. 1987): inferior olive lesions shift the dose response curve for strychnine seizure to the left, resulting in a $CD_{50}$ of 0.9 mg/kg, compared to the $CD_{50}$ of 2.18 mg/kg in naive rats. In comparison, buspirone, gepirone and ipsaperone pretreatment resulted in $CD_{50}$s of 0.7, 0.97 and 1.65 mg/kg strychnine, respectively. In inferior olive lesioned animals treated with buspirone, the $CD_{50}$ for strychnine-induced seizure was 0.1 mg/kg; gepirone also produced a similar effect. This suggests that the proconvulsant effects of azaspirodecanediones and inferior olive lesions could be additive.

The major efferent pathways from the inferior olive are climbing fibers which synapse on Purkinje cells in the cerebellum. In intact animals, Purkinje cell excitation occurs via parallel fibers from granule cells, while the net effect of climbing fiber input to Purkinje cells is inhibitory and modulatory (Colin et al. 1980). We have previously proposed that the proconvulsive effect for strychnine induced seizures produced by inferior olive

lesions may be mediated by increased Purkinje cell excitation resulting in GABA release by Purkinje cell nerve terminals in the cerebellar deep nuclei (Anderson et al. 1987). A direct action of azaspirodecanediones on the inferior olive and climbing fibers can be excluded since the proconvulsive effects of azaspirodecanediones were still present in inferior olive lesioned rats and the effects of inferior olive lesions and azaspirodecanediones on strychnine seizures are additive.

The azaspirodecanediones and 8-OH DPAT have high affinity for the 5-$HT_{1A}$ receptor (Peroutka 1985, Traber et al. 1984) as well as agonist activity at 5-$HT_{1A}$ sites which are negatively coupled to adenylate cyclase (Bockaert et al. 1987, Yocca and Maayani 1986). 5-$HT_{1A}$ receptor activation also produces behavioral phenomena which includes flattened body posture and forepaw treading (Tricklebank et al. 1984). We observed that treatment with azaspirodecanediones and 8-OH DPAT produced these as well as other components of the 5-HT syndrome (hindlimb abduction and tremor). These findings are consistent with previous reports (Smith and Peroutka 1986, Eison et al. 1986). In this study, 8-OH DPAT was used in a concentration that produced a similar incidence of serotonin syndrome symptomes as the azaspirodecanediones. At this dosage, 8-OH DPAT is considered to have a relatively specific agonist action on $5HT_{1A}$ receptors

(Tricklebank et al. 1984). Azaspirodecanediones may be less specific for serotonin receptor subtypes compared to 8-OH DPAT. For example, Smith and Peroutka (1986) observed that buspirone and ipsaperone could antagonize elements of the 5-HT syndrome (forepaw treading, headweaving and tremor) induced by other 5-HT agonists, suggesting a mixed agonist/antagonist action for these compounds. Since 8-OH DPAT had no effect on strychnine seizures, at a dose that produces comparable serotonin syndrome behavior, it seems unlikely that strychnine seizure potentiation by azaspirodecanedione anxiolytics is mediated by a direct agonist action on $5\text{-HT}_{1A}$ receptors. The proconvulsive effect for strychnine of buspirone, gepirone and ipsaperone may be due to either their action on 5-HT receptor subtypes other than $5\text{-HT}_{1A}$, or their mixed agonist/antagonist action on $5\text{HT}_{1A}$ receptors.

5-HT innervation of the cerebellum, originates mainly from the nucleus reticularis gigantocellularis and the lateral reticular nucleus in the reticular formation (Bishop and Ho 1985) and form a dense plexus around the body of the Purkinje cell. Two types of 5-HT receptors regulating glutamate and 5-HT release have been identified in the cerebellum (Raiteri et al. 1986). $5\text{-HT}_1$ receptors are present on parallel fibers and inhibit glutamate release. 8-OH DPAT is an agonist at this site.

A $5\text{-}HT_{1B}$ autoreceptor is present on 5-HT nerve terminals and inhibits 5-HT release; 8-OH DPAT has no effect on these receptors (Raiteri et al. 1986). One possibility is that azaspirodecanediones might inhibit 5-HT release by stimulating these autoreceptors. Recent studies show that more Purkinje cells are inhibited by 5-HT than excited, by a ratio of 6:1 (Strahlendorf et al. 1984). Therefore inhibition of 5-HT release by azaspirodecanediones could create a Purkinje cell firing pattern similar to that seen following inferior olive lesion (Batini and Billiard 1985).

