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MECHANISMS OF CEREBELLAR GRANULE CELL
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JAYME DANELLE MANCINI

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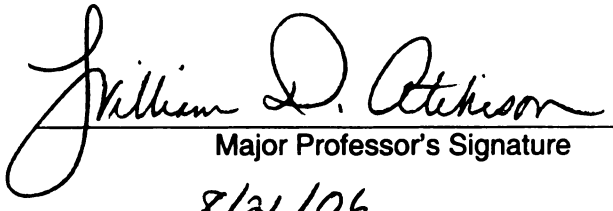

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MECHANISMS OF CEREBELLAR GRANULE CELL MIGRATION IMPAIRMENT BY METHYLMERCURY

By

Jayme Danelle Mancini

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ABSTRACT

MECHANISMS OF CEREBELLAR GRANULE CELL IMPAIRMENT BY METHYLMERCURY

By

Jayme Danelle Mancini

The cerebellum is crucial for motor and cognitive functions. Development of cerebellar granule cells is necessary for maturation of the cells with which they normally interact. Methylmercury (MeHg) prevents development of cerebellar granule cells by impairing their migration from the external germinal cell layer to the internal granule cell layer. Previous studies in rats determined that migration is dependent on N-type voltage-dependent Ca^{2+} channels (VDCC)- and N-methyl-D-aspartate (NMDA) receptor-mediated Ca^{2+}-oscillations. Both of these protein channels are dependent on membrane depolarization for activation, however, the source of which is not clear. The $GABA_A$ receptor depolarizes the membrane in immature neurons and is known to be a target of MeHg even at low micromolar levels. The objective of this study was to determine mechanisms involved in regulating the Ca^{2+}-oscillations of migrating cerebellar granule cell that may be altered by MeHg. Migration was studied using fluorescently-tagged bromodeoxyuridine (BrdU) pulse-track labeling of granule cells in organotypic slice cultures of developing cerebellum. The interactions between the $GABA_A$ receptor and VDCCs and/or NMDA receptors were investigated using acute slice preparations of developing cerebellum. The slices were loaded with a fluorescent Ca^{2+} -indicator dye. Laser confocal microscopy was used to analyze both the organotypic slice cultures and the $[Ca^{2+}]_i$ in the acute slice preparations.

The results suggest that the predominant subtype of NR2 subunit found in cerebellar granule cells at this stage of development, NR2B, is critical to migration. Stimulation of the $GABA_A$ receptor by muscimol increased intracellular calcium ($[Ca^{2+}]_i$) by 50.07% ± 6 in the external germinal cell layer and opened N- and L-type VDCCs, but not P/Q- type VDCCs or NR2B-containing NMDA receptors. $GABA_A$ receptor stimulation did not cause a significant release of Ca^{2+} from intracellular stores of the smooth endoplasmic reticulum or mitochondria. During continuous exposure to MeHg, the concentration at which granule cell death occurred with MeHg appears to be time- and concentration- dependent. Migration was significantly impaired by exposure to 3.0 μM MeHg for 3 days and 0.5 μM MeHg for 7 days, suggesting that MeHg impairs migration in a time-dependent manner at lower concentrations than are required to cause cell death. MeHg caused a time- and concentration-dependent increase in $[Ca^{2+}]_i$ in granule cells of all stages of development. Immature granule cells in the external germinal cell layer showed that an initial pulse of muscimol caused an increased $[Ca^{2+}]_i$ by 154% relative to controls, and was significantly greater than the response to caused by application of muscimol in the absence of MeHg. The $[Ca^{2+}]_i$ following subsequent pulses of muscimol in the presence of MeHg was greater than muscimol alone, but not as high as that in the presence of MeHg alone. In postmigratory granule cells of the internal granule cell layer pulses of muscimol in the presence or absence of MeHg did not increase $[Ca^{2+}]_i$. MeHg may stimulate, and then block the mature $GABA_A$ receptor as described by other studies. Effects of MeHg on the $GABA_A$ receptor at different stages of development may be responsible for the differential changes in $[Ca^{2+}]_i$ during MeHg exposure and disrupt the $GABA_A$ receptor-mediated activation of N- and L- type VDCCs.

DEDICATION

I would like to dedicate my dissertation thesis to my family and friends: my mom who encouraged my childhood interest in science, my Dad and Ma who helped make my university years possible, my siblings who were always supportive, my husband, Dave, for sticking by me during difficult times, and my friends (especially Cindy, Min, John M., Sally, Rana, DJ, and Jennifer) both for listening to me and for making me laugh.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

A, α : alpha

ACSF : artificial cerebrospinal fluid

B, β : beta

BDNF : brain-derived neurotrophic factor

BrdU : bromodeoxyuridine

Ca^{2+} : calcium

$[Ca^{2+}]_i$: intracellular calcium concentration

CaMKII : Ca^{2+}/calmodulin-dependent protein kinase II

cAMPdpk : cyclic adenosine monophosphate-dependent protein kinase

CREB : cyclic AMP response element binding protein

d : days

D-APV : D-(-)-2-amino-5-phosphonopentanoic acid

DIV : days *in vitro*

GC : granule cell

D, δ : delta

EGL : external germinal cell layer

EphB : ephrinB receptor

Fc : fusion protein

FMD : fetal Minamata Disease

Fyn : a tyrosine kinase

GABA : gamma amino butyric acid

$GABA_A$: gamma amino butyric acid receptor type A

γ : Gamma

GAD: glutamic acid decarboxylase

IGL : internal granule cell layer

IPSP: inhibitory post-synaptic potential

KCCl2 : K^+-dependent Cl^- co-transporter

MAPK : mitogen-activated protein kinase

MD : Minamata Disease

MeHg : methylmercury

ML : molecular layer

NCAM: neuronal cell adhesion molecule

NMDA : N-methyl-D-aspartate

NR2B : N-methyl-D-aspartate subunit subtype 2B

Ω, ω : omega

VDCC : voltage-dependent calcium channel

PKC : Protein kinase C

Src : a tyrosine kinase

TrkB : tyrosine kinase receptor B

VGAT: vesicular neurotransmitter transporter

CHAPTER ONE

GENERAL INTRODUCTION

Postnatal cerebellar granule cell migration is dependent on intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations, which may be triggered by the gamma-aminobutyric acid receptor type A ($GABA_A$). Ca^{2+} influxes during cerebellar granule cell migration are mediated through voltage-dependent Ca^{2+} channels (VDCC) (Komuro and Yacubova, 2003; Kumada and Komuro, 2004), and the dually voltage- and ligand-activated N-methyl-D-aspartate (NMDA) receptors (Komuro and Rakic, 1993; Rossi and Slater, 1993; Vallano, 1998; Mascos et al., 2001). However, the hypothesis that $GABA_A$ receptor modulates cerebellar granule cell migration via activation of VDCC and/or NMDA receptor remains to be tested (Fig. 1.1).

During normal mammalian brain development, neuroblasts proliferate and migrate to their appropriate destinations where they form synapses and mature; unused neurons apoptose. Different types of neurons often proliferate and migrate at different times or stages of development. In humans, the majority of cerebral cortical neurons have migrated by 5 months gestation, while cerebellar neurons migrate during the third trimester of gestation and infancy. A small number of cerebellar granule cells are still migrating up until approximately 4 years old. These developmental stages are critical time points which, when perturbed, can lead to major malformations of the central nervous system. The signaling mechanisms and control of neuronal migration are not well understood. Some neurons which fail to migrate all the way to their final destination undergo apoptosis, while others do not. Those remaining are referred to as heterotopic neurons, and are associated with mental retardation and seizures (Kuzniecky et al., 1988; Gressens, 2000; Rafalowska et al., 2001). Severe disorders of neuronal migration

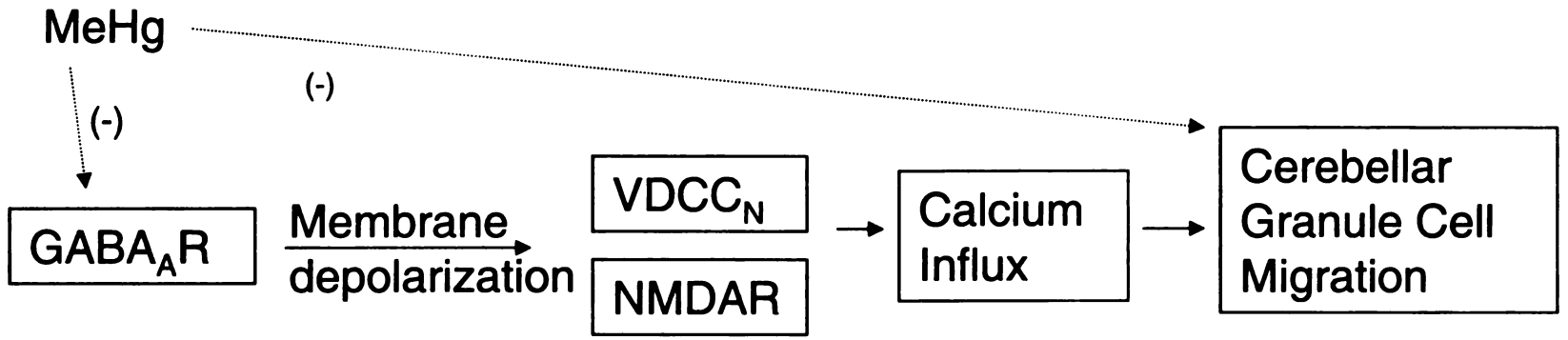


Figure 1.1. Does MeHg affect the putative $GABA_A$ receptor gating of VDCC- and/or NR2B-NMDA receptor-mediated Ca^{2+} -oscillations in non-synaptic, migrating cerebellar granule cells?

usually involve the entire brain. A few pathological features of neuronal migration disorders that can occur throughout the brain include schizencephaly, porencephaly, or lissencephaly (agyria). In schizencephaly, there are areas of the brain in which clefts extending from the cortical surface to the ventricular cavity form where neurons failed to migrate. The presentation of porencephaly is similar to schizencephaly, but is distinguished by a history of a destructive insult such as infection (Tardieu et al., 1981; Miller, 1984; Barkovich and Kjos, 1992; Faina, 1997). Lissencephaly, meaning smooth brain, is caused by a failure of neurons in the cortical plate to migrate during gestational weeks 12-16. It can result from genetic defects such as in a defective LIS1 gene, which is a specific disorder named Miller-Dieker Syndrome, or by an insult from a toxicant such as methylmercury (MeHg) (Steward et al., 1975; Alvarez, 1986; Aicardi, 1991; Barkovich et al., 1991; Dobyn, 1993; Lo Nigro, 1997).

Disorders also exist which are more specific to cerebellar development. The cerebellum consists of a central lobe, which is the vermis, and two lateral lobes, or hemisheres. Congenital cerebellar malformations include hemispheric hypoplasia, vermal aplasia, hemispheric and vermal hypoplasia, or complete agenesis of the cerebellum. Hemispheric hypoplasia is usually an autosomal recessive trait characterized by an absence of granule cells with preservation of Purkinje cells. If persistent, it causes progressive cerebellar dysfunction during infancy (Ramaekers et al., 1997). Vermal aplasia is relatively common. It is frequently associated with other cerebral malformations. Partial agenesis may be asymptomatic or only display a mild gait ataxia with upbeating nystagmus, or it may cause severe ataxia. Complete vermal agensis causes titubation (uncoordinated movement) of the head and truncal ataxia. Agenesis of

the cerebellar vermis can result from genetic defects or toxic insult (Ramaekers et al., 1997). Two examples of complete agenesis are Dandy-Walker Malformation and Joubert's Syndrome. Joubert's Syndrome can cause generalized hypotonia, decreased deep tendon reflexes, delayed motor milestones, abnormal eye movements, and ataxia (Anderson et al., 1999; Maria et al., 1999). X-chromosome-linked cerebellar hypoplasia involves both the cerebellar hemispheres and the vermis. The symptoms are hypotonia, mild dysphasia, delayed motor development at birth with progressive symptoms of ataxia and tremor, but normal cognitive development (Bertini et al., 2000). Dandy-walker Syndrome is a result of congenital anomalies of the posterior fossa, which can interfere with cerebellar development. Chiari Malformation involves cerebrospinal fluid build up displacing the cerebellar tonsils and posterior vermis through the foramen magnum (Sarnat et al., 2002).

Heterotopic neurons are also a prominent feature of Fetal and Non-Fetal Infantile Minamata Disease, or MeHg poisoning. Much of the damage in Non-Fetal Infantile Minamata Disease is concentrated in the cerebellum where cerebellar granule cells fail to migrate. Malformation of the cerebellar cortex in MeHg poisoning can cause deficits in psychomotor development, ataxia, and epilepsy (Rustam and Hamdi, 1974; Reuhl and Chang, 1979a; Bakir et al., 1980; Gressens, 2000).

A. Cerebellar Function

The cerebellar cortex processes feedback about current and intended movements for motor learning, regulation of balance and eye movements, regulation of body and limb movements, planning movement, evaluation of sensory information for action, and

cognitive functions such as timing, rhythm, and word association (Cook et al., 2004). Cerebellar cortical cells have been well studied because they form a distinctly organized neuronal network that is repeated throughout the cerebellar cortex (Fig. 1.2). Input from the vestibular, somatic, and cerebrocortical system afferents terminate as mossy fibers in the internal granule cell layer where they form excitatory synapses with cerebellar granule cells. Golgi cells in the internal granule cell layer form inhibitory synapses on granule cell dendrites as soon as the synaptic glomeruli begin to mature. Golgi cells release GABA onto $GABA_A$ receptors containing the α_6 subunit subtype in adult granule cells, therby inducing phasic inhibition (Rossi et al., 2003). The mature cerebellar granule cells have T-shaped axons, known as parallel fibers, in the molecular layer, which form excitatory synapses with Purkinje cell dendrites. Bergman's glial somas lie near Purkinje cells. Their radial fibers extend through the molecular layer and form knobby end-feet on the pia by birth. The glial fibers have lateral spines and varicosities as well as lateral outgrowths that separate Purkinje cell somas from adjacent parallel fibers. The Bergman's glia mature in parallel with the maturing cortex. The Purkinje cell dendrites also receive input from inhibitory interneurons and climbing fibers (Altman and Bayer, 1997). Mature granule cells are the only excitatory input to Purkinje cells, and Purkinje cell axons are the only output from the cerebellar cortex. Therefore, proper functioning of cerebellar granule cells is important, and investigating the mechanisms of their development may help us understand disorders and diseases of the cerebellum. The migration of granule cells in rats is well characterized (Komuro and Yacubova, 2003), and serves as a good model for studying the mechanisms of normal and aberrant migration.

B) Cerebellar Development

a)Cellular Aspects of Development

In the rat, cerebellar granule cells migrate postnatally. The postnatal cerebellum is easily accessible for slice preparations. Multiple slices can be prepared from each cerebellum, which reduces the number of rats used for each experiment.

In the embryonic stage of rat cerebellar development, the deep cerebellar nuclei are the first neurons to be generated, during embryonic days 12-17 (E 12-17). During that time, Purkinje cells begin to form; their birth peaks at E15. The Purkinje cells migrate from the neuroepithelium at E15 – 20. While the Purkinje cells are migrating, the lateral rhombencephalon or germinal trigone becomes the outermost cortical layer at approximately E17. This layer is the external germinal cell layer, which consists of secondary proliferative cells. Birth of Golgi cells follows at E19- postnatal day 2 (P2) (Altman and Bayer, 1997).

The postnatal stage of cerebellar development mainly involves cells generated in the external germinal cell layer and maturation of the Purkinje cell layer. At birth, the Purkinje cell layer is approximately 6 cells thick. By P3-4, Purkinje cells spread into a monolayer. The outer external germinal cell layer is a densely packed 4-5 cell thick layer of mitotic cells. These progenitor cells undergo asymmetrical mitosis generating basket cells, granule cells, and stellate cells; peak production of basket cells is at P6-7, granule cells at P8-9, and finally stellate cells at P8-11 in rats. Shortly after generation, the immature neurons proceed to migrate. Peak migration occurs at P7-10, P9-11, and P12-21 for basket cells, granule cells, and stellate cells, respectively (Altman and Bayer, 1997). The basket and stellate cells are indigenous, inhibitory interneurons of the

Figure 1.2.

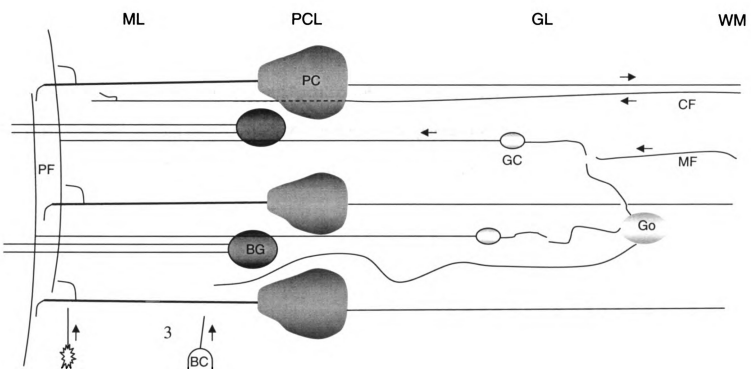


Figure 1.2. Neuronal distribution of normal adult cerebellar cortex. PC: Purkinje Cell, GC: granule cell, Go: Golgi Cell, BC: basket cell, ST: stellate cell, CF: climbing fiber, PF: parallel fiber, BG: Bergman's Glia, MF: mossy fiber GCL: granule cell layer, ML: molecular layer, PCL: Purkinje cell layer

molecular layer, and make up approximately 5% of the cell population in the external germinal cell layer and molecular layer. Granule cells migrate through the inner region of the external germinal cell layer and molecular layer, and into the internal granule cell layer in an "inside out" pattern. They have the furthest distance to migrate, and the other cells of the cerebellar cortex do not fully mature until the granule cells have formed synapses. The final mature distribution of neurons in the cerebellum is shown in Figure 1.2.

Proliferation and tangential migration

Migration of cerebellar granule cells has been broken down further into stages based on morphology and Ca^{2+} -oscillation frequency (See Fig. 1.3). The newly formed granule cells are stationary for 20 to 48 hours. During this time, they extend two processes in parallel to the longitudinal axis of the folium aligned with previously formed parallel fibers. The terminals of these processes are growth cones with lamellopodia. Granule cells initially migrate tangentially within the inner external germinal cell layer following the direction of the larger of the two processes, which may be in mediolateral or anterior-posterior planes (Komuro and Rakic, 1993; Komuro et al., 2001).

Radial migration and synaptogenesis

At the outer border of the molecular layer, granule cells come into contact with Bergman's glia. The Bergman's glia are radially aligned throughout the molecular layer. They function as a radial track. The granule cells begin radial migration by transposition of their somas along the glia (at a right angle to the parallel fibers). Migration slows as the granule cells reach the upper strata of the Purkinje cell layer where they separate and remain immotile for a brief period, but then continue to migrate radially through the

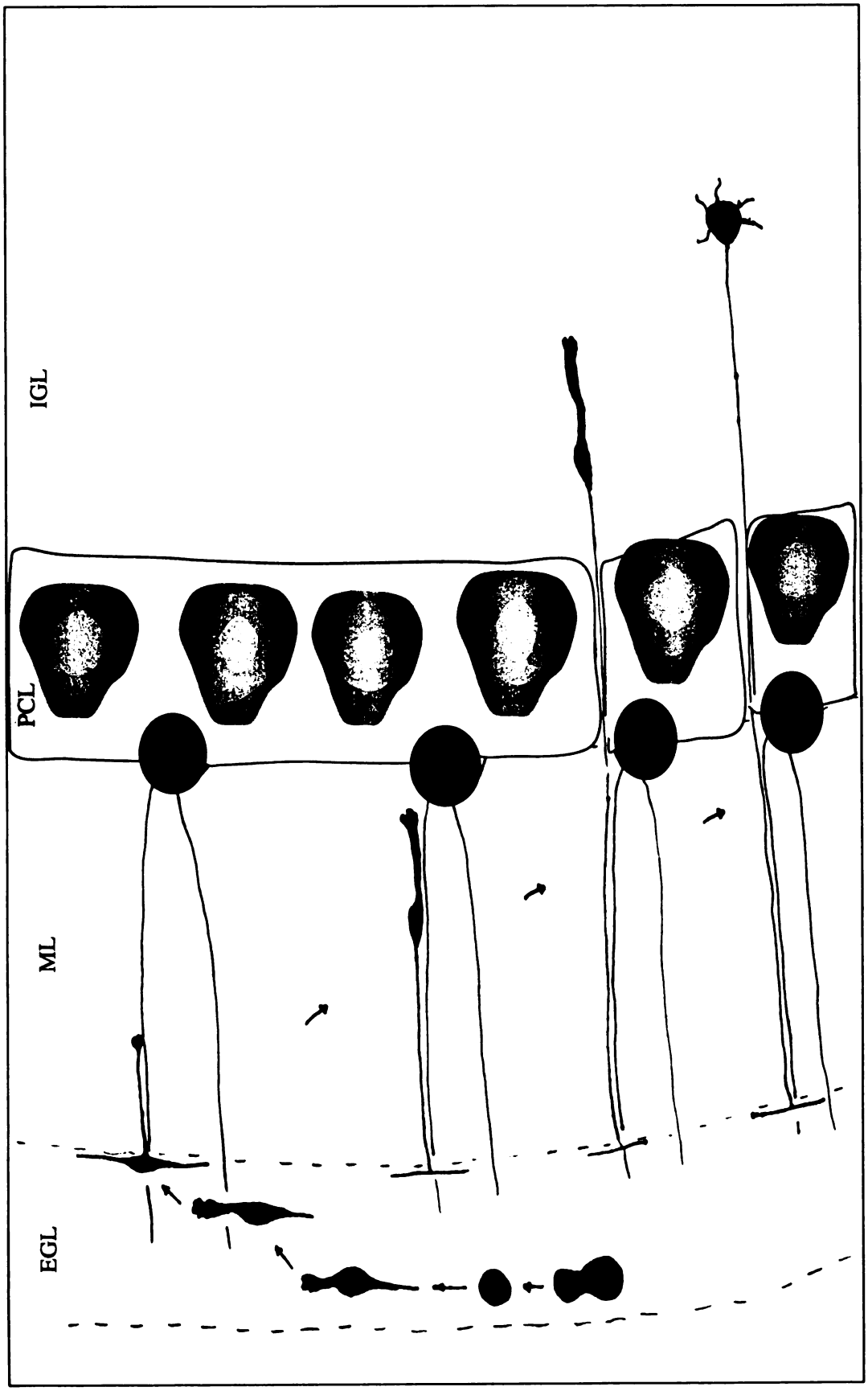


Figure 1.3.

Figure 1.3. Steps in cerebellar granule cell migration. 1. Asymmetrical mitosis in external germinal cell layer (EGL). 2. Post-mitotic stationary phase. 3. Bipolar morphology. 4. Calcium transient-dependent tangential migration through the EGL. 5. Extension of third process into molecular layer (ML). 6. Calcium transient-dependent radial migration along Bergmann's Glia through ML. Granule cells separate from the glia in the upper strata of the Purkinje cell layer (PCL) and remain immotile for a brief period. 7. Radial migration continues through the internal granule cell layer (IGL) to its final destination. 8. Arborization and synaptogenesis. Migration takes approximately 2 days per cell. The majority of cerebellar granule cells migrate during PD 7-14 in rats.

internal granule cell layer (See Fig. 1.3). Migration peaks on P10-11 (Komuro and Yacubova, 2003). The majority of migration is completed by P16 in rats and the external germinal cell layer ceases to exist (Altman, 1972). The developmental stage of the central nervous system in 16 d old rats approximately corresponds to human fetal development at 30 weeks gestation (Altman, 1972). Synaptogenesis begins as the granule cells complete migration (Altman and Bayer, 1997).

b) Molecular aspects of development

i) Ca^{2+} -oscillations

The intracellular mechanisms determining a cell's migratory path or rate of migration are not all known or fully understood. The cells respond to external cues by releasing or activating second messengers and, subsequently, undergo different patterns of gene expression. The second messengers and patterns of gene expression interact with the cytoskeleton causing specific morphological changes of the granule cells as well as distinct Ca^{2+} -flux patterns. When immature cerebellar granule cells are excited, the intracellular Ca^{2+} ($[Ca^{2+}]_i$) increases, and the cells migrate forward. However, continuous high levels of $[Ca^{2+}]_i$, such as 100 μM, kill cells. Cerebellar granule cells have, as most cells do, ways of quickly reducing normal physiologic increases in $[Ca^{2+}]_i$ (Lee et al., 1999; Fonnum and Lock, 2004). Thus, the migration is saltatory due to the transient increases in $[Ca^{2+}]_i$, or Ca^{2+} -oscillations (Komuro and Kumada, 2005). When the $[Ca^{2+}]_i$ is high, cerebellar granule cells move forward, and when the $[Ca^{2+}]_i$ drops, granule cells stop or even move backwards. Ca^{2+} functions as a secondary signal to multiple signaling systems such as phospholipase C, protein kinase C, cyclic adenosine monophosphate, or Ca^{2+}/calmodulin-mediated pathways, or release of internal Ca^{2+} stores (Kumada and

Komuro, 2004). The cerebellar granule cells and Bergmann's glia are bound together by cell adhesion molecules such as NCAM, L1, and astrotactin, while cadherin and integrin facilitate locomotion via the cytoskeleton (Rakic et al., 1994; Komuro and Yacubova, 2003). The processes of the granule cells sequentially express actin-containing microfilaments, microtubules, and neurofilaments (Komuro and Yacubova, 2003), which are regulated by second messengers.

The rate of Ca^{2+} -oscillation-mediated movement is specific to the stage of migration. In the external germinal cell layer, the rate of migration is 12 – 15 μm/hr. Granule cells slow down to about 4 μm/hr at the external germinal cell layer/molecular layer border (Komuro et al., 2001). The molecular layer rates average 9.6 μm/hr at P7 and 18.0 μm/hr at P13 (Komuro and Rakic, 1995). Each granule cell stops at the Purkinje cell layer for 30-220 min, and then continues to migrate radially through the internal granule cell layer at the same rate as the cell migrated through the molecular layer (Komuro and Rakic, 1998). These rates were observed whether studied in slice preparations or explant cultures.

A continuous increase in $[Ca^{2+}]_i$ from intracellular stores by caffeine or thimerosal treatment disrupted migration in a non-uniform manner; it accelerated the cell movement in the outer region of the internal granule cell layer, changed the direction of migration and induced backward movement of the cells (toward the Purkinje cell layer–internal granule cell layer border), and/or significantly delayed the completion of migration (Kumada and Komuro, 2004; Komuro and Kumada, 2005). Neither caffeine nor thimerosal changed the Ca^{2+} transient frequency or the cell motility at the top of the internal granule cell layer. Therefore, a sustained increase in $[Ca^{2+}]_i$ does not necessarily

lead to a normal completion of migration and maturation of cerebellar granule cells. In fact, comparison of the effects of drugs and neurotrophic factors on migration rate at the top and bottom of the internal granule cell layer or other layers has suggested that $[Ca^{2+}]_i$ transients are likely to be a differential response to specific extrinsic factors (Yacubova and Komuro, 2002; Yacubova and Komuro, 2003).

The effects of thimerosal are of interest because it is a mercurial compound. Thimerosal is an ethylmercury containing compound that is 49.6% Hg^{2+} by weight (Ball et al., 2001). Thimerosal was used as a preservative in childhood vaccines until 2001 (2003a). Some studies suggest that thimerasol in vaccines is correlated with autism or possibly other neurodevelopmental disorders (Wakefield et al., 1998; Kawashima et al., 2000; Bernard et al., 2001). However, other data suggest that if mercurials are a significant cause of the epidemic increase in the prevalence of autism and/or other neurodevelopmental disorders (Bertrand et al., 2001; Chakrabarti and Fombonne, 2001; Dales et al., 2001; Fombonne, 2001; Halsey and Hyman, 2001; Croen et al., 2002; Gurney et al., 2003; Lingam et al., 2003; Destefano et al., 2004) (Bertrand et al., 2001; 2003b; Gerlai and Gerlai, 2003; Yeargin-Allsopp et al., 2003; Blaxill, 2004; Gerlai and Gerlai, 2004), the source of mercury is more likely to be industrial practices releasing mercury into the environment than to be from thimerosal in vaccines (Parker et al., 2004; Palmer et al., 2006).

The horizontal processes that granule cells extend prior to migration become T-shaped axons. These axons make up the parallel fibers of the molecular layer. The majority of granule cells have developed the T-shaped axon by P15 in rats (Altman and

Bayer, 1997). The full migration process for one cell takes about 2 d (Komuro and Yacubova, 2003).

The synaptic maturation of parallel fibers with Purkinje cells depends on granule cell migration into the internal granule cell layer. Purkinje cells are necessary for the differentiation and maintenance of granule cells, while mossy fibers appear only necessary for granule cell differentiation (Altman and Bayer, 1997). During normal development, 50% of granule cells apoptose in the external germinal cell layer during the first 2 weeks of postnatal life (Wood et al., 1993). Apoptosis of cerebellar granule cells in organotypic slice culture was increased significantly by exposure to 10.0 μM MeHg for 3 d. While apoptosis of cerebellar granule cells was less evident following 3.0 μM MeHg exposure for 3 d, their migration was significantly impaired (Kunimoto and Suzuki, 1997). The cerebellum may recover the appropriate number of granule cells by increasing proliferation following an acute exposure to MeHg.

Although there are numerous pathways by which Ca^{2+} can enter the cytoplasm of granule cells, migration and the associated Ca^{2+} oscillations are directly dependent on NMDA receptors that contain the NR2B subunit subtype and N-type VDCC (Rakic and Komuro, 1995; Yacubova and Komuro, 2003) (See Chapter Three).

1) Molecular Biology of Glutamate Receptors

The NMDA receptor is one of two classes of ionotropic glutamate receptors with integral channels. Glutamate also binds to 1 class of metabotropic receptor (mGluR). NMDA receptors are both ligand- (glutamate and co-agonist, glycine) and voltage- (by Mg^{2+}) gated (Nowak et al., 1984), with preferential permeability to Ca^{2+} (MacDermott et al., 1986). In mature neurons, NMDA receptors have slow activation and inactivation

kinetics (tens to hundreds of msec) (Hestrin et al., 1990; Putney, 1999). In the internal granule cell layer, glutamate is released by mossy fibers onto mature granule cells at glomerular synapses. Glutamate uptake systems exist in both nerve terminals and surrounding glia, keeping the extracellular level of glutamate at approximately 1.0 μM (Nicholls and Attwell, 1990; Fonnum and Lock, 2004). The predominant glutamate uptake system in cerebellar astrocytes is the transporter protein, EAAT-1 in humans or GLAST in rat. The NMDA receptor channel is permeable to cations, and all receptor subtypes, except the NR2C, are blocked by Mg^{2+} at resting membrane potential (Mori and Mishina, 1995). In newly forming mossy fiber-granule cell synapses, both AMPA receptors and NMDA receptors contribute to action-potential evoked currents, while multi-quantal release is required for NMDA receptor activation in more mature synapses (Farrant et al., 1994; Cathala et al., 2003). However, in immature cerebellar granule cells, NMDA receptors are not located at synapses, and AMPA receptors are not expressed until migrating granule cells begin developing dendritic arbors in the internal granule cell layer (D'Angelo et al., 1993).

NMDA receptor antagonists that discriminate between glutamatergic effects on NMDA receptors and KA/AMPA receptors include D -AP5 (competitive inhibitor at the glutamate site, D-(-)-2-amino-5-phosphonopentanoic acid), dizocilpine, or MK-801(non-competitive inhibitor of the channel, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine hydrogen maleate), 7-CKN (glycine site antagonist, 7-chlorokynurenic acid), $MgCl_2$, PCP (channel blocker), ibotenate (glutamate analog), and enzymatic degradation of endogenous glutamine by glutamine pyruvate transaminase (converts amino levalinic acid and α-ketoglutarate to glutamate and pyruvate) (Komuro

and Rakic, 1993; Rossi and Slater, 1993; Vallano, 1998; Hirai et al., 1999). Treatment with NMDA receptor antagonists impairs cerebellar granule cell migration in neocortex resulting in heterotopic neurons, while glycine, on the other hand, enhances granule cell migration distance and rate (Komuro and Rakic, 1993). However, the specific subunit subtype composition of the NMDA receptors that is critical to cerebellar granule cell migration is not known.

The molecular and biophysical properties of NMDA receptor subunits are developmentally regulated, and any neuron can contain a heterogeneous population of receptor subtypes (Farrant et al., 1994; Gottmann et al., 1997; Kew et al., 1998). NMDA receptors in migrating granule cells are non -synaptic. Non-synaptic, or paracrine released, glutamate may come from glia or parallel fibers in the molecular layer, and glia may release D-serine which could bind to the glycine binding site (Schell et al., 1997). Paracrine transmission from endogenous sources of glutamate elicits tonic activity that is lowest in the external germinal cell layer, higher in the molecular layer, and highest in the internal granule cell layer and white matter. The frequency of spontaneous, tonic, single-channel activity was reversibly inhibited by non-subtype specific NMDA receptor antagonists or the glutamine uptake inhibitor L-α-aminoadipate and potentiated by glycine according to patch-clamp recordings (Rossi and Slater, 1993).

The NMDA receptor is heteromeric. The essential NMDA receptor subunit NR1 is ubiquitous during development. NR1 is expressed at its lowest levels in mice at E13, and it steadily increases after that. NR1 knockouts die as neonates (Nakazawa et al., 2001). However, the NR1 subunit may not be essential for migration since NR1 -/- stem cells migrate and differentiate normally at a variety of sites, including cerebellum and

hippocampus. The NR1 subunit has 8 variants made by alternate splicing of three exons. The NR1 subunit is thought to contain the glycine binding site. Functional NMDA receptors consist of 3-5 subunits. Each receptor contains at least one, but may contain as many as three NR1 subunits. The rest of the subunits are of the NR2 subtype (Hollman and Heinemann, 1994).

The specific modulatory receptor subunits of the NMDA receptor NR2A-D show distinct spatial and temporal distribution (Farrant et al., 1994; Vallano, 1998). The glutamate binding site is thought to be on NR2 subunits (Benveniste and Mayer, 1991). These are transcribed from separate genes (Hollman and Heinemann, 1994). Embryonic and neonatal forebrain and cerebellum express proton-sensitive (activity-sensitive), non-synaptic NMDA receptors composed of NR1a plus NR2B. This receptor subtype is sensitive to and positively modulated by polyamines such as spermine, spermidine, and putrescine (Reynolds, 1995; Zukin and Bennett, 1995; Johnson, 1996), as well as histamine (Bekkers, 1993). They are negatively modulated by Zn^{+} (Christine and Choi, 1990; Legendre and Westbrook, 1990; Hollman et al., 1993), and protons (Tang et al., 1990; Traynelis et al., 1995).

NR2B expression is highest during embryonic development of the forebrain and postnatal development of the cerebellum. NR2B expression decreases and NR2A expression increases as cerebellar granule cells form immature synapses. During synaptic maturation granule cells decrease expression of NR2A and increase expression of NR2C, reducing the potential for excitotoxic damage (Akazawa et al., 1994; Bergmann et al., 1996; Fonnum and Lock, 2004). Upon cerebellar maturation NMDA receptor expression includes NR1b plus NR2C with some NR2A (Akazawa et al., 1994).

The subunit composition of NMDA receptor in cerebellar granule cells varies from much of the central nervous system. Most other mature brain regions mainly express NR2A (Thompson et al., 2000; Fonnum and Lock, 2004). The NR2C subunit is relatively Mg^{2+} insensitive and has a low affinity for MK-801. It also has a higher affinity for glycine and lower affinity for glutamate and Mg^{2+} than NR2A does (Buller et al., 1994; Sucher et al., 1996; Fonnum and Lock, 2004). An NR3 subunit may also exist with two possible variants (Fu et al., 2005). Cerebellar granule cells that fail to switch from NR2A to C expression have been suggested to play a role in heterotopic foci of epilepsy (Fonnum and Lock, 2004).

The NR2 subunits are suspected to be the critical players in migration because they are highly developmentally regulated by intracellular mechanisms and neurotrophic factors. NR2 subunits have particularly long C-terminals that extend into the cytoplasm (Nakazawa et al., 2001). The C-terminus is a major site of receptor modification by phosphorylation. The cytoplasmic C-terminal of the NR2B subunit appears integral for synaptic localization and intracellular regulation of NMDA receptor functions. The NR2B subunit is phosphorylated on the C-terminal by cyclic AMP-dependent protein kinase (cAMPdpk), calcium/calmodulin dependent protein kinase II (CaMKII), and protein kinase C (PKC): the last of which may reduce voltage-dependent block (Chen and Huang, 1992) by an unknown mechanism. The NR2B subunit is also tyrosine phosphorylated by Src-family non-receptor protein kinases and the kinase, Fyn. Ca^{2+}/calmodulin-dependent phosphatase dephosphorylates NMDA receptors, desensitizing them.

NMDA receptors are associated and co-localized with clusters of tyrosine kinases

Figure 1.4

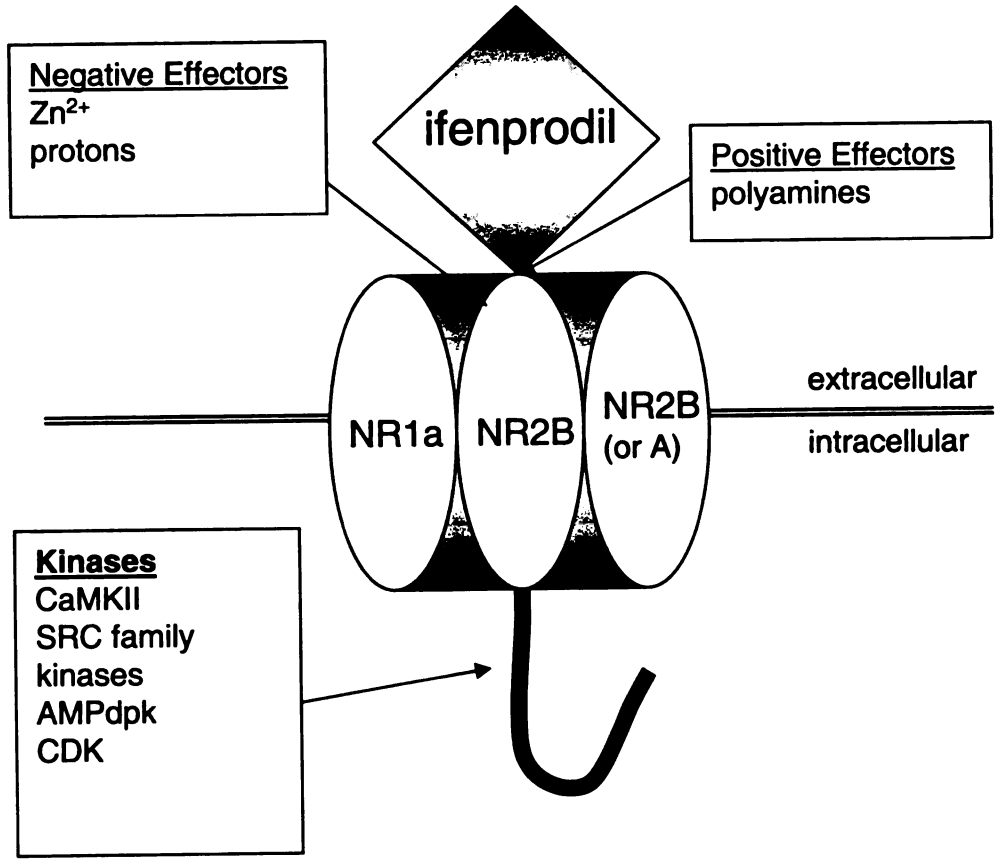

Negative Effectors
Zn^{2+}
protons
ifenprodil
Positive Effectors
polyamines
NR1a
NR2B
NR2B
(or A)
extracellular
intracellular
Kinases
CaMKII
SRC family
kinases
AMPdpk
CDK

Figure 1.4. The NR2B-NMDA receptor. The NR2B-subunit subtype-containing NMDA receptor is inhibited or enhanced by Zn^{+} and protons or polyamines, respectively, and the intracellular tail of the NR2B subunit is modified by several kinases and phosphatases. Ifenprodil is a glutamate-dependent partial antagonist of NR2B-NMDA receptors.

phosphorylating the Ephrin-B receptor, EphB. Ephrin is a membrane bound ligand which is involved in initiating interaction between neurites and have been shown to be essential for migration of many cell types (Flanagan and Vanderhaeghen, 1998; Klein, 2001), boundary formation, cell adhesion, migration of the neural crest, and axon guidance (Drescher, 1997; O'Leary and Wilkinson, 1999). EphrinB2/Fc activation of EphB in primary cortical neurons potentiates NMDA receptor-dependent Ca^{2+} influx leading to both NR2B subunit tyrosine phosphorylation and enhanced NMDA receptor-dependent gene expression (Takasu et al., 2002). The fusion protein, Fc, alone or brain derived neurotrophic factor (BDNF) activates the receptor tyrosine kinase, TrkB, but does not increase glutamate-stimulated Ca^{2+} increases (Takasu et al., 2002). Protein synthesis induced by BDNF in cooperation with ephrinB2 is associated with increasing size of dendritic spines (Miyata et al., 2005). NMDA receptors also interact with the cytoskeleton (Ackermann and Matus, 2003).

2) VDCCs

The other significant source of Ca^{2+} in the oscillations of cerebellar granule cell migration are the VDCCs (Komuro and Rakic, 1993). These proteins are heterogeneous in structure and regulation. They exhibit differential regional expression. All VDCCs have four principal subunits. Each contains an α_1 subunit which is the transmembrane, pore-forming component (Williams et al., 1992; Brust et al., 1993). The other three subunits are of various subtypes, which regulate expression, localization, kinetics, and modulate Ca^{2+} current. In neurons, VDCCs contain α_1, a cytoplasmic β subunit, the integral membrane γ subunit, and $\alpha_2\delta$. The β subunit regulates the channel properties and targeting of α_1 (McEnery et al., 1998; Hajela et al., 2003). The α_2 and δ subunits are

linked by a disulfide bond. Each type of VDCC has a unique subunit subtype composition. A_1 subunits can be of 10 types (α_A-α_I and α_S), α_2 subunits can be of types A-E, and β subunits can be of types 1-4. N- and L -type VDCCs contain α_{1B} and $\alpha_{1C, 1D, 1F, or 1S}$, respectively (Putney, 1999; Bell et al., 2001). Neurons typically co-express multiple types of VDCCs that are spatially regulated for specific cellular functions and temporally regulated during development (McEnery et al., 1998). Channel-mediated Ca^{2+} entry can interact with ATPases and intracellular Ca^{2+} stores causing locally confined Ca^{2+} increases such as Ca^{2+} spikes or waves (Audesirk et al., 2000). VDCCs are phosphorylated by kinases such as CaMdpks, and PKA and C, and they are modulated by G proteins. Mature cerebellar granule cells in primary culture express VDCC types Q-, N-, R-, L-, and P- which constitute 35 %, 20 %, 19 %, 15 %, and 11%, respectively, of the whole cell VDCC current (Randall and Tsien, 1995). VDCC-mediated Ca^{2+} currents are blocked by heavy metal neurotoxicants such as Cd^{2+}, Pb^{2+} , and MeHg in the presence or absence of stimulation at both extra- and intracellular surfaces (Sirois and Atchison, 1996; Shafer, 1998; Sirois and Atchison, 2000; Shafer et al., 2002). VDCCs can also allow entry of heavy metals into the cell at lower concentrations than are required to block the channel (Audesirk et al., 2000).