# CHAPTER SEVEN

## Conclusions and Discussions

## 7.1 Conclusions and Discussion

1: A decreased threshold for strychnine-induced seizures is produced by systemic administration of the neurotoxin 3AP at a dose which causes complete degeneration of the inferior olivary nucleus, in the rat. Bilateral electrothermic lesions of the inferior olive produce a similar effect. 3AP treatment had no effect on the threshold for seizures induced by picrotoxin, bicuculline and pentylenetetrazole (PTZ).

2: Abnormal motor behavior including myoclonus, backward movement and hyperextension, produced by all the convulsants tested (strychnine, brucine, picrotoxin, bicuculline and PTZ) was significantly aggravated in the 3AP treated rats.

These findings suggests that removal of the inferior olivary climbing fibers alters a strychnine-sensitive mechanism in the neural circuitry postsynaptic to the lesion, but does not effect GABAergic seizure mechansims (picrotoxin and bicuculline are GABA antagonists, and PTZ acts at GABA sites, as well as other unspecified sites in the brain). Data presented in chapter 2 show that convulsant doses of bicuculline (2.5 mg/kg) and PTZ

(40 mg/kg), produced a similar incidence of seizures in control and 3AP treated rats, while 2.0 mg/ kg picrotoxin was subconvulsant in both 3AP treated and control rats. In addition, subconvulsant doses of bicuculline and PTZ were tested in inferior olive lesioned and control rats. At 1.5 mg/kg, bicuculline failed to produce seizures in control or 3AP treated rats, n=6 per group; 20 mg/kg PTZ produced a seizure incidence rate of 0/6 in control rats, and 1/6 in 3AP treated rats.

Assuming that the primary action of strychnine to produce seizure is antagonism of inhibitory glycinergic synapses, potential sites of action would involve glycinergic transmission. There are no known glycinergic synapses or glycine releasing neurons directly postsynaptic to the climbing fibers, however there are poly-synaptic connections involving glycinergic neurons, which may be affected by the inferior olive lesion. Purkinje cell bodies and distal dendrites in the molecular layer of the cerebellum; the dentate, interpositus and fastigial nuclei of the cerebellum; and the vestibular nuclei all receive climbing fiber projections. While [$^3$H]strychnine binding sites have not been demonstrated in any of the cerebellar structures (Frostholm and Rotter 1986), Deiters neurons of the lateral vestibular nucleus display electrophysiologic sensitivity to strychnine (ten Bruggencate and Engberg 1979).

Cerebellar efferent nuclei receive a primary input from the climbing fibers via a collateral, which is excitatory, and they receive input from Purkinje cells, which are also directly innervated by climbing fibers. Purkinje cells are inhibitory and receive excitation from two main sources, climbing fibers and parallel fibers. Climbing fibers produce a characteristic complex spike resulting in some GABA release by Purkinje cells and reduced responsiveness of Purkinje cells to glutamatergic excitation from parallel fibers. Parallel fibers produce simple spikes in Purkinje cells and a greater GABA release in cerebellar efferent nuclei. Following inferior olive lesions, Purkinje cells no longer display complex spikes, resulting in hyperexcitation due to unimpeded simple spike production, in response to parallel fiber input. As a result, cerebellar efferent nuclei are more inhibited. The effect of inferior olive lesion on cerebellar efferent nuclei then, is twofold, primary excitatory input from the climbing fiber collateral is lost and inhibition from Purkinje cells is increased. Over time, the mean firing rate for both simple spike activity for Purkinje cells and cerebellar efferent nuclei attempts to return to a 'normal' level, however, the rate is never fully restored, and the pattern of firing for both Purkinje cells and cerebellar efferent nuclei is erratic (Batini and Daniel 1986, Batini et al. 1985). Both the diminished excitatory

cerebellar efferent output, and the altered pattern of remaining excitation could affect sensitivity to strychnine-induced disinhibition, in the 3AP-induced inferior olive lesioned rat.