Determining which factors lead to activation of VDCC and the NMDA receptor in immature, non-synaptic cerebellar granule cells would aid in the understanding of the mechanisms regulating their migration. Both the N- type VDCC and the NMDA receptor are dependent on membrane depolarization. The membrane depolarization following $GABA_A$ receptor stimulation in immature neurons activates VDCCs in every part of the developing brain studied thus far (Yuste and Katz, 1991). In developing hypothalamic

neurites, stimulation leads to a rise in $[Ca^{2+}]_i$ that is sometimes greater than that evoked by glutamate application alone (Obrietan and Van den Pol, 1996, 1997). However, the type of VDCC functioning in response to the $GABA_A$ receptor may differ depending on neuron type. The source of the depolarization that activates N-type VDCC in migrating cerebellar granule cells appears to be the $GABA_A$ receptor (See Chapter Three), however the source of voltage activation of the NMDA receptor is not clear. Research in other regions of the brain suggest that the $GABA_A$ receptor also activates the NMDA receptor (Represa and Ben-Ari, 2005). GABAergic regulation of granule cell migration is particularly of interest when considering Fetal and Non-Fetal Infantile Minamata Disease because the $GABA_A$ receptor is the most sensitive receptor to MeHg yet studied in cerebellar granule cells (Yuan et al., 2005).

3) GABA

The ligand, GABA, is synthesized from glutamate through an enzymatic reaction with glutamic acid decarboxylase 65 and 67 (GAD65 and GAD67). It is loaded into synaptic vesicles by the vesicular neurotransmitter transporter (VGAT), which is not highly expressed or co-localized with GABA early in development (Takayama and Inoue, 2004). GABA is mainly secreted by Ca^{2+}-dependent exocytosis, but sometimes it is secreted by non-vesicular means such as by reverse transporters (Wu et al., 2003). Plasma membrane GABA transporters (GATs) take up GABA into nerve terminals and/or surrounding glia, within which, GABA-transaminase (GABA-T) metabolizes it (Owens and Kriegstein, 2002).

GABA appears to play a role in neuronal development, but its function is not clear. Represa et al. (2005) showed that paracrine, diffuse, non-synaptic GABA

functions as an epigenic factor controlling cell proliferation, neuroblast migration, and dendritic maturation in hippocampal cells (Represa and Ben-Ari, 2005). GABA appears to enhance embryonic migration of hippocampal neurons through the first three layers of hippocampus (ventricular zone, subventricular zone, and intermediate zone, but impair migration into the final layer (cortical plate) (Behar et al., 2000). GABA increases cerebellar granule cell proliferation in dissociated culture (Fiszman et al., 1999), but it decreases proliferation in cortical epithelium and directs subsequent migration (Barker et al., 1998). GABA was determined to be a chemoattractant for E13 spinal cord neurons in rats, and the chemoattraction was mediated by the $GABA_A$ receptor (Behar et al., 1994). In sum, the function of GABA appears to be spatially distinct. Therefore, the function of GABA in development is likely to vary depending on which GABA receptor(s) is involved, whether the neuron is in the cerebellum or cerebrum, and which the stage of development the neuron is in. There are at least 4 types of GABA receptors, A-D. Types A and C are ionotropic while type B is metabotropic. Very little is known about the $GABA_D$ receptor. They are spatially localized and their subunit subtype compositions are developmentally and spatially regulated.

The $GABA_B$ receptor inhibits cAMP formation and inositol phosphate turnover (Kuriyama, 1994), and is coupled to K^+ and Ca^{2+} channels through G- proteins in the cerebellum (Billinton et al., 1999). $GABA_B$ receptors have also been shown to modulate Ca^{2+} spikes in pyramidal cell dendrites (Perez-Garci et al., 2006). $GABA_B$ receptors in developing cerebellum are found in the highest levels at the glutamatergic synapses between parallel fibers and Purkinje cells (Lujan and Shigemoto, 2006). The role of the

$GABA_B$ receptor in cerebellar granule cell migration is not certain, but it does appear to have a facilitory, although non-critical, effect (Komuro and Rakic, 1993).

The $GABA_A$ receptor is a heteropentameric ligand-gated membrane receptor found throughout the mammalian nervous system. In adults, stimulation of the $GABA_A$ receptor is widely known to produce IPSPs, or inhibitory responses. The IPSPs are a result of Cl^- flowing intracellularly through the open receptor-associated channel causing hyperpolarization. Each $GABA_A$ receptor contains at least one α, β, and, usually, a γ subunit, plus either π, δ, ε, or θ subunits. Any given neuron has a heterogenic population of $GABA_A$ receptor subunits and subunit subtypes. Upon synaptogenesis, the expression switches from α_2 to α_1 or α_6 in cerebellar granule cells (Gao and Fritschy, 1995). The expression of α_6 subunits in $GABA_A$ receptors is rather unique to adult cerebellar granule cells (Fritschy et al., 1994) and kidney cells. A_6 subunits begin to be detectable between P8-14 in rats. The α_1, α_6, $\beta_{2,3}$ receptor subunit subtypes are highly localized to type II Golgi cell terminal-granule cell synapses (Nusser et al., 1998; Nusser et al., 1999).

The subunits and physiology of the $GABA_A$ receptor in immature neurons differ from the adult. The predominant receptor subunit subtypes in immature cerebellar granule cells is $\alpha_{2,3}\,\beta_3\,\gamma_{1,2}$ (Mellor et al., 1998). The type α_2 is only transiently expressed during migration (Gao and Fritschy, 1995). The immature receptor subtype is not synaptic. It has a relatively high affinity for GABA and a very low desensitization rate (Owens and Kriegstein, 2002). These are optimal conditions for receptors functioning in response to paracrine release of ligand. The immature subtype has a different response to several agonists and antagonists, including a decreased response to benzodiazepines. Stimulation of these receptors causes a fast excitatory response, due to the Cl-

electrochemical gradient. In mature cells, the Cl- concentration is lower intracellularly than extracelulary, with a reversal potential of -60-70 mV. Immature granule cells have a high intracellular Cl^- concentration, which drives Cl^- extracellularly through the $GABA_A$ receptor-associated Cl^- channel, leading to depolarization. Both the mature and immature Cl^- channel can be blocked by picrotoxin or bicuculline. As the expression of K^+ coupled Cl^- transporter (KCC12) increases, the depolarizing effect of $GABA_A$ receptor stimulation decreases. Increasing $GABA_A$ receptor stimulation leads to the increase in KCC12, and chronic $GABA_A$ receptor block delays the GABA inhibitory response of mature cerebellar granule cells (Ganguly et al., 2001). However, the spontaneous miniature depolarizations at GABAergic synapses are sufficient to drive the switch *in vivo*.

The developmental role of GABA in the cerebellum is multi-faceted and not well understood. $GABA_A$ receptor stimulation in immature cerebellar granule cells causes depolarization and promotes neurite extension, increases the complexity of dendritic arborization, and synaptogenesis, while decreasing late-stage neurogenesis (Owens and Kriegstein, 2002; Borodinsky, 2003). The receptor is found both in the cell body as well as in neurites. Neurotrophic factors are also expressed in response to $GABA_A$ receptor stimulation (Van den Pol et al., 1998; Obrietan et al., 2002). Manent et al. (2005) found that L -type VDCCs were the predominant channel-type responding to $GABA_A$ receptor stimulation in the embryonic development of olfactory bulb, cortex, medulla, striatum, hippocampus, colliculus, and hypothalamus. However, cerebellar granule cells differ by age at which migration and maturation occur, as well as by their changes in cell morphology in response to manipulation of the $GABA_A$ receptor. In the developing

cerebellum, $GABA_A$ receptors antagonists did not significantly impair granule cell migration *in vitro* (Komuro and Rakic, 1993).

In summary, cerebellar granule cell migration is dependent on N-type VDCC- and NR2B-NMDA receptor- mediated Ca^{2+} -oscillations. The N-type VDCC is activated, at least in part, by the $GABA_A$ receptor. The interactions of $GABA_A$ receptors may modulate the direction, frequency and/or rate of granule cell migration by modulating N-type VDCC activity. Recent evidence suggests that $GABA_A$ receptor and VDCC function in mature granule cells is altered by submicromolar levels of MeHg , and that submicromolar levels of MeHg disrupt Ca^{2+} regulation in both immature and mature cerebellar granule cells (See Chapter Four). It has also been shown that low-level exposure to MeHg can impair cerebellar granule cell migration (Kunimoto and Suzuki, 1997). Altered function of the $GABA_A$ receptor or VDCCs may be a significant mechanism by which heavy metals such as MeHg impair migration.

C) MeHg Poisoning

MeHg became well recognized as a neurotoxicant following the world's first mass poisoning in Minamata Bay, Japan in the 1950's. Residents of Minamata Bay who consumed fish or shellfish experienced concentric constriction of their visual fields, hearing loss, tremors, cerebellar incoordination, and sensory impairment of the legs and arms and/or tongue and lips (Hunter et al., 1940; Hunter and Russell, 1954; Tokuomi et al., 1982). The intention tremor, myoclonus, and static tremor observed by Rustam et al (1974) and the postural tremor and action tremor observed by Tokuomi et al (1968) in MeHg poisoned people were correlated with cerebellar granule cell death and the preservation of Purkinje cells, dentate nucleus, brachium conjunctivum, basal ganglia,

and nuclei of the brainstem according to computed tomography (CT) scan studies. In the cerebellum, MeHg causes preferential loss of granule cells, particularly in the central position (Takeuchi, 1968) and inferior vermis (Tokuomi et al., 1982). Later cases of MeHg poisoning (1973-81) were more difficult to diagnose due to the atypicality and mildness of the symptoms (Tokuomi et al., 1982). Studying patients with 10 years of chronic MeHg poisoning outside of Minamata Bay revealed high frequencies of hypo-esthesia in the distal extremities, which is known as a sign of slight MeHg intoxication (Bakir et al., 1973; Berlin, 1986), as well as ataxia, hearing impairment, visual changes, and dysarthria (Ninomiya et al., 1995).

The symptoms of MD were a result of ingesting fish from mercury contaminated seas. Elemental mercury is methylated by microorganisms in soil sediment and bioaccumulates in the food chain. Fish or other animals higher on the aquatic food chain contain relatively higher levels of MeHg (Chang and Verity, 1995).

Many infants born after 1955 in or around Minamata Bay had mental retardation and cerebral palsy (Kitamura et al., 1960). The disease was named Fetal Minamata Disease (FMD) (Matsumoto and Takeuchi, 1965; Snyder, 1971; Harada, 1977; Marsh et al., 1980) and has been characterized by 1) bilateral cerebral atrophy and hypoplasia with decreased cortical nerve cells, 2) cerebellar atrophy and hypoplasia with decreased cerebellar granule cells, 3) abnormal cytoarchitecture with atopic and disorientated neurons implying impaired neuronal migration and maturation, 4) hypoplasia of the corpus collosum, 5) dysmyelination of white matter, and 6) hydrocephalus (Matsumoto and Takeuchi, 1965; Chang and Guo, 1998). Non-fetal infantile Minamata Disease has some generalized central nervous system features as in the FMD, but most of the lesions

were more focused in the central cerebellum, sensory cortex, and occipital cortex (Chang and Guo, 1998).

Mass MeHg poisonings also occurred in Iraq in 1956 and 1960 as a result of consumption of bread made from grain treated with the mercury-containing anti-fungal, Granosan M (Jalili and Abbasi, 1961; Kantarjian, 1961; Damluji, 1962). Another outbreak occurred in Iraq in 1971- 1972. The adult cases developed paraesthesia at an approximate body burden of 25 mgHg/Kg and ataxia at 50 mgHg/Kg (Bakir et al., 1980). Studies of infants exposed *in utero* or through breastfeeding showed that the blood-Hg levels were higher in infants and children than in adults (Bakir et al., 1980). The children displayed mental retardation with delayed onset of speech and impaired motor, sensory, and autonomic function. Severely affected children were blind and deaf. The Iraqi epidemic differed from that in Minamata Bay because, in Iraq, it was severe, prolonged, and continuous.

Today, elemental mercury is regularly released by agricultural, paper, lumber, and leather industries, gold-mining, and manufacturing of electrical equipment, paint, and combustion of fossil fuels (Chang and Verity, 1995). The methylated metal is chronically taken in at a low-level by regular ingestion of contaminated fish or shellfish (See Chapter Four). At low-level concentrations, overt clinical symptoms have not been reported in adults (Harada, 1978; Reuhl and Chang, 1979a; Sakamoto et al., 1998; Miyamoto et al., 2001); subtle behavioral abnormalities have been demonstrated in children exposed chronically to concentrations that do not result in abnormalities in similarly exposed adults (Bakir et al., 1980; Takeuchi, 1982; Grandjean et al., 1997; Grandjean et al., 1998; Grandjean et al., 1999). However, there is some controversy

regarding this as the Seychelles study does not consistently identify effects of MeHg (For more information See Chapter Three).

Once MeHg is ingested, it is readily absorbed into the blood and distributed throughout the body. MeHg passes through the blood brain barrier (Steinwall and Klatzo, 1966; Chang and Hartmann, 1972; Aschner and Aschner, 1990). MeHg accumulates in the nervous system. The areas of greatest accumulation during chronic exposure in rats are the spinal dorsal root ganglia, cerebral cortex, and cerebellum (Somjen et al., 1973). Histopatholoical studies showed that chronic MeHg causes gross atrophy of the cerebrum particularly within the calcarine cortex, cerebellar granule cell layer, and axonal degeneration secondary to the loss of myelin sheaths around sensory branches of the peripheral nervous system (Hunter and Russell, 1954). The half-life of MeHg in humans is 70 d (Clarkson, 1972), however, the half-life for MeHg in the brain may be much longer during chronic exposure (Rice, 1989).

While research into the mechanisms of MeHg neurotoxicity in adults has provided some understanding of how the symptoms arise, less is known about the mechanisms causing Fetal and Non-Fetal Infantile Minamata Disease. This is, in part, because the mechanisms involved in normal development are not all known.
The objectives of this study were to investigate particular mechanisms (Fig. 1.1) in granule cell migration that, when perturbed, lead to impairment of migration. Chronic, low level MeHg exposure is the perturbing agent of focus in this thesis for the reasons mentioned above.

CHAPTER TWO

THE NR2B SUBUNIT SUBTYPE IN NMDA RECEPTORS IS CRITICAL TO CEREBELLAR GRANULE CELL MIGRATION

A) ABSTRACT

Migration of granule cells from the external germinal cell layer to the internal granule cell layer within the cerebellar cortex is a crucial developmental process early in life. Antagonists to NMDA receptors impair cerebellar granule cell migration significantly, but studies to determine which subunit subtypes control or affect migration have been controversial. Migrating granule cells transiently express NMDA receptor subunit subtypes NR1a plus NR2B. Grafted NR1-/- subunit knockout cells continue to migrate, indicating that the NR1 subunit is not necessary for migration. In the present study, the functional importance of the NR2B subtype in developing cerebellum was investigated using organotypic slice cultures prepared from P8 rats. Granule cells were labeled with bromodeoxyuridine (BrdU) during the first 20 hrs and then continuously treated with the NR2B-subtype-specific NMDA antagonist, ifenprodil, or the non-specific NMDA antagonist, D-APV, for 7 days (d). Cultures were incubated with fluorescently tagged anti-BrdU IgG and analyzed using laser confocal microscopy. The percent of BrdU labeled cerebellar granule cells that migrated from the external germinal cell layer to the internal granule cell layer during treatment was calculated. Migration into the internal granule cell layer was significantly impaired by treatment with 0.5 and 1.0 μM ifenprodil. Fewer cells had migrated to the internal granule cell layer in 1.0 μM ifenprodil than in 0.5 μM ifenprodil; there was no significant difference between the percent impairment caused by 1.0 μM ifenprodil and 50 μM APV. Untreated controls had few, if any, granule cells in the external germinal cell layer at DIV 8. The percent of granule cells remaining in the external germinal cell layer following treatment with antagonists significantly increased, indicating impairment of migration. In conclusion,

the predominant subtype of NR2 found in cerebellar granule cells at this stage of development, the NR2B, appears to be necessary for their migration.

B) INTRODUCTION

Immature granule cells migrate from the external germinal cell layer to form the internal granule cell layer (Komuro and Rakic, 1993). However, the specific mechanisms which orchestrate migration are not clear. This information is critical because granule cells that do not migrate to their appropriate destination usually fail to mature properly. Deficits in neuronal migration are involved in numerous pathological conditions as indicated by misplaced or heterotopic neurons in the cerebellum. Clinical symptoms of malformed cerebellar cortex include deficits in psychomotor development, ataxia, and epilepsy (Gressens, 2000).

Cerebellar Development

(See Chapter One)

Migration is dependent on transient Ca^{2+} fluxes, or Ca^{2+} oscillations (Kumada and Komuro, 2004). The frequency of Ca^{2+} oscillations, and therefore the rate of migration, is influenced by extracellular signals to receptors or channels, which then modulate $[Ca^{2+}]_i$, the secondary messenger (Komuro and Yacubova, 2003). The Ca^{2+} oscillations are known to be dependent on NMDA receptors and N-type VDCCs (Komuro and Yacubova, 2003).

NMDA receptors in immature granule cells differ from those of mature granule cells. Migrating granule cells transiently express NMDA receptors subunit subtypes NR1a plus NR2B. As granule cells approach the internal granule cell layer, expression of the NR2A subtype increases (Watanabe, 1996; Snell et al., 2001). Mature cerebellar granule cells predominantly express the NR2C subtype (Fu et al., 2005; Metzger et al., 2005). Subunit identity is important because functional properties of the channel such as

its conductance and deactivation time of the receptor are a result of its subunit composition. NMDA receptors containing NR2B subunits have the highest affinity for the voltage-dependent blocker, Mg^{2+}, and display slow decay kinetics (Farrant et al., 1994; Vallano, 1998). NMDA receptors receive tonic stimulation by endogenous ligands in acute cerebellar slices, and the frequency of stimulation increases as the granule cells migrate (Rossi and Slater, 1993; Farrant et al., 1994). Non-subunit subtype-specific NMDA receptor antagonists, namely MK-801 and D-APV, significantly impair migration, but do not completely inhibit it (Komuro and Rakic, 1993; Rakic and Komuro, 1995). The functional role of each subunit subtype during development is not clear.

NR1a plus NR2B receptors are likely candidates for the critical source of Ca^{2+} during migration. Interestingly, grafted NR1-/- subunit knockout cells are able to migrate, indicating that the NR1 subunit is not necessary for migration (Maskos and McKay, 2003). On the other hand, NR2B subunits are regulated by numerous trophic factors, and possess an intracellular tail that is known to be regulated by intracellular kinases (See Chapter One) (Vallano, 1998). Metzger et al. (Metzger et al., 2005) found that sustained expression of NR2B subunits in cerebellar slice cultures impaired granule cell migration and Purkinje cell maturation. However, direct and indirect effects of the sustained expression could not be distinguished. Thus, the specific function of the NR2B-containing NMDA receptors in migrating cerebellar granule cells remains unclear.

The objective of the present study was to determine whether inhibition of NR2B-containing NMDA receptors impairs cerebellar granule cell migration. Ifenprodil treatment was used to examine the function of the NR2B-containing receptors in granule cell migration. Ifenprodil is an NR2B subunit-specific partial antagonist that binds to a

polyamine regulatory site in the extracellular region of the subunit (Berger and Rebernik, 1999). Organotypic slice cultures of rat cerebellum were used as the model system. These cultures maintain cortical structure and cell-cell interactions that are similar to or the same as those which occur *in vivo*. This makes them an ideal model system for investigating receptor responses and Ca^{2+} regulation during migration. Pulse-chase labeling with BrdU was used to track the generation and migration of granule cells because BrdU is a marker for DNA synthesis. The results strongly indicate that the NR2B subunit is critical for cerebellar granule cell migration.

C) MATERIALS AND METHODS

Organotypic slice culture

Organotypic cultures were prepared according to previous reports (Komuro and Rakic, 1993; Haydar et al., 1999). The protocol was approved by the IACUC at Michigan State University. Briefly, sagittal slices of cerebellum were prepared from male and female 8-9 d old (P8-9) Charles River rat pups [Harlan, Verona, WI]. Slices were cut approximately 400 μm thick. Sagittal slices through the vermis were obtained to view all cerebellar cortical layers and to avoid monitoring cells migrating out of the section plane. Slices were cultured on porous, collagen-coated membranes [Transwell, Corning, Inc., Corning, NY] suspended in culture medium (Neurobasal medium supplemented with N-2 and B-27 [Invitrogen-Gibco, Carlsbad, CA], 90 U/ml penicillin, streptomycin, and gentamycin [Sigma-Aldrich, St. Louis, MO]) in 12-well culture plates [Corning, Inc., Corning, NY] with one slice per well. Cultures were incubated in BrdU [Molecular Probes, Eugene, OR] at 37°C in 5/95% CO_2/O_2 for 20 hrs.

Following incubation in BrdU, the medium was replaced with BrdU-free medium containing an NR2B-specific antagonist or the non-subunit-subtype specific antagonist D-APV [Sigma-Aldrich, St. Louis, MO]. The culture medium contained 0.5 μM ifenprodil (number of samples (n) = 3), 1.0 μM ifenprodil (n = 3), 50 μM D- APV (n = 3), or vehicle (control) (n = 5) for 7 d. Half the medium was removed and replaced with fresh medium daily. On day 8, cultures were fixed in 4% (w/v) formaldehyde ammonium bromide and incubated overnight in 1: 1000 AlexaFluor 546-tagged anti-BrdU rabbit IgG [Molecular Probes, Eugene, OR]. Cultured slices were mounted in Slow Fade® Light Antifade [Molecular Probes, Eugene, OR] on microscope slides and were examined using a Leica laser confocal microscope (Leica Microsystems Inc., Bannockburn, IL) with an

emitting laser wavelength of 543 nm at 70% power. A TD 488/543/633 filter was used, and wavelengths from 555-630 nm were collected. Fluorescence throughout the entire thickness of a 40x visual field was recorded using z-series stacks.

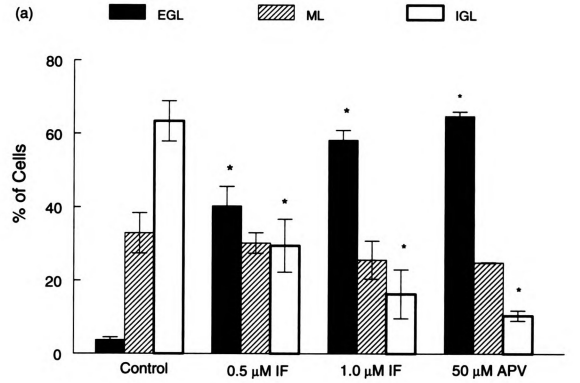
BrdU-labeled cells were identified by: 1) having an absolute intensity greater than 200, 2) having a diameter of at least 4.5 μm, and 3) having a circular or elipical shape. The number of BrdU-labeled cells in each of the external germinal cell layer, molecular layer, and internal granule cell layer within a defined square area was counted, and the percentage in terms of the total number of cells present in the viewed section of cortex was calculated. Treatment effects were tested using a one-way analysis of variance and Tukey-Kramer post-test for multiple comparisons. Data were considered significantly different when $p \leq 0.05$. Images in this dissertation are presented in color.

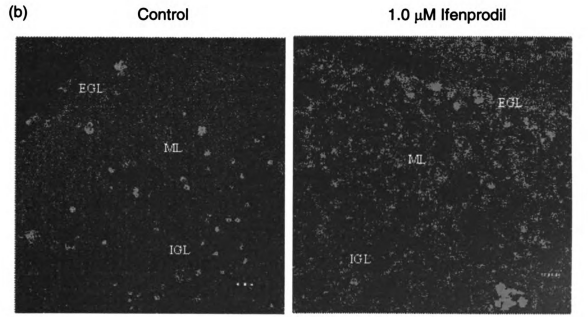
D) RESULTS & DISCUSSION

Cerebellar granule cell migration was impaired in cultures treated with ifenprodil or D-APV (positive controls) (Fig. 2.1.). Control cultures contained few to no cells in the external germinal cell layer at 8 DIV, similar to cortical organization at 16 days *in vivo* (Altman, 1972). Impairment of migration by D- APV is in agreement with previous reports on impairment induced by NMDA receptor antagonists (Rakic and Komuro, 1995). Migration into the internal granule cell layer was significantly impaired following treatment with 0.5 and 1.0 μM ifenprodil. Impairment was greater with 1.0 μM ifenprodil than with 0.5 μM ifenprodil, but there was no significant difference between the percent of migrating cells in the presence of 1.0 μM ifenprodil or 50 μM D- APV treatments. Since impairment by 1.0 μM ifenprodil is not significantly different from that caused by D- APV, it is unlikely that NMDA receptors which do not contain the NR2B subunit play an integral role in migration. The percent of granule cells observed in the external germinal cell layer was significantly increased by treatment with the antagonists. There was no significant difference between the percent of cells remaining in the external germinal cell layer following treatment with 1.0 μM ifenprodil and 50 μM D- APV. Persistance of granule cells in the external germinal cell layer and/or an abnormally thickened one in the cerebellum has previously been correlated with inhibition of migration *in vitro* (Sass et al., 2001). The presence of granule cells in the external germinal cell layer at 8 DIV was significantly increased by 1.0 μM compared to 0.5 μM ifenprodil. This concentration-dependent increase of neurons remaining in the external germinal cell layer distinctly suggests impaired migration from the external germinal cell

Figure 2.1. The NR2B subtype NMDA receptor antagonist, ifenprodil, impaired cerebellar granule cell migration. A. Slices were treated for 7 d with the NR2B-antagonist ifenprodil or the non-specific NMDA receptor antagonist D- APV. Inhibition of NMDA receptor function significantly impairs migration of CGC in organotypic slice cultures. Results are shown as the mean percent (±) SEM of BrdU-labeled granule cells in each cell layer. External germinal cell layer (EGL), molecular layer (ML), internal granule cell layer (IGL), ifenprodil (IF). * = $p \leq 0.05$ with respect to control data for corresponding cell layer). B. Laser confocal images of fluorescently-tagged BrdU-labeled granule cells in organotypic slice cultures of developing cerebellum taken following 7 d of treatment with 1.0 μM ifenprodil or control conditions.

Figure 2.1.


(a)
EGL
ML
IGL
% of Cells
80
60
40
20
0
Control
0.5 μM IF
1.0 μM IF
50 μM APV


(b)
Control
1.0 μM Ifenprodil
EGL
ML
IGL
EGL
ML
IGL

layer into the molecular layer. Fewer cells migrate from the external germinal cell layer, but the percent in the molecular layer does not change.

In conclusion, the results suggest that the NMDA receptors that are essential for granule cell migration contain at least one NR2B subunit. NR2B -containing receptors appear to be a main source of the Ca^{2+} oscillations and/or the regulation of cell movement involved in migration. The only other NR2 subunit previously shown to exist in migrating neurons is NR2A (Monyer et al., 1994; Watanabe et al., 1994), and the binding affinity of ifenprodil to NR1a/NR2B (IC_{50} = 0.34 μM) is 400 fold greater than its affinity to NR1a/NR2A (IC_{50} = 146 μM) (Williams, 1993). Hence, NR1a/NR2A receptors are not likely to be the source of Ca^{2+} oscillations during migration. However, the number of subunits in the NMDA receptors of immature cerebellar granule cells is not known. There may be co-assembly of more than one NR2B or an NR2A or D subunit in a small proportion of receptors (Fu et al., 2005).

CHAPTER THREE

Ca^{2+} SIGNALING IN IMMATURE CEREBELLAR GRANULE CELLS: THE $GABA_A$ RECEPTOR GATES OPENING OF THE N-TYPE VDCC, BUT NOT THE NMDA RECEPTOR

A) ABSTRACT

The migration of cerebellar granule cells is dependent on transient influxes of Ca^{2+} through N-type VDCC and the dually voltage- and ligand-dependent NMDA receptors. Both the NMDA receptor and N-type VDCC are activated by membrane depolarization. However, the factor(s) causing the membrane depolarization during cerebellar granule cell development is unclear. It has been previously reported that $GABA_A$ receptor stimulation triggers opening of VDCCs in all immature neuronal types studied thus far. The $GABA_A$ receptor was also suggested to activate the NMDA receptor in immature hippocampal pyramidal cells. In the present study, this phenomenon was tested in immature, non-synaptic cerebellar granule cells. Stimulation of the $GABA_A$ receptor by 50 μM muscimol increased intracellular Ca^{2+} ($[Ca^{2+}]_i$) by 50.07% ± 6 (mean ± SEM). The muscimol-induced increase in $[Ca^{2+}]_i$ was completely inhibited by the N-type VDCC blocker, ω -conotoxin GVIA, and reduced by the L-type VDCC blocker, nifedipine. It was not inhibited by the P/Q-type VDCC blocker, ω –conotoxin MVIIC, or the NR2B-containing NMDA receptor antagonist, ifenprodil. The results suggest that stimulation of the $GABA_A$ receptor opens N- and L-type VDCCs, but not P/Q- type VDCCs or NR2B-containing NMDA receptors. Pretreatment with thapsigargin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and/or oligomycin to deplete or block intracellular Ca^{2+} stores did not block the muscimol-induced increase in $[Ca^{2+}]_i$. Therefore, $GABA_A$ receptor stimulation is not dependent on a significant release of Ca^{2+} from intracellular stores of the smooth endoplasmic reticulum or mitochondria.

B) INTRODUCTION

Both the N-type VDCC and the NR2B-NMDA receptor are voltage-sensitive. The source of membrane depolarization that activates either of these channels in non-synaptic, migrating cerebellar granule cells is not clear, but may involve the $GABA_A$ receptor. In immature neurons, $GABA_A$ receptor stimulation is excitatory, in contrast to adult neurons in which $GABA_A$ receptors are inhibitory. $GABA_A$ receptor stimulation in immature neurons produces a fast excitatory response in many regions of the central nervous system, including cerebellum (Conner et al., 1987), hypothalamus (Obrietan and Van den Pol, 1995), hippocampus (Fiszman et al., 1999; Ben-Ari, 2002; Manent et al., 2005), cortex (Luhmann and Prince, 1991; LoTurco et al., 1995; Behar et al., 2000; Owens and Kriegstein, 2002), olfactory bulb (Serafini et al., 1995), and spinal cord (Wu et al., 1992; Rohrbough and Spitzer, 1996). The depolarization results from the Cl^- electrochemical gradient. Immature granule cells have a high intracellular Cl^- concentration due to delayed expression of the K^+-dependent Cl^- transporter. As such, the Cl^- is driven extracellularly through the open $GABA_A$ receptor– associated Cl^- channel, leading to depolarization.

The $GABA_A$ receptor-induced membrane depolarization causes large influxes of $[Ca^{2+}]_i$ mediated by VDCCs (Conner et al., 1987; Yuste and Katz, 1991; Leinekugel et al., 1995; LoTurco et al., 1995; Obrietan and Van den Pol, 1995). The role of $GABA_A$ receptor-mediated activation of VDCCs in neuronal migration is not clear. Inhibition of the $GABA_A$ receptor did not impair migration of neocortical neurons, but $GABA_C$ and $GABA_B$ receptors are important chemoattractant receptors during their migration (Behar et al., 2000; Ben-Ari, 2002; Owens and Kriegstein, 2002). The results are in concordance

with Komuro and Rakic's (1993) studies showing that bicuculline, a $GABA_A$ receptor antagonist, did not impair granule cell migration significantly in cerebellar slice cultures (Komuro and Rakic, 1993). The $GABA_A$ receptor-induced membrane depolarization may affect more than just VDCCs depending on neuron type and stage of development. It has been suggested that $GABA_A$ receptor stimulation causes voltage-dependent release of the Mg^{2+} from NMDA receptors during hippocampal pyramidal cell radial migration and post-migrational GDP-dependent maturation (Ben-Ari, 2002; Manent et al., 2005), possibly in a similar manner to that of the kainate/AMPA receptor in the mature brain.

The role of the NMDA receptor in cerebellar granule cell development may be more complex than that of the N-type VDCC. The NMDA receptor is both voltage- and ligand- gated as well as highly developmentally regulated by differential gene expression, neurotrophic factors and intracellular phosphorylation (Leonard and Hell, 1997; Takasu et al., 2002; Miyata et al., 2005). Migrating granule cells transiently express NMDA receptor subunit subtypes NR1a plus NR2B. NMDA receptors containing NR2B subunits have the highest affinity for the voltage-dependent blocker, Mg^{2+}, and display slow decay kinetics (Farrant et al., 1994; Vallano, 1998). The receptors receive tonic stimulation by endogenous ligands in acute cerebellar slices (Rossi and Slater, 1993; Farrant et al., 1994). NR2B-containing NMDA receptor-mediated glutamate-dependent influxes of Ca^{2+} are potentiated by the endogenous neurotrophic factor, EphrinB2/Fc (Takasu et al., 2002). The NR2B subunit appears to be critical for cerebellar granule cell migration (See Chapter Two).

The objective of the present study was to determine whether stimulation of the $GABA_A$ receptor causes an increase in $[Ca^{2+}]_i$ in the immature granule cells as well as to

determine the source of the $[Ca^{2+}]_i$. The hypothesis that $GABA_A$ receptor stimulation causes the N-type VDCCs and NR2B-containing NMDA receptors to open in early development was tested using acutely isolated slices of cerebellum from P9-11 rats. The slices were loaded with fluorescent Ca^{2+} -indicator dye, and Ca^{2+} imaging of granule cells in the inner external germinal cell layer and outer molecular layer was performed in the presence or absence of specific pharmacological probes to assess $GABA_A$ receptor response and interactions.

C) MATERIALS & METHODS

Acute slice preparations

Acute slice preparations of cerebellum were used to investigate changes in $[Ca^{2+}]_i$ among the developing cortical layers in response to receptor manipulation. Cerebella from male and female 8-11 d old (P8-11) Charles River rat pups [Harlan, Verona, WI] were isolated in cold artificial cerebrospinal fluid (ACSF). Sagittal slices of cerebellar vermis were cut 250 μm thick using an OTS-3000-05 FHC Vibratome [Vibratome, Brunswick, ME]. The slices were loaded with Fluo-4, AM ($K_{d\ (Ca2+)}$ = 345 nM) and Fluo-5F, AM ($K_{d\ (Ca2+)}$ = 2.3 μM) [Molecular Probes, Eugene, OR] (1 μM dissolved in 0.01 DMSO, 20% (w/v) plus pluronic acid [Sigma-Aldrich, St. Louis, MO]) for 1 hr. Slices were then transferred to a continuous perfusion chamber [RC-27 Warner Instruments Corporation, Hamden, CT] mounted on the stage of an upright TSL Leica laser confocal microscope [Leica Microsystems Inc., Bannockburn, IL]. A z-series stack of optical frames through the depth of several cells was collected from each visual field through a 63x water immersion objective. The emitting laser wavelength was 488 nm set at a power of 30 %. A long pass 500 nm filter was used, and fluorescence at wavelengths from 500-550 nm were collected. In each experimental condition described below, an averaged 2-dimensional image of the z-series stack was used for analysis. The average intensity for the fluorescence in granule cells in each layer at each interval was normalized to that of the cells prior to treatment. The averages of relative fluorescence intensities from 3-5 different slices per drug treatment were plotted and compared using a two-way analysis of variance with a Tukey-Kramer post-test. $P \leq 0.05$ was considered significant.

Pharmacological applications

Control slices were continuously perfused with ACSF only. Pulses of 50 or 100 μM muscimol or 10 μM bicuculline were used to stimulate or inhibit, respectively, the $GABA_A$ receptor. Ifenprodil (3.0 μM) or ephrinB2/Fc (0.75 μg/ml) were used to partially inhibit or potentiate, respectively, the glutamate-stimulated NR2B-containing NMDA receptor mediated response. N-, L-, and P/Q-type VDCCs are present in mature cerebellar granule cells (Pearson et al., 1995; Randall and Tsien, 1995). Responses and interactions of Ca^{2+} sources through the N-, L-, and P/Q-type VDCCs were assessed by pretreatment with 1.0 μM ω-conotoxin GVIA, 1.0 μM nifedipine, or 1.0 μM ω-conotoxin MVIIC, respectively. Ca^{2+} from the SER or mitochondria were assessed by pretreatment with 5.0 μM thapsigargin, or 5.0 μM CCCP to uncouple oxidative phosphorylation and 10.0 μM oligomycin to dissipate mitochondrial membrane potential, respectively [Sigma-Aldrich, St. Louis, MO]. Each pharmacological condition was tested on three cultures (one slice per culture). Each culture was from a different brain.

D) RESULTS

A 30 s pulse of 50 μM muscimol increased $[Ca^{2+}]_i$ levels by an average of 50.07% ± 6 (mean ± SEM) in granule cells of the inner external germinal cell layer and outer molecular layer. The response differed from that of granule cells of the internal granule cell layer in which $[Ca^{2+}]_i$ did not increase following muscimol treatment (See Chapter Five). Bicuculline alone did not significantly change $[Ca^{2+}]_i$ (Fig. 3.1). Application of 50 μM muscimol had no effect on $[Ca^{2+}]_i$ in the presence of bicuculline (Fig. 3.1), suggesting that bicuculline blocked stimulation of the $GABA_A$ receptor and subsequent influx of Ca^{2+}.

To assess N-type VDCCs response to $GABA_A$ receptor-induced membrane depolarization, 1.0 μM ω-conotoxin GVIA was applied to cerebellar slices prior to muscimol treatment (Fig. 3.2). Ω-conotoxin GVIA alone significantly decreased $[Ca^{2+}]_i$ by an average of 30.5% ± 6 (Fig. 3.3). Application of muscimol in the presence of ω-conotoxin GVIA decreased $[Ca^{2+}]_i$ to a level (36.0% ± 4) that was not significantly different from that of ω-conotoxin GVIA alone (Fig. 3.3). The results suggest that blocking the N-type VDCC inhibited a muscimol-induced increase in $[Ca^{2+}]_i$. Treatment with nifedipine alone did not significantly alter $[Ca^{2+}]_i$, but application of 1.0 μM nifedipine followed by muscimol increased $[Ca^{2+}]_i$ by 10.5% ± 0.5 (Fig. 3.4.), suggesting that blocking L-type channels may have partially inhibited the muscimol-induced increase in $[Ca^{2+}]_i$. Treatment with ω-conotoxin MVIIC alone did not significantly alter $[Ca^{2+}]_i$, nor did 1.0 μM ω-conotoxin MVIIC treatment block the muscimol-induced increase in $[Ca^{2+}]_i$ (Fig. 3.4.). Thus, there is tonic N-type, but no L- or P/Q- type VDCC activity in the inner external germinal cell layer and outer molecular layer within slices,

Figure 3.1.

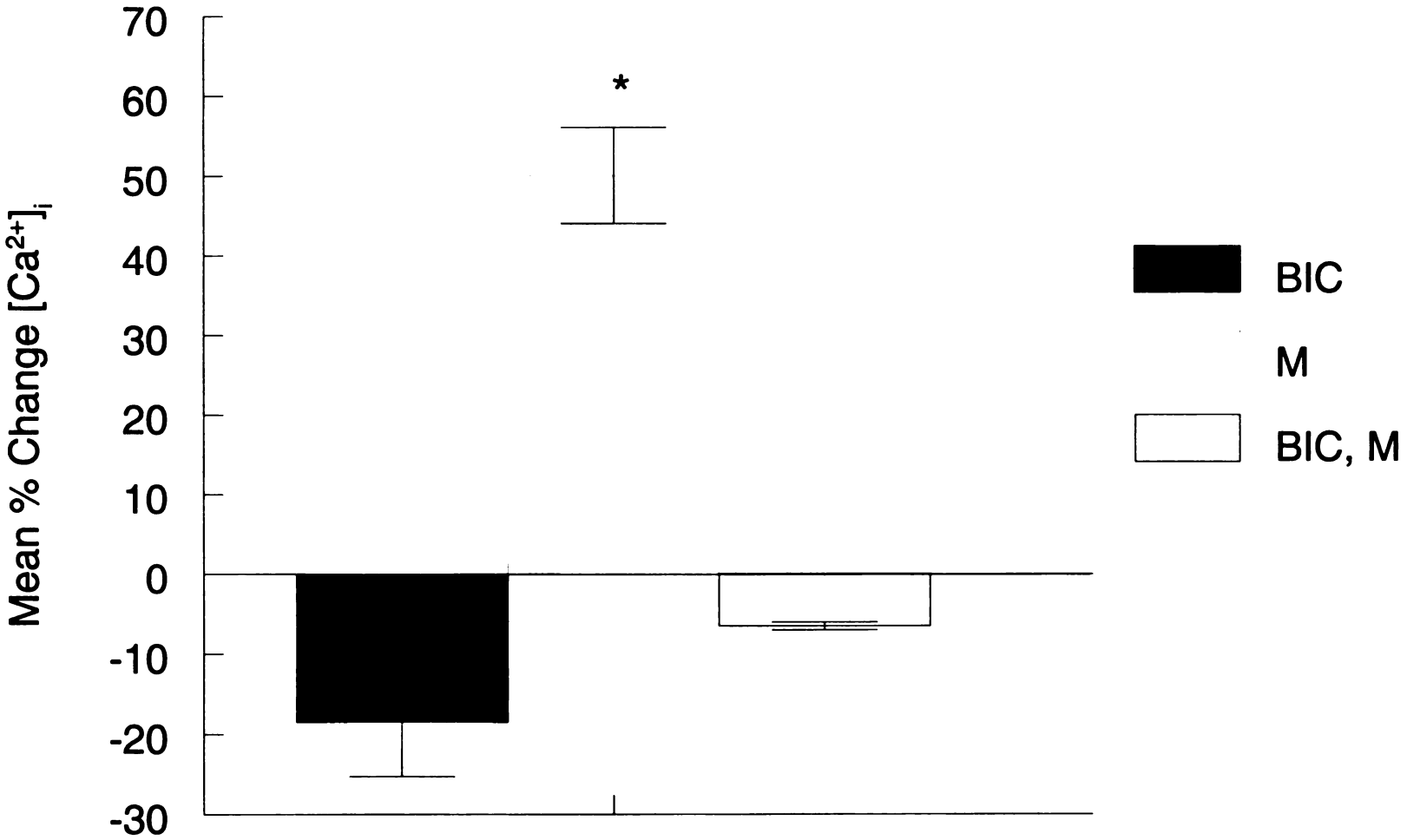


Figure 3.1. The mean percent change of $[Ca^{2+}]_i$ in granule cells of the inner external germinal cell layer and outer molecular layer in response to muscimol (M), bicuculline (BIC), and bicuculline followed muscimol (BIC, M) is shown. * = $p \leq 0.05$ with respect to control conditions

Figure 3.2.