The loss of inferior olivary activity and the changes in Purkinje cell and cerebellar efferent nuclear firing patterns, are accompanied by changes in motor behavior (Anderson et al. 1987, Batini et al. 1985, Anderson and Flumerfelt 1980, D'esclin and Escubi 1977). Within 12 hours of 3AP administration, a period of little to no activity (2-4 days) is observed, followed by impaired walking characterized by a rolling ataxia. A transient tremor occurs from 3 to 6 weeks post lesion, Batini et al. (1985) suggest a correlation between motor activity, for example the tremor, and Purkinje cell and cerebellar efferent firing. The tremor has been attributed to bursting of cerebellar efferent nuclei in an abnormal pattern, due to a temporary loss of Purkinje cell inhibition to some postsynaptic sites. The 'bursting' may occur as synaptic activity through the parallel fiber-Purkinje cell-cerebellar efferent nuclei circuit reestablishes a pattern of activity. Following acute inferior olive lesion, or reversible inhibition, simple spike production increases over two-fold, however, the average simple spike discharge rate declines within 4-6 weeks. This new rate however, is still higher than normal

and the pattern of firing is altered. Supporting this hypothesis, a reduction of monosynaptic inhibition on Deiters neurones of the lateral vestibular nucleus has been observed in 3AP-induced inferior olive lesioned rats (Ito et al. 1979, 1978).

Climbing fiber innervation could indirectly affect all sites which receive input from cerebellar efferent and vestibular nuclei. One system that is affected by 3AP treatment and subsequent climbing fiber degeneration is the red nucleus (Billard and Daniel 1986). In the rat, the red nucleus receives dense projections from both the interposed and dentate nuclei of the cerebellum (Gwyn and Flumerfelt 1974). A multi-synaptic projection from the red nucleus, the rubrobulbospinal path produces 1) excitation of fusimotor neurons in the spinal cord and 2) inhibition of poly-synapytic segmental and ascending pathways activated by flexor reflex afferents in hindlimb nerves (from Ito 1984). Following 3AP treatment and resultant inferior olive lesion, there is a dramatic decrease in the firing rate of neurons from the red nucleus. This is in response to the lack of excitatory input from the dentate and interposed nuclei. A progressive compensation in neural activity emanating from the red nucleus occurs over time, however, the pattern of firing is unlike that observed in non-lesioned rats, and normal rubral firing is never fully restored (Billard and

Daniel 1986). Decreased rubrobulbospinal activity will affect spinal motor systems in two ways, first there will be less direct excitation of fusimotor neurons, and less control of output; and secondly there will be diminished excitation from rubral neurons to inhibitory neurons in supraspinal structures, which project to inhibitory neurons in spinal cord. The lack of excitation to inhibitory systems will create hyperexcitation. The effect of 3AP-induced inferior olive lesion on poly-synaptic inhibitory systems can create a situation with heightened responsiveness to disinhibition.

Thus, more specifically, increased inhibition of cerebellar efferent nuclei by Purkinje cells can produce decreased excitation which is transmitted to the red nucleus. These rubral neurons are now less excited than they would be in a non-lesioned rat, and efferents projecting from the red nucleus now fail to excite inhibitory neurons in the bulbar region. Assuming that bulbar-spinal projections emanating from these neurons act to inhibit glycinergic interneurons in spinal cord, failure of the bulbar neurons to release their inhibitory neurotransmitter allows for the release of glycine and inhibition of the motoneuron. The action of strychnine at this synapse then inhibits the inhibition of the motor neuron by the interneuron. The motoneuron now responds differently to the other inputs it receives, both

excitatory and inhibitory. Thus in inferior olive lesioned rats, a different pattern of excitation may occur at the motoneuron, and this is transmitted to the neuromuscular junction. This is just one proposed action of 3AP-induced inferior olive lesion that could affect response to glycinergic disinhibition by strychnine.

In addition, efferents from the fastigial nucleus, will also be affected by 3AP-induced inferior olive lesion. These fibers project mainly to vestibular nuclei and the major nuclei of the reticular formation, where they exert a monosynaptic excitation (Ito et al. 1970). In the reticular formation, fastigioreticular fibers give off both ascending and descending fibers. Cerebellar influence, then can be exerted over supraspinal structures and the spinal cord. Indeed, unilateral lesions of the fastigial nucleus in the cat, result in ipsilateral hypotonia and contralateral atonia of the limbs (Moruzzi and Pompeiano 1956). During seizure states induced by cortical stimulation in cats and monkeys, increased firing of the fastigial nucleus has been observed (Gartside 1979), and electrical stimulation of the fastigial nucleus, or the reticular formation will diminish and ultimately inhibit seizure activity localized to the hippocampus (Babb et al. 1974). This might occur by stimulation of a fastio-septal connection, which has been demonstrated in cats and monkeys (Heath and Harper 1974).

In the case of bilateral inferior olive destruction, fastigial nuclear output would be inhibited thus preventing activation of the fastigial-septal pathway and possible endogenous 'anti-convulsant ' activity. While these observations have not been made in rats, it is possible that similar anatomical and physiological processes are present.

. In addition, while not present in abundance, direct fastigiospinal projections have been demonstrated. It is important to keep in mind that all areas receiving afferents from cerebellar nuclei, and the vestibular nuclei will be effected by 3AP-induced inferior olive lesions.

3: 3AP-induced inferior olive lesions did not alter specific [$^3$H]-strychnine binding in medulla or spinal cord. There also was no difference in [$^3$H]GABA binding to cerebellar nuclei in inferior olive lesioned rats.

4: 3AP-induced inferior olive lesions produced an apparent increase in QA displaceable [$^3$H]AMPA binding to cerebellar membranes.

Binding was measured using 10 uM QA to displace 5 nM [$^3$H]AMPA, since the $IC_{50}$ for QA inhibition of

[$^3$H]AMPA binding is 60 nM (Olsen et al. 1987), 10 uM will displace >90% of the AMPA binding sites. The difference in binding observed between control and 3AP treated rats may represent either an increase in the number of receptors, or an increased affinity for AMPA.

5: Pretreatment with GDEE (glutamate diethylester), reversed the effects of 3AP-induced inferior olive lesion by restoring the strychnine-seizure dose response curve in 3AP treated rats to control values. The same dose of GDEE also suppressed strychnine-induced myoclonus in inferior olive lesioned rats. In control rats, GDEE had no effect on either strychnine-induced seizure or myoclonus.

6: Pretreatment with MK-801, an NMDA antagonist, produced a change in the type of seizure observed following strychnine administration to 3AP treated rats, as well as a change in the shape of the dose response curve. Since the type of seizure observed was different, it is difficult to compare dose response curves. MK-801 had no inhibitory effects on strychnine-induced myoclonus in 3AP treated rats. Similar to GDEE, MK-801 had no effect on either strychnine-induced seizure or myoclonus in

control rats.

The change in QA displaceable [$^3$H]AMPA binding and the change in the strychnine-seizure dose response curve following administration of GDEE in 3AP-induced inferior olive lesioned rats may be related. One interpretation of these data is that the change in QA displaceable [$^3$H]AMPA binding represents an increase in QA-sensitive glutamate receptors. AMPA has a high affinity for QA-sites, and autoradiographic studies show that the highest concentration of both AMPA and glutamate displaceable [$^3$H]AMPA sites in the cerebellum are localized to the molecular layer of the cerebellar cortex (Monaghan et al. 1984, Rainbow et al. 1984). Quisqualate has been shown to displace 80% of specific [$^3$H]L-glutamate binding in the molecular layer (Greenamyer et al. 1985), which also contains parallel fiber terminals and Purkinje cell dendrites.

Physiological studies show that QA elicits the typical simple spike response from Purkinje cells, characteristic of parallel fiber stimulation (Kano et al. 1988), and can substitute for parallel fiber stimulation in producing the electrophysiologic phenomenon of long-term depression (LTD) (Kano and Kato 1987). LTD is a decrease in simple spike activity recorded from the Purkinje cell, produced by sequential stimulation of climbing and parallel fibers,

that ranges in magnitude from 50 to 80% of normal value, and lasts upward of 1 hour (Ito and Kano 1982). It is possible that climbing fiber deafferentation might induce a sprouting phenomenon by the parallel fibers, as a compensatory mechanism. Heterotypic "replacement" innervation of Purkinje cells by parallel fibers, following climbing fiber deafferentation in weanling rats, and in rats weighing up to 50 grams at the time of 3AP administration has been demonstrated by the presence of ectopic spines (Anderson and Flumerfelt 1986). The increase in QA-displaceable [$^3$H]AMPA binding may represent sprouting by parallel fibers, with the formation of new QA-sensitive glutamate receptors on Purkinje cells.

By interpreting the binding data as representing a 'new' population of QA-sensitive glutamate receptors on Purkinje cells, behavioral responses of 3AP treated rats following systemic administration of GDEE can be interpreted thusly: In 3AP treated rats, the dose response curve for strychnine-seizures is shifted to the left, an effect which may be mediated in part, by increased excitation of inhibitory Purkinje cells. The increased excitation of Purkinje cells may proceed through the 'new' QA-sensitive glutamate receptors, which may have been induced by compensatory, increased parallel fiber innervation following climbing fiber deafferentation. Further, activation of these receptors by parallel fibers,

in the absence of climbing fiber-induced complex spikes will result in increased Purkinje cell firing. Systemic administration of GDEE restores the strychnine-seizure dose response curve to control values in 3AP treated rats, and may do this by antagonizing QA sites, particularly the 'new' population of QA-sensitive glutamate receptors on Purkinje cells.