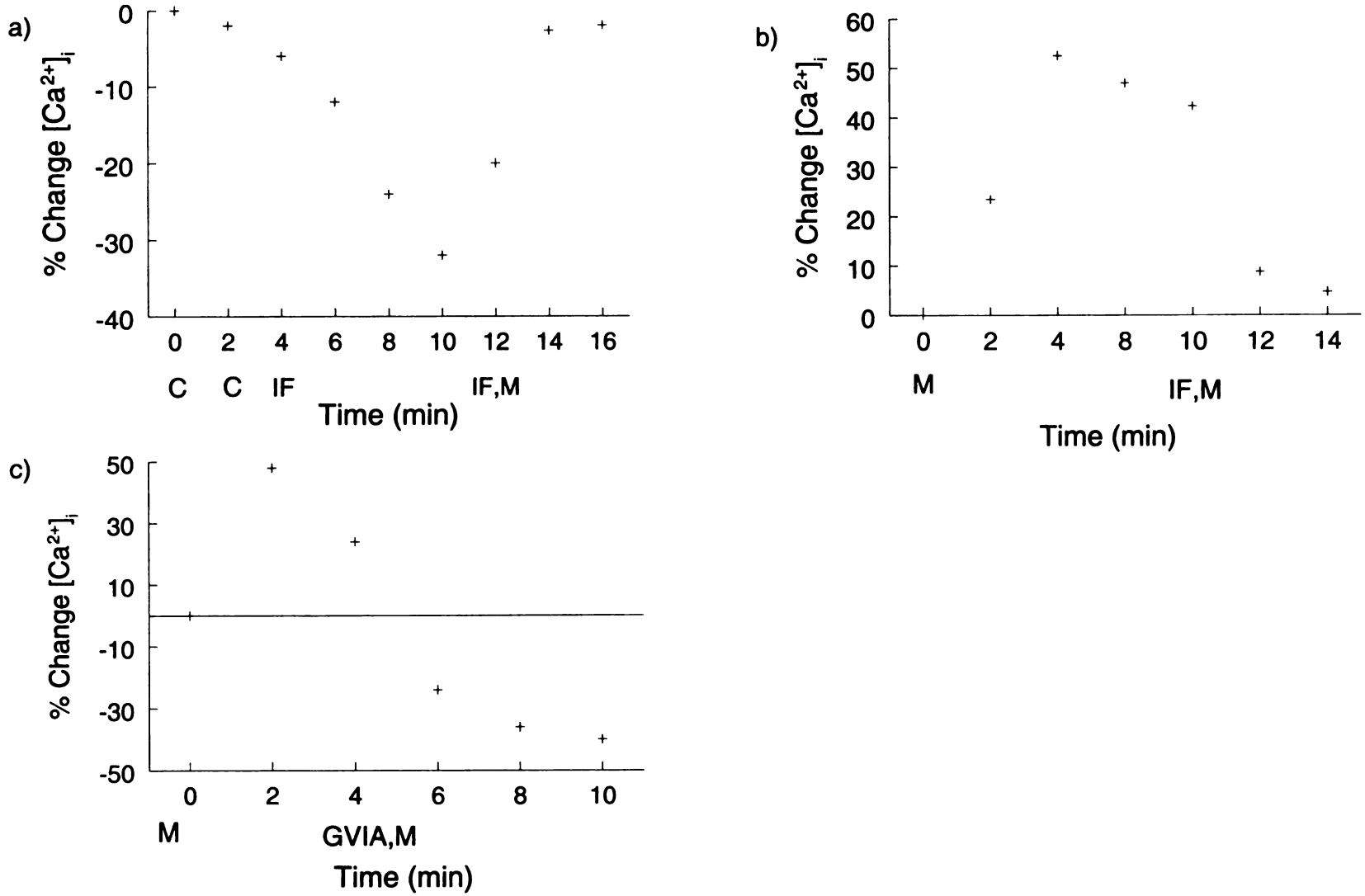

a)
% Change $[Ca^{2+}]_i$
0
-10
-20
-30
-40
0 2 4 6 8 10 12 14 16
C C IF IF,M
Time (min)
b)
% Change $[Ca^{2+}]_i$
60
50
40
30
20
10
0
0 2 4 8 10 12 14
M IF,M
Time (min)
c)
% Change $[Ca^{2+}]_i$
50
30
10
-10
-30
-50
0 2 4 6 8 10
M GVIA,M
Time (min)

Figure 3.2. Application of ω-conotoxin GVIA, but not ifenprodil inhibited the muscimol-induced increase in $[Ca^{2+}]_i$ in non-synaptic, immature cerebellar granule cells. Acute slice preparations of developing cerebellar vermis were loaded with Fluo4-AM Ca^{2+} indicator dye. Continuous 2 min interval measurements of the percent change in Fluo-indicated Ca^{2+} fluorescence within cerebellar granule cells of the inner EGL and outer ML were determined using a Leica laser confocal fluorescent microscope and plotted over time. Changes in $[Ca^{2+}]_i$ within individual slices treated with a) ifenprodil (IF) followed by muscimol (M), b) muscimol followed by ifenprodil, or c) muscimol followed by ω - conotoxin GVIA (GVIA) and muscimol over time are depicted compared to baseline control (C).

Figure 3.3

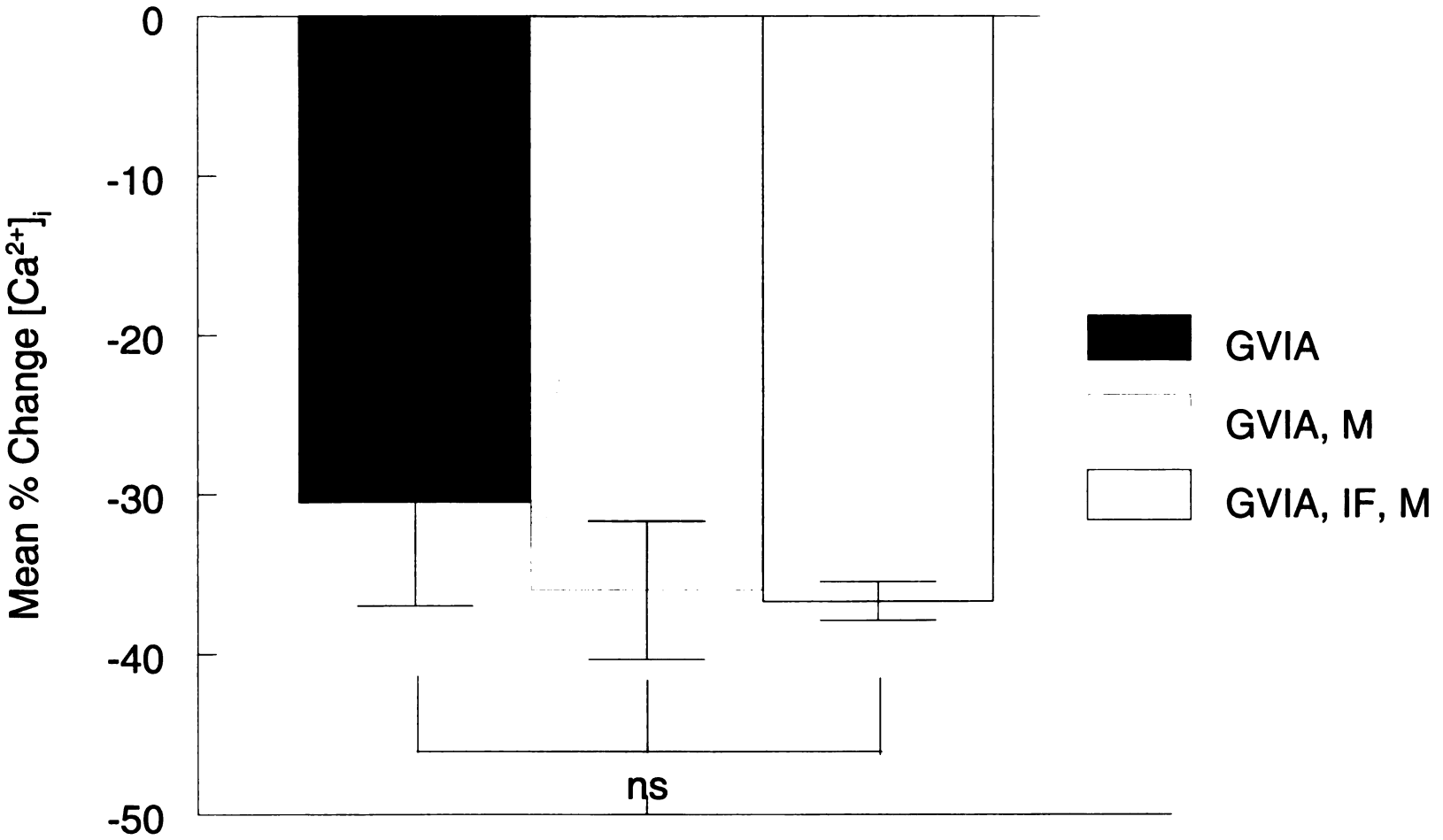


Figure 3.3. The mean percent change of $[Ca^{2+}]_i$ in granule cells of the inner external germinal cell layer and outer molecular layer in response to ω -conotoxin GVIA (GVIA), ω -conotoxin GVIA followed by muscimol (GVIA, M), or ω -conotoxin GVIA and ifenprodil (IF) followed by muscimol (GVIA, IF, M) is shown. * = $p \leq 0.05$ with respect to control conditions

and $GABA_A$ receptor-induced depolarization activates N- and possibly L- type VDCC, but not P/Q-type.

Ifenprodil and ephrinB2/Fc were used to study the presence of and tonic activity of NR2B-containing NMDA receptors. Ifenprodil significantly reduced $[Ca^{2+}]_i$ both in the presence and absence of ephrinB2/Fc (Fig. 3.5). Treatment with 3.0 μM ifenprodil decreased $[Ca^{2+}]_i$ by 31.8% ± 9 (Fig. 3.5). EphrinB2/Fc, alone, significantly increased $[Ca^{2+}]_i$ (Fig. 3.5). Thus, there is tonic activity of the NR2B-containing NMDA receptors that can be enhanced by ephrinB2/Fc. Pretreatment with bicuculline did not prevent ephrinB2/Fc-induced enhanced opening of the NMDA receptor. Therefore, blockade of the $GABA_A$ receptor has no significant effect on ephrinB2/Fc-mediated receptor potentiation. The hypothesis that $GABA_A$ receptor stimulation gates the activation and/or opening of the NR2B-containing NMDA receptors was tested (Figs. 3.2 and 3.5). However, ifenprodil does not appear to block a muscimol-induced increase in a $[Ca^{2+}]_i$. The combined effect of ifenprodil plus muscimol led to a $[Ca^{2+}]_i$ of 2.11% ± 2 (Fig. 3.5). Following treatment with both ω-conotoxin GVIA and ifenprodil The $[Ca^{2+}]_i$ decreased and prevented a muscimol-induced increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ in cells treated with ω-conotoxin GVIA and ifenprodil, and then muscimol was not significantly altered from that observed with either antagonist alone (Fig. 3.3).

To eliminate intracellular Ca^{2+} stores as a source of Ca^{2+} following pulses of muscimol, slices were treated with oligomycin, thapsigargin, and/or CCCP prior to muscimol. Pretreatment to deplete and/or block intracellular Ca^{2+} stores did not block the muscimol-induced increase in $[Ca^{2+}]_i$ (Fig. 3.4). The results indicate that the muscimol-induced increase in $[Ca^{2+}]_i$ is not dependent on release of Ca^{2+} from intracellular stores.

Figure 3.4.

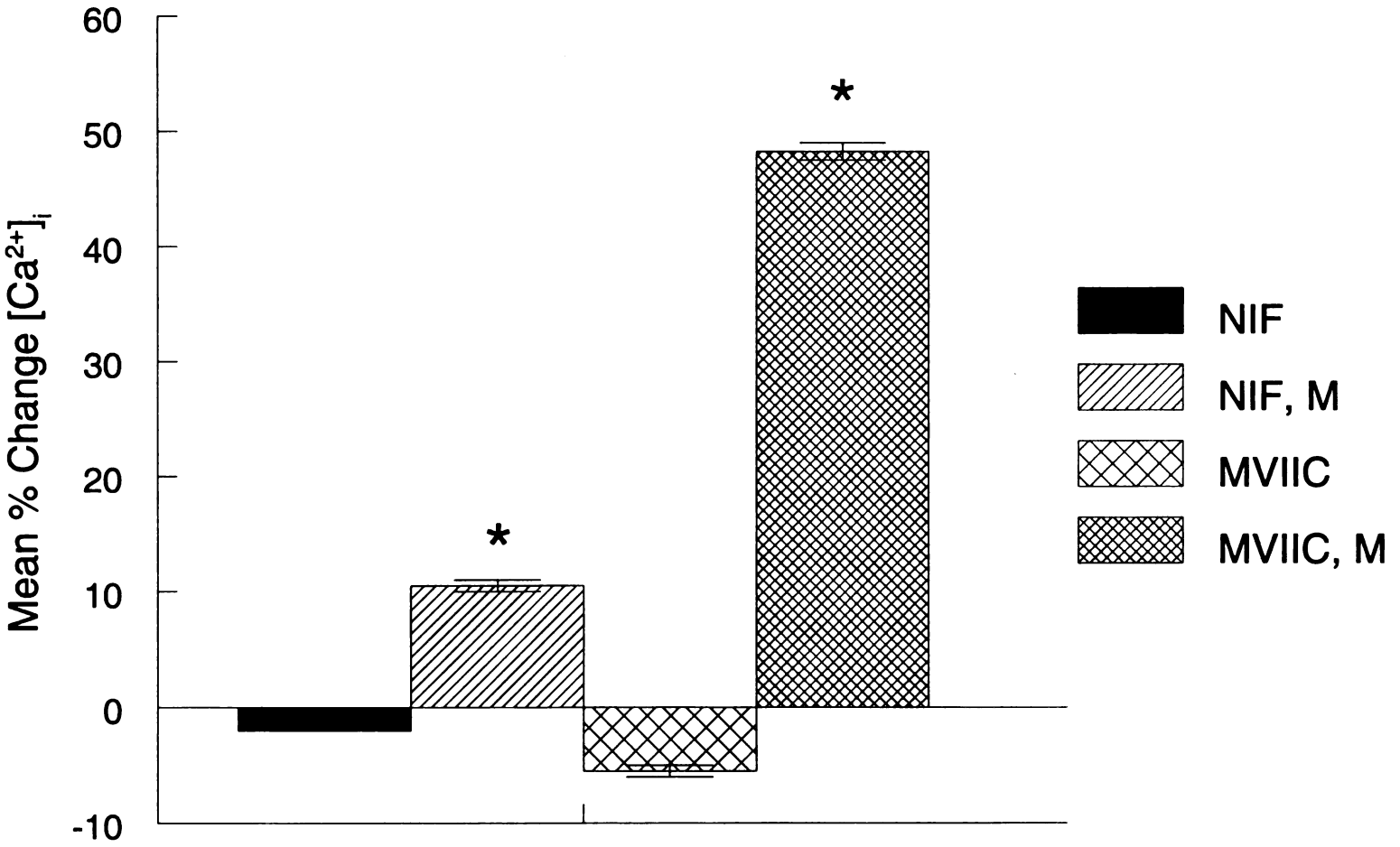


Figure 3.4. The role of L- and P/Q- type VDCCs in $[Ca^{2+}]_i$ following application of muscimol in non-synaptic, immature granule cells. Acute slice preparations of developing cerebellum were loaded with Ca^{2+} -indicator dye. The mean percent change of $[Ca^{2+}]_i$ in granule cells of the inner external germinal cell layer and outer molecular layer in response to the antagonists for P/Q -type VDCCs, ω -conotoxin MVIIC (MVIIC), or L-type VDCCs, nifedipine (NIF), and antagonist treatment before muscimol (M) application is depicted. * = $p \leq 0.05$ with respect to control.

Figure 3.5.

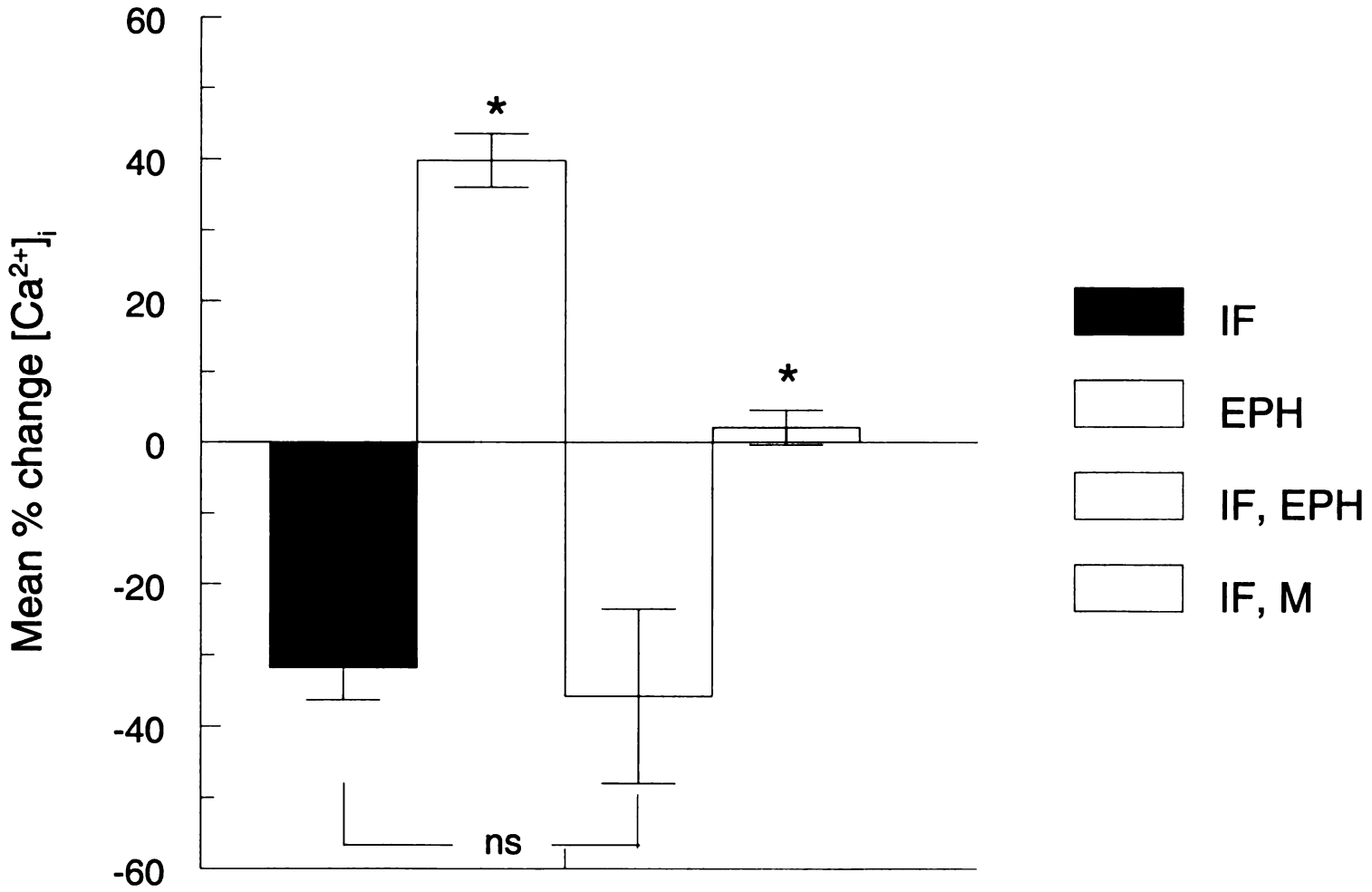


Figure 3.5. The mean percent change of $[Ca^{2+}]_i$ in granule cells of the inner external germinal cell layer and outer molecular layer in response to ifenprodil (IF), ephrin2Bc/Fc (EPH), ifenprodil followed by ephrin2Bc/Fc (IF, EPH), or ifenprodil followed by muscimol (IF, M) is shown. * = $p \leq 0.05$ with respect to control conditions

Figure 3.6.