Since GDEE does not affect strychnine-seizure threshold in control rats, blockade of endogenous QA-sensitive glutamate receptors in the cerebellar cortex does not prevent seizure production in the normal rat. However, following inferior olive lesion, the pattern of incoming excitation to the Purkinje cell is altered. This change is due to the lack of climbing fiber input, which allows for unimpeded simple spike production by the Purkinje cell in response to parallel fiber stimulations, causing increased release of GABA on efferent nuclei of the cerebellum. These deep nuclei are now inhibited, and a different pattern of synaptic activity is transmitted outward to various motor systems. Inactivity of GDEE in control rats suggests that the anti-convulsant site of action for GDEE in 3AP treated rats may be distinct from areas affected by GDEE in non-lesioned rats. Failure of MK-801 to elicit the same response as GDEE suggests that blockage of specific glutamate sensitive sites (QA-receptors) is required to reverse the effect of the

3AP lesion.

To test these hypotheses for the mechanisms underlying the decrease in strychnine-seizure threshold produced by 3AP-induced inferior olive lesion, would require further, extensive experimentation. In this dissertation 1) the phenomenon was discovered and characterized behaviorally, and 2) an attempt was made to identify the target populations of neurotransmitter receptors affected by the lesion, which might be responsible for the change in strychnine-seizure threshold. Testing the anti-convulsant efficacy of other glutamate analogs in this system would be of interest. However, the major problem with this line of experimentation is that there are few selective antagonists for QA- and KA-sensitive glutamate receptors, and none, other than GDEE, readily cross the blood-brain barrier. While more selective NMDA antagonists are available, such as AP5 and AP7, they also do not readily cross the blood brain barrier.

Although MK-801 does not affect strychnine-induced myoclonus, it is not without activity in 3AP-induced inferior olive lesioned rats. The effect is complex, since the type of seizure induced by strychnine is changed and the shape of the strychnine-seizure dose response curve is altered. This does not occur in control rats treated with MK-801. Control rats treated with MK-801 display typical strychnine seizures, in the typical

progression when challenged with increasing concentrations of strychnine. In addition, no [$^3$H]MK-801 binding has been observed in rat cerebellar membranes (Wong et al. 1986), indicating that this drug is not acting directly in the area postsynaptic to the inferior olive. However, as discussed earlier, the ramifications of climbing fiber degeneration are far reaching, and it is quite possible that the resultant change in cerebellar efferent activity and input to sites outside of the cerebellum could result in the pharmacological action of MK-801 observed in these experiments.

7: Systemic administration of the azaspirodecanedione anxiolytics; buspirone, gepirone and ipsaperone, shifted the dose-response curve for strychnine-induced seizures 1.4 to 3.3 fold to the left.

8: Buspirone administration further lowered the threshold for strychnine-induced seizure in 3AP-induced inferior olive lesioned rats.

9: Systemic administration of 0.5 mg/kg 8-OH DPAT had no effect on the strychnine-seizure dose response curve.

My experiments show that azaspirodecanedione pretreatment lowers the threshold for strychnine seizures. Buspirone has also been reported to lower the threshold for seizures induced by picrotoxin (Riblet et al. 1983). While azaspirodecanediones have high affinity binding for the 5-$HT_{1A}$ receptor (Peroutka 1985, Traber et al. 1984) and agonist activity at 5-$HT_{1A}$ sites which are negatively coupled to adenylate cyclase (Bockaert et al. 1987, Yocca and Maayani 1986), they may have mixed agonist/antagonist activity at these sites, as suggested by their participation in the '5-HT syndrome'. For example, 5-$HT_{1A}$ receptor activation by 8-OH DPAT, produces both flattened body posture and forepaw treading (Tricklebank et al. 1984). We also observed that treatment with 8-OH DPAT (0.5 mg/kg, i.p.) or azaspirodecanediones (40 mg/kg, i.p.) produced these as well as other components of the 5-HT syndrome (hindlimb abduction and tremor), consistent with previous reports (Smith and Peroutka 1986, Eison et al. 1986). However, Smith and Peroutka (1986) also found that buspirone and ipsaperone could antagonize elements of the 5-HT syndrome (forepaw treading, headweaving and tremor) induced by other 5-HT agonists, suggesting a mixed agonist/antagonist action for these compounds.

We tested 0.5 mg/kg 8-OH DPAT as a proconvulsant, and found that it had no affect on strychnine-induced

seizures. This result suggests that activation of 5-$HT_{1A}$ receptors alone does not facilitate strychnine as a convulsant. Higher doses of 8-OH DPAT were not tested; a positive result at a higher dose (either seizure facilitation or inhibition), could suggest 5-HT participation in the process, but would not provide information regarding the identity of the receptors subtype since higher doses are less specific, for the 5-$HT_{1A}$ receptor.

The effects of buspirone in lowering the threshold for strychnine-induced seizures were additive when the drug was administered to 3AP treated rats. This result shows that buspirone does not act at the inferior olive in mediating proconvulsant activity. These results further suggest that the proconvulsant effects of the two treatments may be mediated by separate mechanisms.

Possible sites of action for buspirone, gepirone and ipsaperone in the cerebellum include 5-HT receptors that are present on parallel fibers, 5-HT terminals and Purkinje cells. 5-$HT_1$ receptors, which have not been subclassified as 5-$HT_{1A}$ or 5-$HT_{1B}$, are present on parallel fibers and inhibit glutamate release (Raiteri et al. 1986). Azaspirodecanediones, with antagonist activity at these receptors, could prevent inhibition of parallel fiber glutamate release. This would result in hyperexcitation of the Purkinje cell, and increased simple

spike formation.

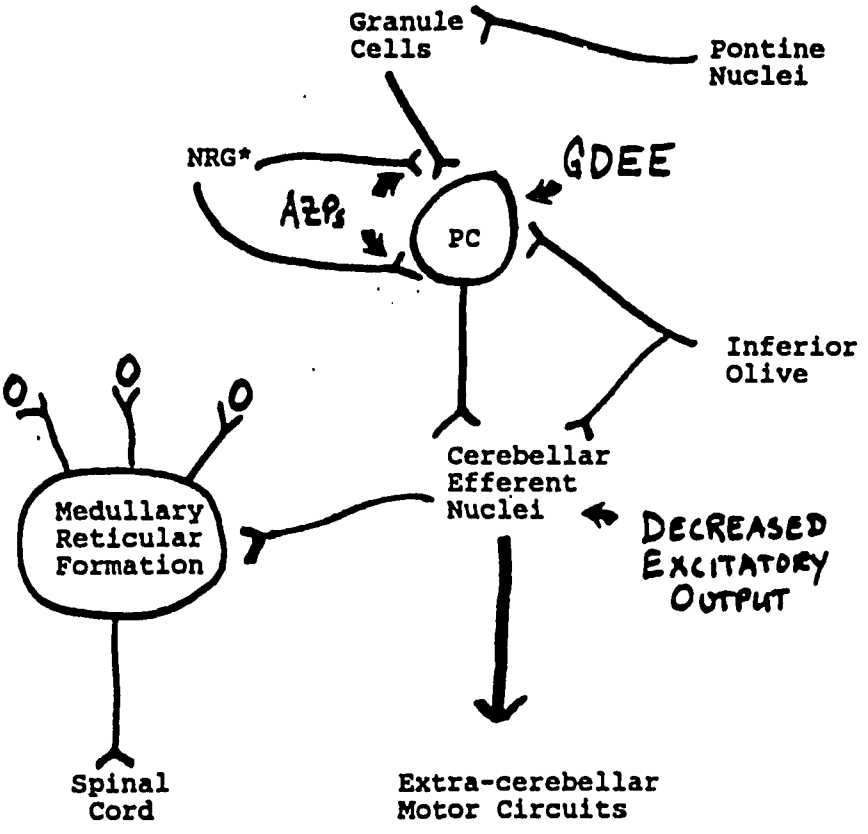
Another site in the cerebellum, where azaspirodecanediones might act is on 5-HT terminals. Serotonergic fibers, arising mainly from the nucleus reticularis gigantocellularis and the lateral reticular nucleus in the reticular formation, form a dense plexus around the body of the Purkinje cell (Bishop and Ho 1985). Presynaptic 5-HT receptors are present on 5-HT nerve terminals, and regulate 5-HT release. These are called 'autoreceptors', since activation of the receptor by the neurotransmitter regulates release of the same neurotransmitter by the same neuron. In the cerebellum, presynaptic, 5-HT autoreceptors have been classified as $5\text{-HT}_{1B}$ on the basis of differential sensitivity to (-)-propanolol and lack of response to 8-OH DPAT (Raiteri et al. 1986). Azaspirodecanediones may have agonist activity at autoreceptors, and could act to inhibit 5-HT release in the cerebellum. Since postsynaptic 5-HT receptors present on Purkinje cells are mainly inhibitory (Strahlendorf et al. 1984), blockage of 5-HT release by azaspirodecanediones could prevent 5-HT inhibition of Purkinje cells. This might create a Purkinje cell firing pattern similar to that seen following an inferior olive lesion. Assuming that azaspirodecanediones act within the cerebellum, then both actions discussed above could result in an increase in Purkinje cell activity and diminished

cerebellar efferent input to motor circuitry.