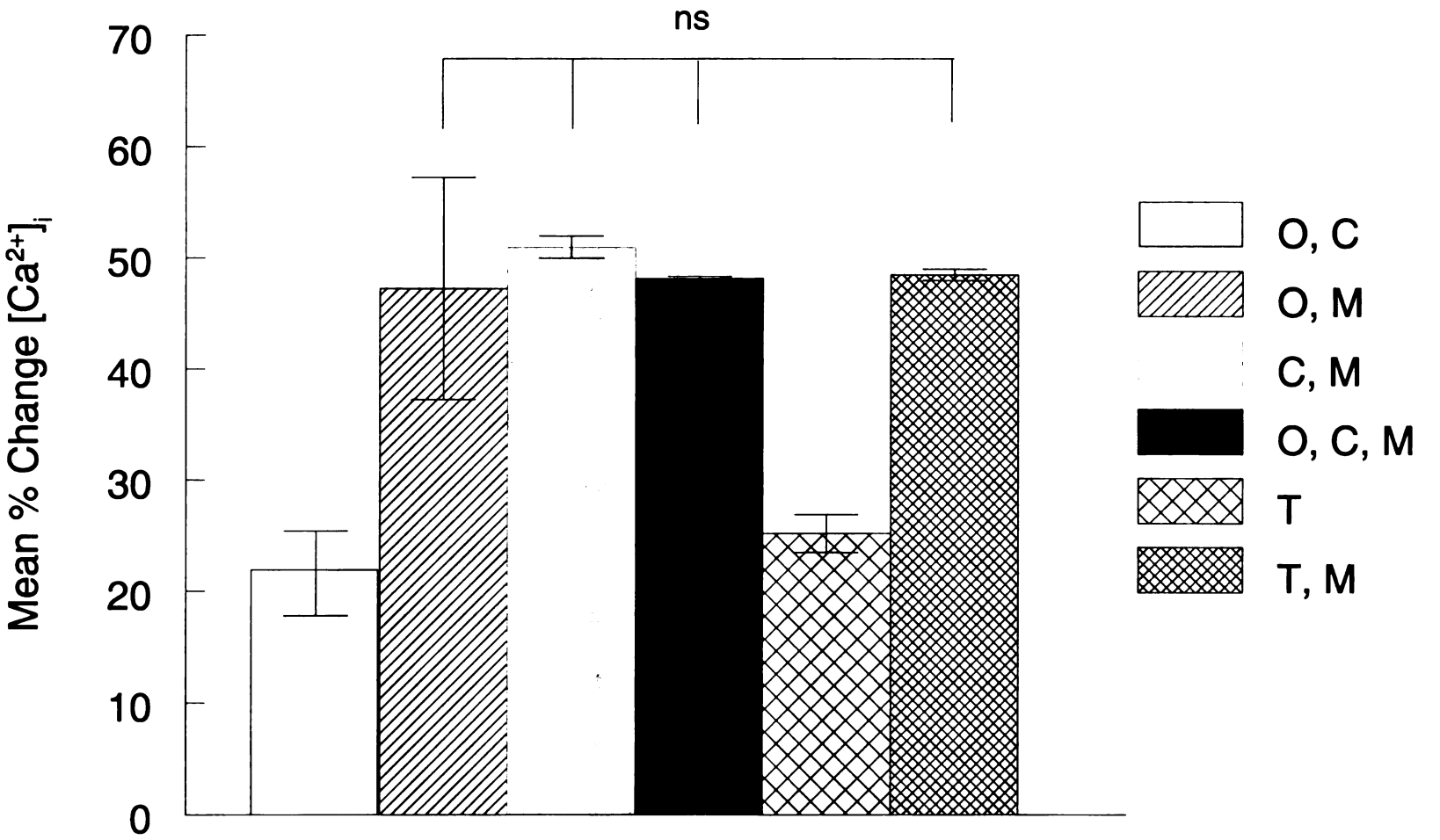


Figure 3.6. The role of intracellular stores of Ca^{2+} in responding to muscimol application in non-synaptic, immature cerebellar granule cells. Acute slice preparations of developing cerebellum were loaded with Ca^{2+} -indicator dye. The mean percent change of $[Ca^{2+}]_i$ in granule cells of the inner external germinal cell layer and outer molecular layer during treatment with 5.0 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to uncouple oxidative phosphorylation and 10.0 μM oligomycin (OLIGO) to dissipate mitochondrial membrane potential is shown as well as that during treatment with 5.0 μM thapsigargin (THAPS), which depletes intracellular Ca^{2+} stores. The mean percent change of $[Ca^{2+}]_i$ in immature cells in response to muscimol (M) following depletion of intracellular stores by OLIGO, CCCP, and/or THAPS is depicted. The results of muscimol treatment following depletion of intracellular stores were not significantly different from treatment with muscimol alone. ns: not significantly different.

E) DISCUSSION

As reported in previous studies of immature neurons, stimulation of the $GABA_A$ receptor increases $[Ca^{2+}]_i$; the main source of the Ca^{2+} appears to be the N-type VDCC. This result supports the hypothesis that the $GABA_A$ receptor modulates the $[Ca^{2+}]_i$ oscillations that are necessary for cerebellar granule cell migration. A portion of the muscimol-induced $[Ca^{2+}]_i$ increase originates through the L-type VDCC. Previous reports suggest that $GABA_A$ receptor stimulation may activate L-type VDCC in growth cones (Borodinsky, 2003). The $[Ca^{2+}]_i$ increase also occurred following depletion of and/or blocked release of Ca^{2+} from the SER and mitochondria indicating that the $GABA_A$ receptor evoked little or no release of Ca^{2+} from intracellular sources.

The $GABA_A$ receptor does not appear to affect significantly the NR2B subunit subtype containing NMDA receptors in developing, non-mitotic, non-synaptic granule cells. $GABA_A$ receptor activation may not have removed the Mg^{2+} block or the two receptors may not be co-localized because blocking NR2B-containing receptors did not prevent the muscimol-induced increase in $[Ca^{2+}]_i$. The source of activation of the NR2B subtype is not clear, however, ephrinB2/Fc application did potentiate the receptors. Cambray et al. (1990) showed that immature granule cells of the external germinal cell layer and outer molecular layer do not express KA/AMPA receptors, which usually activate the NMDA receptor in mature neurons by membrane depolarization (Cambray-Deakin et al., 1990). The combined results suggest that neurotrophic factors other than GABA may be removing the Mg^{2+} block, thereby activating the receptor and allowing the paracrine released glutamate to open the associated channel. GABA as a neurotrophic factor could still affect the NMDA receptors indirectly as a result of second messengers

or associated channels of the $GABA_B$ receptor (Komuro and Rakic, 1993; Van den Pol et al., 1998) or BDNF (Miyata et al., 2005) or altering gene expression.

The present results are in concordance with previous studies showing that VDCC blockers alone or NMDA receptor antagonists alone impair the Ca^{2+} -oscillations of migration, but that bicuculline did not (Komuro and Rakic, 1993). In addition, the dynamics between the two receptors in response to $GABA_A$ receptor stimulation may change as the granule cells develop immature synapses.

CHAPTER FOUR

CONTINUOUS EXPOSURE TO LOW CONCENTRATIONS OF METHYLMERCURY IMPAIRS CEREBELLAR GRANULE CELL MIGRATION IN ORGANOTYPIC SLICE CULTURE

A) ABSTRACT

Chronic, low-level MeHg exposure in children has been associated with cognitive and motor deficits are associated with cerebellar dysfunction. Neuropathological studies suggest that these deficits result from impaired cerebellar granule cell migration in humans. The majority of cerebellar granule cell migration in rats occurs during postnatal days 8 to 14 (P8 to P14); Migration peaks on P10 and P11. Although neuronal migration *in vivo* and *in vitro* has been shown to be impaired during acute and/or high level exposure to MeHg, the cellular effects of chronic exposure to submicromolar and micromolar levels of MeHg during development are not clear. In the present study, organotypic cultures of parasagittal slices of cerebellum from P8 rat were exposed to 0.0, 0.2, 0.5, 1.0, 3.0, 5.0, and10 μM MeHg for 3 or 7 days; both granule cell viability and migration were assessed. Culture viability declined significantly to 80.4 % ± 1 (mean ± SEM) in 3.0 μM MeHg after 3 days. Cultures treated with 1.0 μM MeHg for 7 days showed a significant decline in granule cell viability (73.1 % ± 4). Therefore, the concentration at which granule cell death occurred during continuous MeHg exposure appeared to be time and concentration- dependent. Granule cell migration was assessed by BrdU pulse-chase labeling. Migration was impaired in cultures exposed to 3.0 μM MeHg for 3 days. A significant decline in migration was observed in cultures exposed to 1.0 μM MeHg for 7 days. Granule cells remained in the external germinal cell layer of cultures treated with 0.5 μM MeHg for 7 days, suggesting that MeHg impairs cerebellar granule migration in a time-dependent manner at lower concentrations than are required to cause cell death. The results suggest that chronic, submicromolar levels of MeHg significantly impair the development of the cerebellar cortex in organotypic culture.

B) INTRODUCTION

MeHg is derived from methylation of elemental mercury released by combustion of fossil fuels as well as by agriculture, paper, lumber, and leather industries, gold-mining and refining, and manufacturing of electrical equipment and paint. Elemental mercury is methylated by aquatic and soil microorganisms (Chang and Verity, 1995). The lipophilic nature of MeHg allows it to diffuse through the blood brain barrier more easily than other heavy metals, making it more effectively neurotoxic (Aschner and Aschner, 1990; Evans, 2002; Limke et al., 2004a). MeHg crosses the placenta, and accumulates to 30% more MeHg in erythrocytes of fetal blood than in those of maternal blood (Kuhnert et al., 1981). In addition, the concentration of MeHg in fetal brain has been shown to be more than twice that of their mothers. Infants may also receive substantial exposure from ingesting breastmilk (Amin-Zaki et al., 1976).

Studies following incidences of epidemic high levels of MeHg exposure in Minamata, Japan in the 1950's and in Iraq in 1972-3 have established that the cerebellum is among the primary targets of acute and high-level exposure to MeHg. Effects associated with overt clinical manifestations include cerebellar-based ataxia, blindness, and other sensory and motor deficits (Berlin, 1976; Chang et al., 1977; Takeuchi, 1982; Castoldi, 2000; Limke et al., 2004a). The cerebellar-based ataxia appears to be a direct result of cerebellar granule cell death, even though the highest concentrations of MeHg are found in the Purkinje cells (Hunter and Russell, 1954; Chang et al., 1977). In the developing cerebellum, high levels of MeHg also impair granule cell migration and proper laminar cortical organization *in vivo* (Rustam and Hamdi, 1974; Reuhl and Chang, 1979a; Reuhl and Chang, 1979b; Choi, 1989; Limke et al., 2004a). However, acute

exposure to high-levels of MeHg is not as common among humans today as is chronic exposure to low concentrations of MeHg.

Chronic, low-level MeHg intake occurs mainly through regular ingestion of contaminated seafood. At low-level concentrations, overt clinical symptoms have not been observed in adults (Harada, 1978; Reuhl and Chang, 1979a; Sakamoto et al., 1998; Miyamoto et al., 2001); subtle behavioral abnormalities occur in children exposed chronically to concentrations that do not result in abnormalities in similarly exposed adults (Bakir et al., 1980; Takeuchi, 1982; Grandjean et al., 1997; Grandjean et al., 1998; Grandjean et al., 1999). Studies of children from communities having seafood as a major source of food such as the Great Lakes Region (Gilbertson, 2004), Amazon basin (Lebel et al., 1998; Grandjean et al., 1999; Santos et al., 2002), Faroe Islands (Weihe et al., 1996; Grandjean et al., 1997; Grandjean et al., 1998), Seychelles (Myers et al., 1997; Myers and Davidson, 1998; Palumbo et al., 2000), as well as Greenland's Inuit (Hansen, 1990; Dewailly et al., 2001) and North American native (Dellinger et al., 1996) children suggest that neuropsychological deficits in language, motor function, attention, memory, and visuospatial performance are decrementally correlated with hair-mercury levels as low as 3 μg/g body weight (Grandjean et al., 1997; Grandjean et al., 1998; Grandjean et al., 1999). It is unclear if other toxicants in seafood plays a role in these deficits. The neuropsychological deficits are directly or indirectly attributable to cerebellar dysfunction (Schmahmann, 1997; Cook et al., 2004). Although, significant impairment of cerebellar granule cell migration in the developing brain has been shown in primary culture (Sass et al., 2001) and in organotypic slice culture (Kunimoto and Suzuki, 1997) under acute and/or high-levels of MeHg exposure, pregnant or breastfeeding mothers ingesting fish

regularly expose their off-spring to low-levels of MeHg throughout cerebellar development.

The purpose of this study was to determine the effect of subchronic, micro- or submicromolar levels of MeHg on immature cerebellar granule cell viability and migration. Organotypic slice cultures were used in this study because they maintain *in vivo*-like cortical structure, cell-cell interactions, and allow for treatment with MeHg throughout the developmental process. Slices for the cultures were obtained from cerebellar vermis of P8-9 rat pup because granule cell mitosis is prevalent in P8 and 9 pups, and granule cell migration peaks at P10 and 11 (Altman, 1972; Kunimoto and Suzuki, 1997). Granule cell migration in rats occurs postnatally over approximately 7 days (Altman, 1972). Four clearly recognizable layers can be seen from P9-12 (Komuro et al., 2001; Davids et al., 2002). During this time, granule cells are in all stages of development.

Pulse-chase labeling with BrdU was used to track granule cell generation and migration. BrdU is substituted for thymidine in mitotic cells during DNA replication in the external germinal cell layer and therefore, permanently labels the granule cells for tracking during subsequent migration (Kunimoto and Suzuki, 1997). No other neurons are known to be generated in the cerebellar cortex postnatally. D-APV is a non-specific (i.e. NR1) NMDA receptor antagonist used as a positive control in this study; other non-subtype specific NMDA receptor antagonists have been shown to impair migration, but not inhibit it completely (See Chapter Two). The results of the present study show that exposure to chronic, submicromolar MeHg impairs cerebellar granule cell migration without causing significant cell death.

C) MATERIALS & METHODS

Organotypic slice culture

The organotypic cultures were prepared in the same way as in Chapter Two with slight changes. Cultures were incubated in BrdU at 37°C in a humidified Isotemp incubator [Fisher Scientific, Asheville, NC] with CO_2/O_2 (5%/95%) for 20 hrs. Beginning on day *in vitro* (DIV) 2, the culture medium used contained only 33% of the concentration of the antibiotics (30 U/ml streptomycin, 30 U/ml penicillin, and 30 U/ml gentamycin). These slices maintained viability for over 8 days.

Viability

The viability of slice cultures exposed to 0, 0.2, 0.5, 1.0, 3.0, 5.0, and 10.0 μM MeHg [Aldrich Chem, Co., Milwakee, WI] was evaluated to ascertain a concentration range which did not induce cell death. The MeHg was added to the culture medium beginning at 20 hrs *in vitro*. This time point was chosen for comparison to the BrdU-labeled cultures described later in the migration assay. Half of the culture medium was replaced each day with MeHg-containing medium.

Viability of the slice cultures after MeHg incubation was assessed by co-labeling with fluorescent, membrane-permeable calcein AM to stain live cells, and membrane-impermeant ethidium homodimer to stain dead cells. On DIV 4 and DIV 8, 3 slices treated with each MeHg concentration were incubated in both 4 μM ethidium homodimer-1 and 2 μM calcein AM [Molecular Probes, Eugene, OR] for 45 min.

Fluorescent images were collected using a TSL Leica laser confocal microscope [Leica Microsystems Inc., Bannockburn, IL] as a z-series at 40x and analyzed using a cytofluorogram analysis software package from Leica Microsystems. The cytofluorogram calculates several aspects of the recorded images including the sum of the

intensity of each fluorophore as well as the mean intensity. The ratio of live tissue versus dead tissue was calculated from the sum of the intensities of ethidium-labeled dead and calcein-labeled live cells above the background threshold.

Nissl staining

Organotypic slice cultures of cerebellum were incubated in medium as described above for 8 days. The slices were then fixed for 30 min at room temperature in formalin-ammonium bromide 4% (w/v), which doesn't cause molecular cross-linking or form precipitates, and dehydrated in sequentially increasing concentrations of ethanol. Slices were then cleared in xylene. Cultures were embedded in paraffin, sectioned 20 μm thick, placed on gelatin-coated slides, and dried overnight on a hot-plate. The tissue was cleared of paraffin using xylene, and then ethanol, and subsequently stained with cresyl violet. The maintenance of cortical structure in the sections was assessed.

MeHg-induced impairment of migration

BrdU pulse-chase labeling was used to track the granule cells. After 20 hrs, the medium was replaced with BrdU-free medium, and the cultures were maintained in the incubator for 3 or 7 days. The medium for each slice contained either 0 (n = 5), 0.2 (n = 3), 0.5 (n = 4), 1.0 (n = 4), 3.0 (n = 4), or 5.0 (n = 4) μM MeHg, or 50 μM D-APV (n = 3) as a positive control. At the end of DIV 4 or 8, the slices were fixed in formalin-ammonium bromide 4% (w/v) for 30 min at room temperature. The slices were incubated in primary mouse anti-BrdU IgG [1:500] for 72 hrs, and then fluorescein-tagged goat anti-mouse secondary IgG [1:1000] for 2 hrs [Roche Diagnostics Corp., Indianapolis, IN] to visualize BrdU-labeled cells. Cultured slices were mounted in Slow Fade® Light Antifade [Molecular Probes] on microscope slides and fluorescent imaging

of stained sections was performed using the TSL Leica laser confocal microscope with an emitting laser wavelength of 488 nm at a power of 30%, a long pass 500 nm filter, and collection wavelength range of 500-550 nm.

Fluorescence was recorded throughout the full thickness of a 40x visual field using z-series stack. The percent of labeled cells in each layer out of the total number of cells within the section of cortex viewed was calculated the same way as in Chapter Two. Impairment of granule cell migration in organotypic cultures due to continuous exposure to MeHg was evaluated to determine the extent of impairment of granule cell migration. A concentration-response curve was plotted to determine if the number of granule cells which migrate to their final destination declined when cultures were treated with MeHg. The replication number is shown in Table 4.1. Differences in the percent of cells observed in each cortical layer compared among MeHg concentrations was tested using a one-way analysis of variance with a Tukey Kramer post-test of multiple comparisons. Data were considered significantly different when $p \leq 0.05$. Images in this dissertation are presented in color.

D) RESULTS

Viability

Cell viability following 3 and 7 days (d) of MeHg treatment is shown in Figure 4.1. There were no significant changes in viability over time in the absence of MeHg. A significant decline in viability was evident with 5 μM MeHg treatment for 3 d. Nearly all cells were dead after 3 d at 10 μM MeHg. However, viability began to decline significantly at 1.0 μM MeHg when cultures were exposed for 7 d compared to controls and to 3 d MeHg cultures treated with 1.0 μM MeHg.

In addition to the viability of cells in the organotypic cultures, the maintenance of cortical structure was evaluated. The survival and structural integrity of Purkinje cells is an indicator of cytoarchitectural maintenance and quality in the cerebellar cortex (Davids et al., 2002). Thionine (Nissl) staining was used to visualize the external germinal cell layer, molecular layer, Purkinje cell layer, and internal granule cell layer. The Purkinje cell layer was present in the cultures at 8 DIV, with cell morphology consistent with the Purkinje cell layer in Nissl-stained acutely obtained brain slices.

MeHg-induced impairment of migration

In the absence of MeHg, 75.2% of the BrdU-labeled cells migrated from the external germinal cell layer following 3 d of treatment. The positive control (block of NMDA receptors) yielded a very low percentage of migration (Table 4.1). Overall migration into the molecular layer or internal granule cell layer following treatment with MeHg for 3 d significantly declined in 3.0 μM MeHg. These results are comparable to those reported by Kunimoto and Suzuki (Kunimoto and Suzuki, 1997). However, cultures treated for 7 d had a more dramatic impairment at lower concentrations. In both

the 3 and 7 d MeHg treatments, migration decreased in a concentration-dependent manner (Table 4.1).

The average percent of granule cells that migrated to each cortical layer is depicted in Figure 4.2. Migration into the internal granule cell layer within cultures treated for 3 d MeHg was impaired at 3.0 μM. However, when cultures were treated with MeHg for 7 d, migration into the inernal granule cell layer alone was markedly decreased at 0.5 μM MeHg, and significantly decreased at 1.0 μM MeHg compared to 0 and 0.2 μM MeHg treatments over the same duration. Therefore, the number of cells that migrated was reduced, and those which did migrate did so at a slower pace. The percent of granule cells that migrated into the internal granule cell layer increased significantly over time in control samples. Thus, the duration of MeHg exposure played a role in impairment of granule cell migration.

In untreated cultures, few or no cells remained in the external granule cell layer at 8 d. This is similar to the *in vivo* cortical organization of rat cerebellum at 16 d (Altman, 1972). The proportion of cells leaving the external germinal cell layer tended to decrease inversely with MeHg concentration (Fig. 4.3). The presence of granule cells in the external germinal cell layer following 3 d of MeHg exposure became significant at 3.0 μM. The concentration-dependent increase of the presence of granule cells in the external germinal cell layer at 7 d MeHg increased significantly with concentration from 0.5 to 1.0 μM MeHg. Above 1.0 μM MeHg, the viability of the granule cells declined. The concentration-dependent increase in neurons remaining in the external germinal cell layer distinctly suggests impaired migration out of the layer into the molecular layer. The proportion of BrdU-labeled granule cells found in the molecular layer following 3 d of

Figure 4.1.

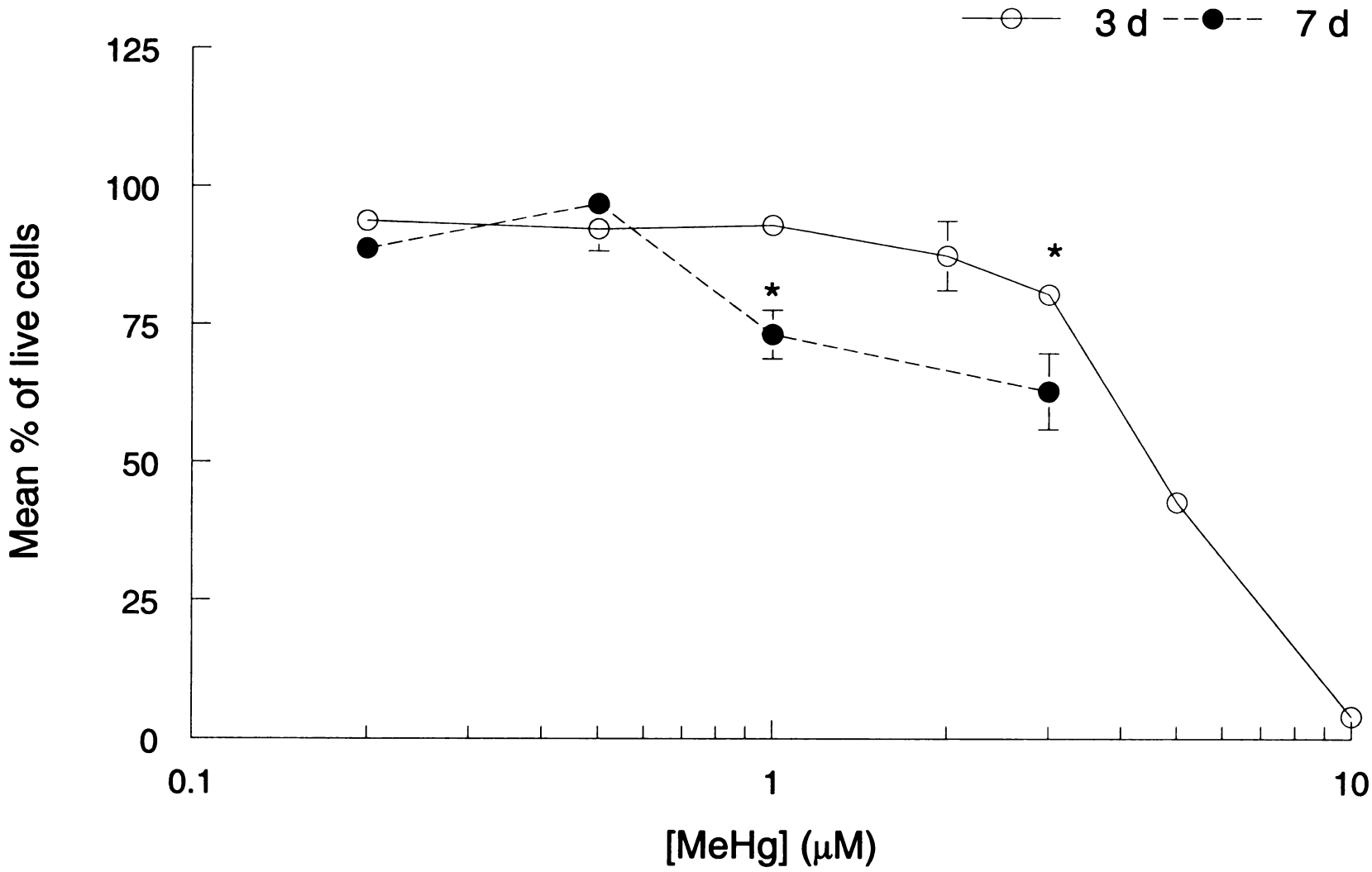


Figure 4.1. Viability of organotypic slice cultures of developing cerebellum continuously exposed to MeHg for 3 or 7 days (d) decreased in a time- and concentration- dependent manner. n=4, * = $p \leq 0.05$ with respect to control

3.0 μM MeHg exposure was 14.9% less than controls, while cultures assessed following 7 d of 1.0 μM MeHg had 23.2% more granule cells in the molecular layer than controls. There was no accumulation of granule cells at the borders between cortical layers suggesting that MeHg's actions are not highly specific to developmental stage.

Table 4.1.

	Mean percent of BrdU-labeled cells					
[MeHg]	**3 d MeHg**			**7 d MeHg**		
	EGL	**Migration**	**n**	**EGL**	**Migration**	**n**
0	24.8	75.2	5	3.7	96.3	4
0.2	14.3	85.7	3	2.1	98	3
0.5	24.6	75.4	3	19.7	80.3	3
1.0	33.3	66.7	5	35.5	64.5	3
3.0	52.1	47.9	3	nd	nd	
5.0	44.8	55.2	2	nd	nd	
D-APV	40.5	59.5	3	64.7	35.3	4

Table 4.1. The percent of total BrdU-labeled cells migrating out of the EGL into the ML and IGL combined. Migration decreased in a MeHg concentration-dependent manner in slice cultures exposed to MeHg for 3 d MeHg or 7 d MeHg. The corresponding number of samples (n) at each MeHg concentration is given in the table. nd: not determined.

Figure 4.2.

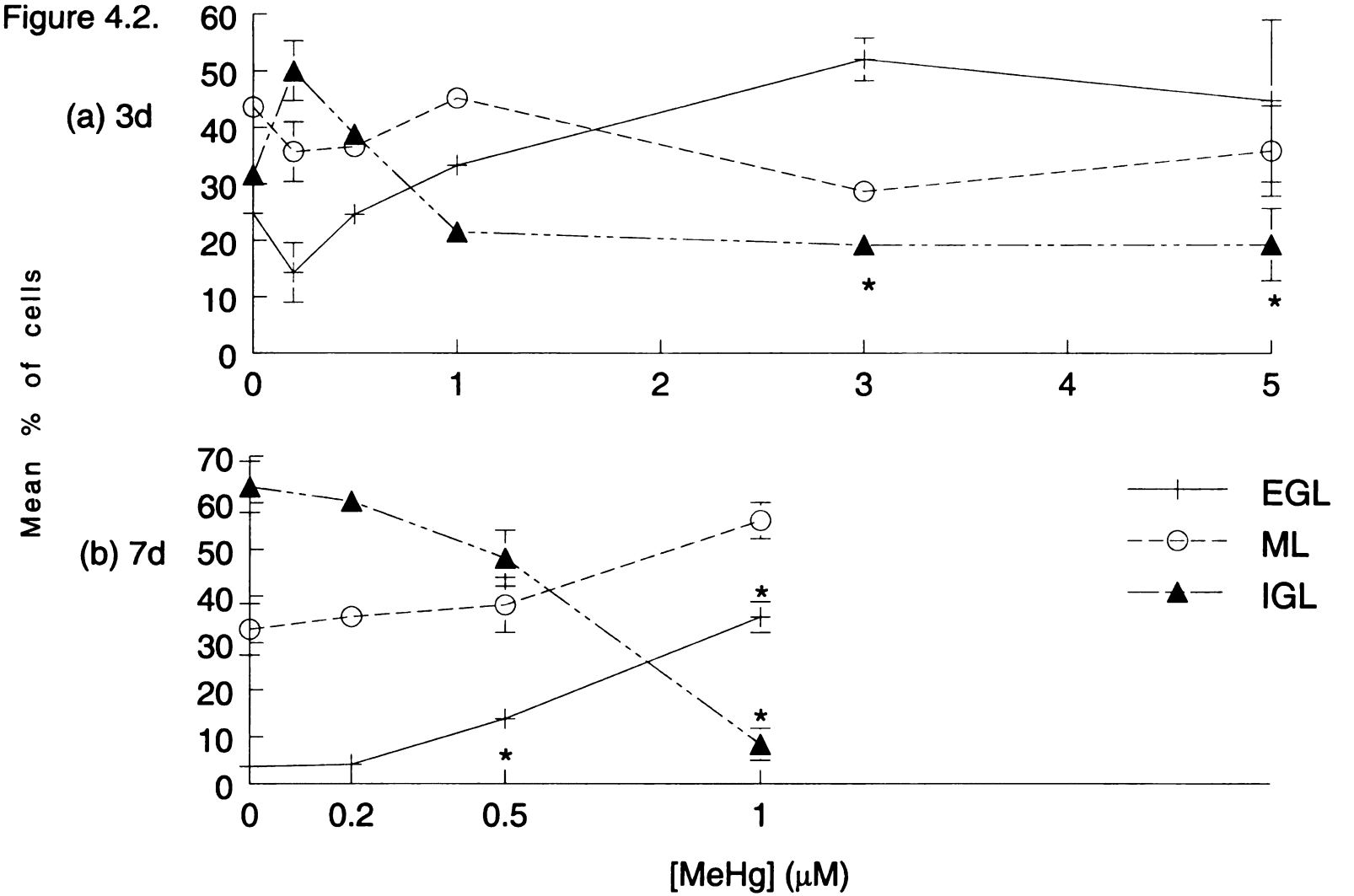
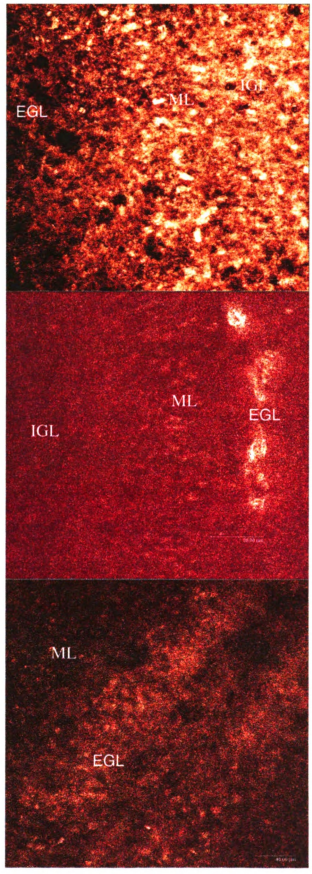

(a) 3d
(b) 7d
Mean % of cells
[MeHg] (μM)
EGL
ML
IGL
0
10
20
30
40
50
60
70
0
0.2
0.5
1
2
3
4
5
*

Figure 4.2. Continuous, low-level MeHg exposure impaired cerebellar granule cell migration in organotype slice cultures of developing cerebellum. The mean percent of BrdU-labeled cells ± SEM observed in each of the external germinal cell layer (EGL), molecular layer (ML), or internal granule cell layer (IGL) following (a) 3 d MeHg or (b) 7 d MeHg exposure. * = $p \leq 0.05$ with respect to control

Figure 4.3. Laser confocal images of fluorescently-tagged BrdU-labeled cerebellar granule cells in cerebellar slice cultures following 7 days of exposure to a) 0, b) 0.5, or c) 1.0 μM MeHg. The external germinal cell layer (EGL), molecular layer (ML), and internal granule cell layer (IGL) are labeled in each image.

Figure 4.3.



Control

0.5 μM methylmercury

1.0 μM methylmercury

E) DISCUSSION

The modified organotypic slice culture protocol and medium used were effective in demonstrating that chronic, low-level MeHg impairs cerebellar granule cell migration. MeHg caused death of immature granule cells exposed to 3.0 μM MeHg or greater for 3 d and1.0 μM MeHg or greater for 7 d. The extent of cell death was concentration-dependent. The percent of granule cells that failed to migrate increased with increasing concentration of MeHg. The lowest concentration at which MeHg significantly impairs migration depends on the duration of the exposure. Here, the lowest concentration given for 3 d and 7 d which induced significant impairment was 3.0 and 0.5 μM MeHg, respectively. MeHg appeared to affect migration in all cortical layers because there was no accumulation of cells at any cortical layer border, a phenomenon which has previously been shown to occur in response to layer-specific trophic factors (Kumada and Komuro, 2004; Komuro and Kumada, 2005).

Experimental models using chronic, low-level MeHg treatment better mimic the current existing patterns of MeHg intoxication. The medium had to be appropriate for both the stage of cell development and MeHg treatment. MeHg binds to thiol groups on proteins. Previous studies of migration have generally used serum-based media in MeHg-free studies of migration (Tanaka et al., 1994; Yuan et al., 1998; Davids et al., 2002) as well as in MeHg studies of migration (Kunimoto and Suzuki, 1997). The serum-free medium, Neurobasal (Brewer and al, 1993; Haydar et al., 1999), was used to minimize binding of MeHg to cysteine- or methionine-containing proteins of the medium, and thereby, reduced the chances of quenching MeHg's effects. In addition, the interactions of antibiotics and ion channels were considered. At high concentrations, the

antibiotics generally used in cell culture interact with membrane receptors. Specifically, penicillin affects $GABA_A$ receptor-mediated inhibitory function of mature cells. Streptomycin acts presynaptically and possibly postsynaptically on voltage-gated Ca^{2+} channels (Fujimoto et al., 1995; Sugimoto et al., 2002). In order to avoid confounded results, exposure of the slices to these antibiotics was minimized to one third the concentration normally used, without significantly compromising slice viability due to increased microbial contamination.

Characterization of MeHg effects on viability along with migration was an important consideration because MeHg affects numerous cell functions simultaneously, and it was necessary to assess whether impairment was due to an effect on neurobiological processes that regulate migration rather than due to a general decline in viability. At such low concentrations as 0.5 μM MeHg, disruption of Ca^{2+} homeostasis is one of only a few cellular phenomenon of significance that is extensively reported in the literature (Limke et al., 2004a). While dysregulation of Ca^{2+} alone is highly likely to disrupt granule cell migration, there may also be cumulative effects of MeHg neurotoxicity over time. An insignificant amount of damage to cellular proteins or other molecules may occur each day, but over 1 week, the cumulative damage may exceed the ability of the cells to repair themselves. The overwhelmed cell may then undergo apoptosis or necrosis. The notion that MeHg could have a cumulative effect over time may apply more to migration at lower concentrations than it applies to viability.

The results of the present study are in concordance with previous research showing impairment of neuronal migration by MeHg. However, the chronicity of treatment differed from previous reports. Kunimoto and Suzuki (1997) have shown that

treating organotypic cultures of postnatal rat cerebellar slices with 3 μM MeHg for 3 d selectively impaired cerebellar granule cell migration by approximately 20% without impairing or killing surrounding neurons (Kunimoto and Suzuki, 1997). If organotypic cultures are not treated with MeHg for the entire duration of the developmental window, affected granule cells can recover and migrate, or more granule cells can be generated and migrate to the internal granule cell layer. Chronic exposure to MeHg throughout the duration of migration caused impairment at concentrations as low as 0.5 μM MeHg. MeHg treatment throughout the specific stage of development appears to cause greater deficits than those found by less chronic treatment paradigms.

CHAPTER FIVE

INVOLVEMENT OF THE $GABA_A$ RECEPTOR IN METHYLMERCURY-INDUCED DISRUPTION OF Ca^{2+} HOMEOSTASIS IN DEVELOPING CEREBELLAR SLICES

A) ABSTRACT

Perinatal exposure to MeHg impairs development of cerebellar granule cells causing motor dysfunction. The mechanism by which this occurs is unknown. Acute cerebellar slice preparations were used to investigate changes in $[Ca^{2+}]_i$ during development in response to the $GABA_A$ receptor agonist, muscimol, and/or MeHg. Sagittal cerebellar slices (200 μm thick) of 9-11 d old rats were loaded with Ca^{2+} indicator dyes Fluo4-AM or Fluo5-AM. Flourescent changes in the external germinal cell layer, molecular layer, and internal granule cell layer were simultaneously recorded within one visual field by confocal laser microscopy. Recordings through the depth of the slice were obtained using z-series stacked. MeHg caused a time- and concentration-dependent increase in $[Ca^{2+}]_i$ in granule cells of all stages of development. Immature granule cells in the external germinal cell layer showed an increased $[Ca^{2+}]_i$ in response to muscimol pulses. Within the external germinal cell layer, application of a 100 μM muscimol pulse in the presence of 20 μM MeHg increased the average $[Ca^{2+}]_i$ by 154% relative to controls. This is significantly greater than that caused by application of muscimol in the absence of MeHg. Application of a 50 μM muscimol pulse in the presence of 10 μM MeHg in the external germinal cell layer increased $[Ca^{2+}]_i$ more so than that induced by 10 μM MeHg alone. The $[Ca^{2+}]_i$ at subsequent pulses of muscimol in the presence of MeHg was not as high as that in the presence of MeHg alone. Postmigratory granule cells in the internal granule cell layer are presumed to generate inhibitory responses to $GABA_A$ receptor activation. Within the internal granule cell layer, pulses of 100 μM muscimol alone did not increase $[Ca^{2+}]_i$. Muscimol pulses in the presence of MeHg led to $[Ca^{2+}]_i$ levels that were not as high as MeHg alone. Thus,

effects of MeHg on the $GABA_A$ receptor at different stages of development may be responsible for the differential changes in $[Ca^{2+}]_i$.

B) INTRODUCTION

As described previously (Introduction and Chapter Three), MeHg is a neurotoxicant to which cerebellar granule cells are particularly sensitive (Leyshon and Morgan, 1991); Granule cells *in vitro* are more sensitive to MeHg than are Purkinje cells (Marty and Atchison, 1997; Marty and Atchison, 1998; Edwards et al., 2005). Some neurotoxic effects such as disruption of divalent cation homeostasis (Limke et al., 2003; Limke et al., 2004b) are induced at submicromolar concentrations in cerebellar granule cells, but not in juxtaposed Purkinje cells (Edwards et al., 2005), which accumulate an equivalent or even greater concentration of MeHg (Hunter and Russell, 1954). The highest concentrations of MeHg are found in Purkinje and Golgi cells (Glomski et al., 1971; Chang et al., 1977; Leyshon-Sorland et al., 1994).

MeHg can bind with high affinity to sulfhydryl groups of any exposed cysteine or methionine (Castoldi, 2000; Fronfria et al., 2001), and thereby affect cells at multiple sites. MeHg disrupts cell homeostatic processes by production of reactive oxygen species (Sarafian and Verity, 1991), interference with macromolecule synthesis (Sarafian and Verity, 1985), increased spontaneous neurotransmitter release (Atchison, 1986), and disturbance of divalent cation (Ca^{2+} and Zn^{2+}) compartmentalization (Denny et al., 1993; Hare et al., 1993). Disruption of $[Ca^{2+}]_i$ homeostasis is a common aspect of toxicity among MeHg's targets, and has been demonstrated in numerous cell types including neuroblastoma cells (Hare et al., 1993) and cerebellar granule cells (Marty and Atchison, 1997). The disruption of divalent cation homeostasis shown by studies using the Ca^{2+}-indicator Fura-2 have determined that a multiphasic increase in divalent cations, mainly $[Ca^{2+}]_i$, is a consistent consequence of MeHg exposure (Hare et al., 1993; Marty and

Atchison, 1997; Limke and Atchison, 2002). The initial, or first, phase involves a MeHg concentration-dependent increase in $[Ca^{2+}]_i$ from intracellular stores. In the second phase, there is an influx of extracellular Ca^{2+} (Hare et al., 1993; Marty and Atchison, 1997). MeHg also releases Zn^{2+} into the cytosol of NG108-15 cells (Hare et al., 1993; Denny and Atchison, 1994) and rat cerebellar granule cells (Marty and Atchison, 1997); the Zn^{2+} may have been released from cellular proteins that bind it at cysteine sulhydryl groups as MeHg displaced the ion (Hunt et al., 1984; Denny and Atchison, 1995).

Investigation of the intracellular sources of Ca^{2+} in the initial phase of MeHg exposure determined that MeHg acts at the M_3 muscarinic acetylcholine receptor in the plasma membrane inducing release of IP_3. IP_3 appeared to mediate Ca^{2+} release through the IP_3 receptor on the SER (Limke et al., 2004b). The Ca^{2+} released was taken up into the mitochondria. MeHg also opens the mitochondrial transition pore allowing release of the Ca^{2+} previously sequestered (Limke and Atchison, 2002; Limke et al., 2003).

The second phase of MeHg-induced increase of $[Ca^{2+}]_i$ is characterized by an influx of extracellular Ca^{2+}. Phase two was delayed by treatment with the VDCC antagonists nifedipine and ω-conotoxin MVIIC prior to 0.5-1 μM MeHg exposure in primary culture of cerebellar granule cells suggesting involvement of the N-, L- and/or Q-type VDCCs (Marty and Atchison, 1997). MeHg-induced Ca^{2+} influx was also delayed by TTX and nifedipine in NG108-15 neuroblastoma cells (Hare and Atchison, 1995). Antagonists of excitatory amino acid receptor activated channels did not prevent MeHg-induced elevations of $[Ca^{2+}]_i$ (Marty and Atchison, 1997).

$GABA_A$ receptor in MeHg Neurotoxicity

Previous research has shown that MeHg affects the $GABA_A$ receptor (Yuan and Atchison, 1997, 2003a). Compared to excitatory postsynaptic potentials, inhibitory postsynaptic potentials appeared to be more sensitive to MeHg because block of inhibitory postsynaptic potentials occurred before block of excitatory postsynaptic potentials (Yuan and Atchison, 1995), and $GABA_A$ receptor-mediated inhibitory transmission is blocked by MeHg before excitatory transmission in neurons of the CA1 region of hippocampus (Yuan and Atchison, 1997). MeHg appeared to block $GABA_A$ receptor-mediated inhibitory transmission resulting in disinhibition of excitatory synaptic transmission in hippocampus (Yuan and Atchison, 1997). MeHg suppressed the GABA-induced Cl^- current in dorsal root ganglion neurons as well (Arakawa et al., 1991). MeHg's effect on the $GABA_A$ receptors in mature cerebellar granule cells is similar; Yukun and Atchison (2003) found that MeHg suppressed Cl^- -dependent spontaneous IPSCs in slices of rat cerebellum. However, an initial increase in frequency of spontaneous IPSCs was observed prior to the suppression (Yuan and Atchison, 2003a). $GABA_A$ receptor-mediated whole cell currents were blocked at concentrations as low as 0.1 μM MeHg in primary cultures of cerebellar granule cells (Xu and Atchison, 1998). MeHg also blocked responses evoked by bath application of muscimol (Yuan and Atchison, 1997).

In addition to the $GABA_A$ receptor, MeHg affects VDCCs. At 0.125 – 5.0 μM, MeHg caused an initial, rapid component and a subsequent time- and concentration-dependent reduction to block of current through VDCCs in HEK cells (Hajela et al., 2003). For more information on MeHg's effect on VDCCs, see Chapter One.

Chronic, low-level exposure to MeHg impaired cerebellar granule cell migration (See Chapter Three), but the mechanism by which it does so is not clear. The neurotoxicant may disrupt Ca^{2+} -oscillations by increasing $[Ca^{2+}]_i$ and/or the signals that regulate Ca^{2+} -oscillations by altering $GABA_A$ receptor and VDCC function. Few studies of MeHg effects have focused on the non-synaptic $GABA_A$ receptor of immature cerebellar granule cells. As stated previously (See Chapters One and Three), $GABA_A$ receptor stimulation in immature neurons is excitatory and leads to an N- and L-type VDCC -mediated increase in $[Ca^{2+}]_i$. The objectives of the present study were to examine acute slice preparations of developing cerebellum (P9-11) for the characteristic increase in $[Ca^{2+}]_i$ induced by MeHg as seen in cells of primary cultures during previous studies and to investigate the combined Ca^{2+} response of MeHg and $GABA_A$ receptor stimulation and inhibition by muscimol and bicuculline, respectively, within immature cerebellar granule cells.