## 7.2 Summary

A diagram of the cerebellar circuitry affected by 3AP-induced inferior olive lesion and climbing fiber degeneration, is presented in Figure 9. Following

Figure Nine




*nucleus reticularis gigantocellularis

inferior olive lesions there is complete loss of climbing fiber-induced complex spikes, and an acute increase in the Purkinje cell firing rate by production of parallel fiber-induced simple spikes, accompanied by increased inhibition of cerebellar efferent nuclei (the dentate, interpositus, fastigial nuclei, and the vestibular nuclei) (Batini and Billard 1985). The pattern of efferent excitation from the cerebellum is decreased dramatically as a result of 3AP-induced inferior olive lesion, because Purkinje cell simple spike activity is much higher than normal. The net effect is increased inhibition of cerebellar efferent nuclei, and decreased excitation of areas postsynaptic from the cerebellar efferent nuclei. This inhibitory effect diminishes over time, but never returns to normal, in addition excitatory patterns of cerebellar efferent nuclear firing remain abnormal in the 3AP treated rat.

The effect of 3AP lesion on parallel fiber - Purkinje cell - cerebellar efferent nuclear output then is an acute increase in Purkinje cell simple spike production in response to parallel fiber input. More GABA is released on neurons postsynaptic to Purkinje cells, causing inhibition of synaptic activity from the dentate, interpositus and fastigial nuclei, and the vestibular nuclei (Batini and Billard 1985). Supporting this

electrophysiologic observation, increases in glutamic acid decarboxylase activity, the synthetic enzyme for GABA, have been demonstrated in the deep nuclei, following 3AP induced-inferior olive lesions (Oltmans et al. 1985).

Diminished output from cerebellar efferent sources, for example, the dentate nucleus has been traced through to the red nucleus in the brainstem, where changes in rubral output has been recorded (Billard and Daniel 1986). The ramifications of altered synaptic activity from the dentate and interposed nuclei, through rubral neurons has already been discussed, in relation to the decreased threshold for strychnine seizure observed following 3AP treatment or electrothermic inferior olive lesion (page 128). The changes in synaptic output from the fastigial nucleus to motor circuitry in the brain and spinal cord, following 3AP-induced inferior olive lesions, were also considered.

The proposed sites of action for the excitatory amino acid antagonists GDEE and MK-801 are also shown in Figure 9. GDEE may interact with QA-sensitive excitatory amino acid receptors in the cerebellum localized to Purkinje cells (PCs). MK-801, on the other hand is most likely acting outside the cerebellum. The effect of increased excitatory amino acid transmission through Purkinje cells (mediated by QA-sensitive glutamate receptors) due to the

loss of climbing fiber activity (and thus lack of climbing fiber-induced inhibition of the parallel fiber simple spikes) is persistent inhibition of the cerebellar efferent nuclei (due to release of GABA by Purkinje cells). Antagonism of the QA-sensitive glutamate receptors by GDEE, would then tend to 'normalize' the system, and the result would be decreased GABA release and less inhibition of the cerebellar efferent nuclei.

Cerebellar sites that may be activated by the azaspirodecanediones (AZPs) are shown in Figure 9. These are serotonin receptors on parallel fibers, 5-HT terminals and Purkinje cells. As discussed previously, antagonist action by azaspirodecanediones at either parallel fiber 5-HT receptors, or presynaptic 5-HT 'autoreceptors' on 5-HT terminals might create a Purkinje cell firing pattern similar to that seen following an inferior olive lesion. The means by which azaspirodecanediones could accomplish this are 1) by antagonizing 5-HT receptors which inhibit glutamate release by parallel fibers, thus increasing simple spike production by Purkinje cells, and inhibiting cerebellar efferent nuclei, and 2) by acting as 5-HT agonists at presynaptic 'autoreceptors' present on 5-HT terminals. Diminished 5-HT release in the cerebellum would result in decreased 5-HT inhibition of Purkinje cells and a greater response to parallel fiber excitation,

which would also increase simple spike production and provide greater inhibition of cerebellar efferent nuclei.

## 7.3 Directions for Future Research

There are several areas of research that could be pursued to further elucidate the role of the cerebellum in strychnine seizures and myoclonus. Following most directly in the line of research pursued herein, are studies to test the proposed hypothesis that the strychnine specific proconvulsive state seen in inferior olive lesioned rats is due to increased Purkinje cell activity, which results in an increased output of GABA in cerebellar efferent nuclei.

1: To test the hypothesis that the strychnine specific proconvulsive behavior seen in inferior olive lesioned rats is mediated through increased GABA output by Purkinje cells to cerebellar efferent nuclei, experiments to mimic this condition <u>in vivo</u> could be carried out. Gamma-vinyl GABA is an irreversible inhibitor of GABA transaminase, and will elevate GABA levels (Miller et al. 1986). If our hypothesis is correct, local infusion of gamma-vinyl GABA to the cerebellar efferent nuclei

(dentate and/or fastigial) should mimic the effects of inferior olive lesions and lower the threshold for strychnine seizure. In inferior olive lesioned rats, local infusion of gamma-vinyl GABA into cerebellar efferent nuclei should have minimal or no further effect on strychnine seizure threshold. Obviously, this is based on the assumption the inferior olive lesions maximally inhibit cerebellar efferent nuclear output. If the threshold for strychnine seizure is significantly lower in gamma-vinyl GABA-treated rats, it would be worthwhile to also test the effects of other convulsants in this model.

Gamma-vinyl GABA has been used in other seizure models to increase local GABA levels, most notably in the substantia nigra, where increased GABAergic transmission is anti-convulsant (Gale 1986).

If local infusions of gamma-vinyl GABA into cerebellar efferent nuclei produce changes in strychnine seizure threshold, it would be interesting to see whether electrothermic lesions of the cerebellar efferent nuclei produce a similar effect. Since GABA is an inhibitory neurotransmitter, the effect of gamma-vinyl GABA and electrothermic lesions of the same sites might be

expected to have a similar effect.

2: If gamma-vinyl GABA infusions into the cerebellar efferent nuclei produce a strychnine specific proconvulsant state similar to that seen in the inferior olive lesioned rat, then it should be possible to test our proposed hypothesis that
a) the site of action of GDEE (glutamate diethyl ester) is on the Purkinje cell and
b) the site of action of MK-801 is outside of the cerebellum

If our hypothesis is correct, pretreatment with GDEE would not restore the strychnine seizure threshold to control values in rats with gamma-vinyl GABA infused into the cerebellar efferent nuclei. However, pretreatment with MK-801 would have the same effect that it does in 3AP-induced inferior olive lesioned rats.

3: Since the effects of azaspirodecanediones and 3AP-induced inferior olive lesions on lowering strychnine seizure threshold were additive, we concluded that the actions of azaspirodecanediones were not mediated by the inferior olive - climbing fiber pathway. We have proposed that azaspirodecanediones may produce a

proconvulsant state by blocking either

a) presynaptic 5-HT receptors on parallel fiber terminals, which inhibit glutamate release or
b) presynaptic autoreceptors on 5-HT terminals, thus inhibiting 5-HT release.

These hypotheses assume that the inferior olive lesions do not cause maximal Purkinje cell hyperexcitation, i.e., Purkinje cells in the 3AP-induced inferior olive lesioned animals can be <u>further</u> excited by either an additional increase in glutamate release by parallel fibers or by a reduction in 5-HT release. If gamma-vinyl GABA infusions into the cerebellar efferent nuclei causes maximal inhibition of cerebellar efferent nuclear output and if our hypothesis that azaspirodecanediones produce a strychnine specific proconvulsant state by further increasing Purkinje cell activity is correct, then azaspirodecanedione pretreatment would cause no further decrease of strychnine seizure threshold in rats with gamma-vinyl GABA infused into the cerebellar efferent nuclei.

Further investigation of the inferior olive lesion induced changes in the excitatory amino acid receptor population in the cerebellum should be carried out. Certainly, kinetic studies with scatchard analysis of

[$^3$H]AMPA binding displaceable by QA, and unlabeled AMPA to cerebellar membrane preparations from control and 3AP treated rats, should be performed to determine the extent of change in binding parameters between groups. Since the results presented in chapter 5 are somewhat preliminary, thorough analysis of quantitative equilibrium binding studies would determine whether the change in binding detected is due to an increase in binding sites, or a change in binding site affinity. Quantitative autoradiographic receptor studies are also recommended, since the changes in excitatory amino acid receptors are expected to be localized in the molecular layer of the cerebellum where Purkinje cells are present. The ligands that would be most useful in identifying receptors would be labelled MK-801, phencylidine, N-acetylaspartyl glutamate, 2-amino-5-phosphonovaleric acid and AMPA.

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