C) MATERIALS & METHODS

Acute slice preparations

Acute slice preparations of cerebellum from P8-11 rats were used to investigate changes in $[Ca^{2+}]_i$ among the developing cortical layers in response to receptor manipulation. Preparation of the slices was the same as in Chapter Three. In each experimental condition described below, an averaged 2-dimensional image of the z-series stack was used for analysis. The average intensity for the granule cells in each layer at each time interval was normalized to that of the cells prior to treatment. The averages of relative intensities from 3-5 different slices per treatment were plotted and compared using a two-way analysis of variance with a Tukey-Kramer post-test. Data were considered different if $p \leq 0.05$. Images in this dissertation are presented in color.

Pharmacological Applications

Several slices were loaded with both Fluo-4, AM and ethidium homodimer-1, an indicator of dead or dying cells. Slices to be exposed to MeHg were loaded with Fluo5-AM to avoid saturation of fluorescent probe. Control slices were continuously perfused with ACSF only. Experimental slices were perfused with ACSF for 15 min, and then with 10 or 20 μM MeHg in ACSF for 30 min. Pulses (30-60 s) of 100 μM muscimol or 10 μM bicuculline at 10 min intervals were used to stimulate or inhibit the $GABA_A$ receptor, respectively, in the presence or absence of MeHg.

D) RESULTS

Slices incubated in both Fluo-4, AM and in ethidium homodimer-1 showed no co-localization of the two dyes. This suggests that the deviations in the amount of Fluo-fluorescence were not the result of the inclusion of dead or dying cells in the analysis (Fig. 5.1).

Acute application of 10 or 20 μM MeHg increased $[Ca^{2+}]_i$ in both immature (external germinal cell layer and molecular layer) and mature (internal granule cell layer) granule cells in acute sagittal slices (Fig. 5.2). The most dramatic change in relative Fluo-4 intensity was seen in the external germinal cell layer, which increased by 50-100% after 30 min MeHg exposure compared to the MeHg-free control. Increasing the [MeHg] induced a more rapidly occurring and greater amplitude $[Ca^{2+}]_i$ increase in all layers. Muscimol alone significantly increased $[Ca^{2+}]_i$ in granule cells of the external germinal cell layer by 9-14 % (Fig. 5.3). Muscimol application slightly decreased $[Ca^{2+}]_i$ in the internal granule cell layer (Fig. 5.4).

A 30-60 s pulse of 100 μM muscimol at 10 min during continuous perfusion of 20 μM MeHg produced an increase in $[Ca^{2+}]_i$ in the external germinal cell layer that was 1.54 times greater than that induced by 20 μM MeHg alone at the same time point. Subsequent pulses of muscimol at 20 and 30 min caused increases in $[Ca^{2+}]_i$ that were greater than muscimol alone, but the levels were not as high as 20 μM MeHg alone (Fig. 5.3). The Fluo-indicated Ca^{2+} levels in the internal granule cell layer did not increase in the presence of 20 μM MeHg and 100 μM muscimol compared to control conditions at Time = 0 min (Fig. 5.4).

Simultaneous perfusion of both 100 μM bicuculline and 10 μM MeHg did not significantly alter the MeHg-induced increase in Fluo-indicated $[Ca^{2+}]_i$ in the external germinal cell layer. In the internal granule cell layer, bicuculline and 10 μM MeHg together caused a greater increase in relative intensity at 15 min perfusion compared to MeHg only at 15 min as well as a greater absolute intensity compared to the external germinal cell layer perfused with both bicuculline and MeHg (Fig. 5.5).

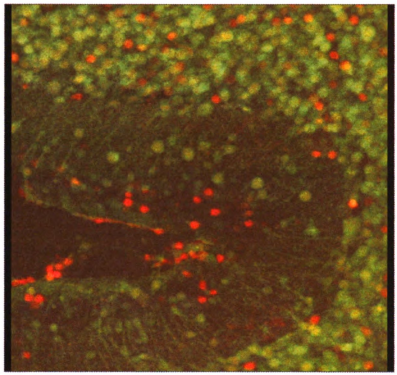


Figure 5.1. Fluo-4, AM calcium indicator dye (green) and ethidium homodimer (red), an indicator of cell death, do not colocalize in an acute preparation of a P10 rat cerebellum sliced sagittally.

Figure 5.2. MeHg perfusion significantly increased the $[Ca^{2+}]_i$ indicated by Fluo-4, AM and Fluo-5F, AM in postmitotic granule cells in the external germinal cell layer (EGL) (A), migrating granule cells in the molecular layer (ML) (B), as well as post-migrational cells in the internal granule cell layer (IGL) (C). There was no significant photobleaching (n=3) (blue) or normal physiologic change in Fluo-fluorescence (n=3) (green) during ACSF only perfusion. * = $p \leq 0.05$ with respect to control

Figure 5.2.

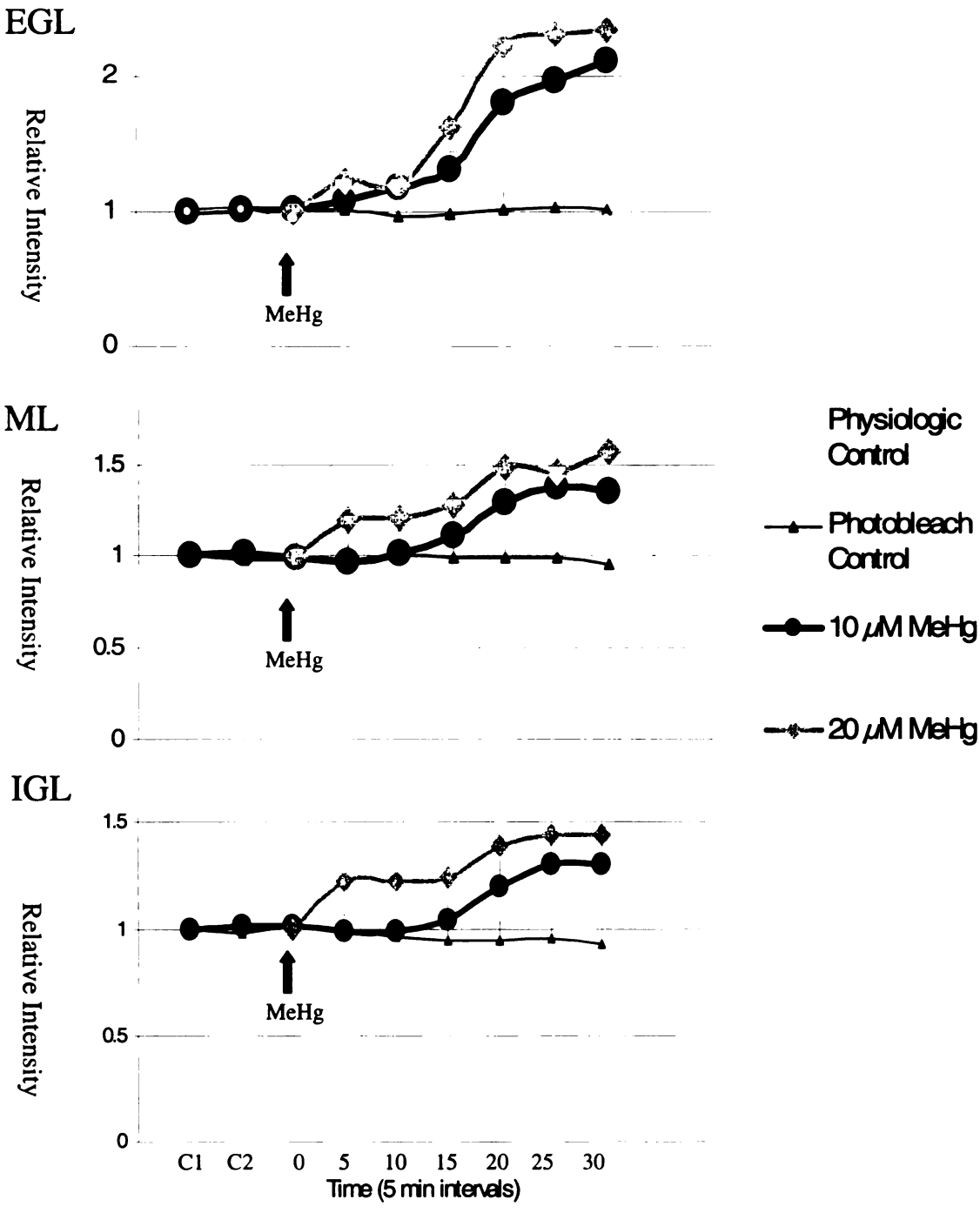

EGL
Relative Intensity
2
1
0
MeHg
ML
Relative Intensity
1.5
1
0.5
0
MeHg
IGL
Relative Intensity
1.5
1
0.5
0
MeHg
C1 C2 0 5 10 15 20 25 30
Time (5 min intervals)
Physiologic Control
Photobleach Control
10 μM MeHg
20 μM MeHg

Figure 5.3.

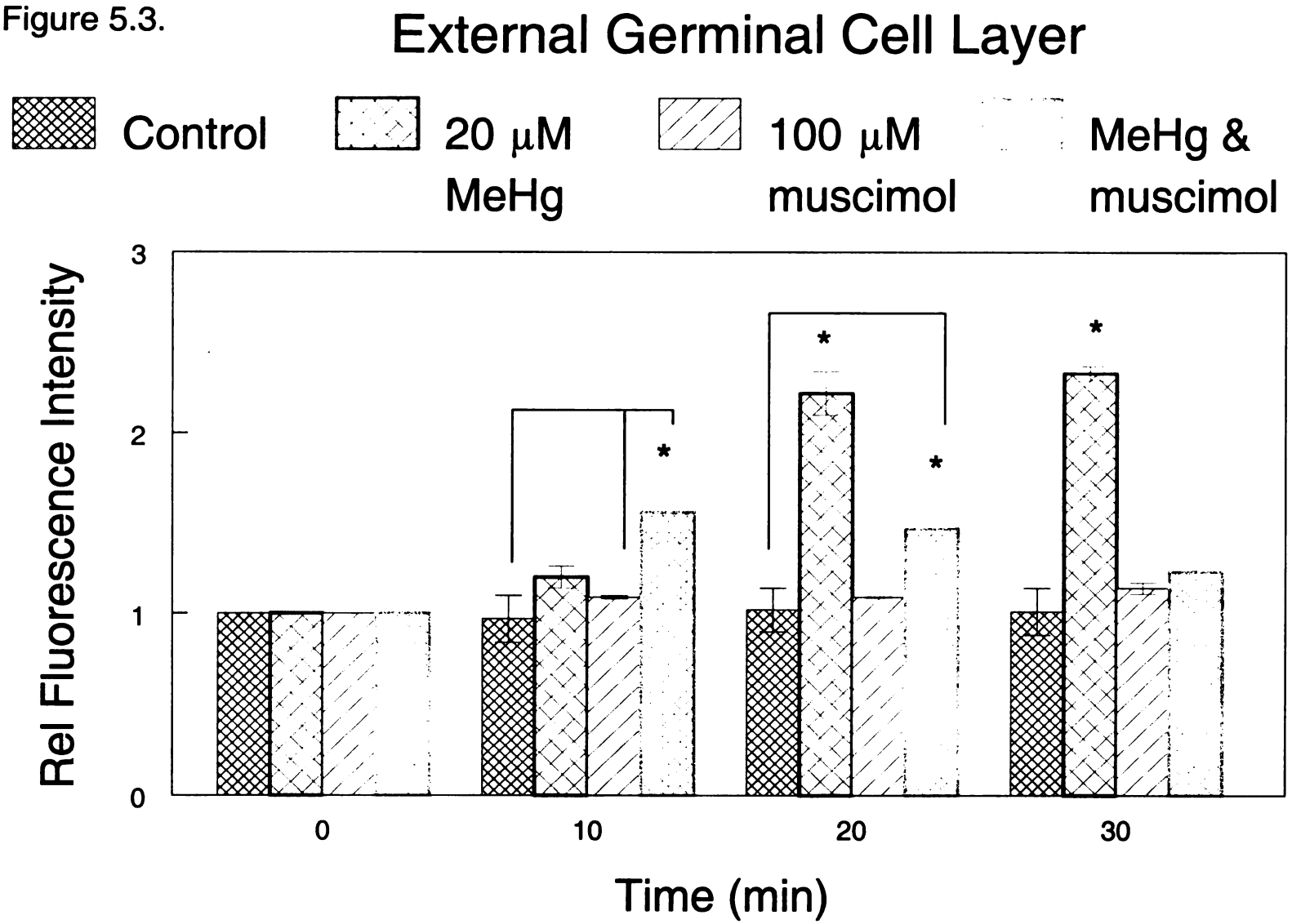

External Germinal Cell Layer
Control
20 μM MeHg
100 μM muscimol
MeHg & muscimol
Rel Fluorescence Intensity
0
1
2
3
*
*
*
*
0
10
20
30
Time (min)

Figure 5.3. MeHg alters $GABA_A$ receptor function in non-synaptic, immature granule cells of the external germinal cell layer. 30-60 sec pulses of muscimol were applied to Fluo-4, AM- loaded acute slice preparations of developing cerebellum in the presence of 20 μM MeHg for 30 min. Fluorescent intensity levels of the cerebellar cortex were recorded. The mean percent changes in $[Ca^{2+}]_i$ in immature granule cells of the external germinal cell layer are shown. * = $p \leq 0.05$ with respect to control

Figure 5.4.

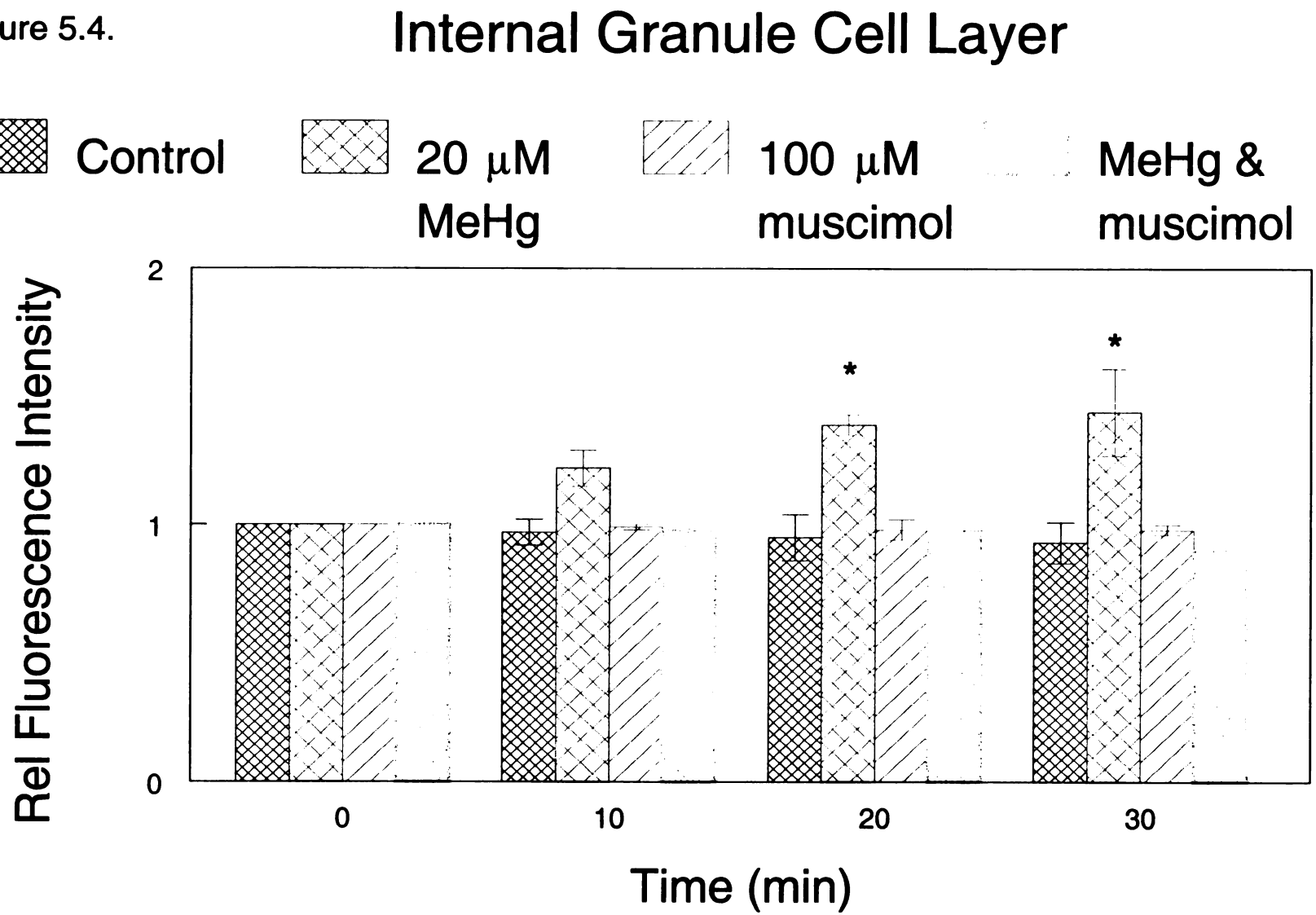
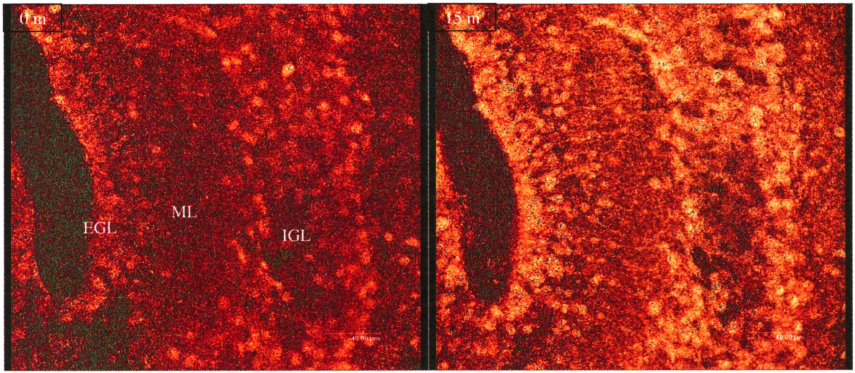

Internal Granule Cell Layer
Control
20 μM MeHg
100 μM muscimol
MeHg & muscimol
Rel Fluorescence Intensity
2
1
0
*
*
0
10
20
30
Time (min)

Figure 5.4. MeHg alters $GABA_A$ receptor function in maturing granule cells of the internal granule cell layer. 30-60 sec pulses of muscimol were applied to Fluo-4, AM- loaded acute slice preparations of developing cerebellum in the presence of 20 μM MeHg for 30 min. Fluorescent intensity levels of the cerebellar cortex were recorded. The mean percent changes in $[Ca^{2+}]_i$ in post-migrational granule cells of the internal granule cell layer are shown. * = $p \leq 0.05$ with respect to control

Figure 5.5.



Cortical Layer	MeHg only: relative intensity	MeHg & bicuculline: Relative intensity (Mean absolute intensity)
IGL	1.04	1.27 (107.13)
EGL	1.31	1.64 (81.41)

Figure 5.5. MeHg and bicuculline images. Simultaneous perfusion of both 100 μM bicuculline and 10 μM MeHg did not significantly alter the MeHg-induced increase in Fluo-indicated $[Ca^{2+}]_i$ in the external germinal cell layer (EGL). In the internal granule cell layer (IGL), bicuculline and 10 μM MeHg together caused a greater increase in relative intensity at 15 min perfusion compared to MeHg only at 15 min as well as a greater absolute intensity compared to the EGL perfused with both bicuculline and MeHg. * = $p \leq 0.05$ with respect to control

E) DISCUSSION

In conclusion, MeHg increased $[Ca^{2+}]_i$ in developing cerebellar granule cells of all levels of maturity in a concentration- and time- dependent manner. The most dramatic change in relative Fluo-4 intensity was seen in the external germinal cell layer, which increased by 50-100% from the MeHg-free condition after 30 min of exposure. The granule cells in the external germinal cell layer and molecular layer, but not the internal granule cell layer are immature. Hence, the greater susceptibility of the developing cerebellum to MeHg-induced neuronal damage may be related to the dramatic changes in $[Ca^{2+}]_i$ of immature granule cells caused by MeHg.

The mechanisms underlying the greater susceptibility of the developing cerebellar granule cells is not clear, however, there is differential expression of Ca^{2+}-regulating proteins among stages of development; For example, expression of SER and plasma membrane ATPases increases as cerebellar granule cells mature (Sepulveda et al., 2005), and changes in gene expression mediated by Ca^{2+}/calmodulin-dependent calcineurin, which may includeNa^+/Ca^{2+} exchanger isoforms, occurs as cerebellar granule cells mature (Li et al., 2000; Sato et al., 2005). Differential expression of Ca^{2+}-regulating proteins is an important consideration because, as stated previously, granule cell migration is dependent on Ca^{2+} -oscillations. Resting $[Ca^{2+}]_i$ are in the 100 nM range, while the extracellular milieu is in the 1-2 mM range (Kass and Orrenius, 1999). Most neurons are not at rest for very long. Localized transient increases such as at nerve terminals active zones can reach 100 μM (Llinas et al., 1992, 1995a, 1995b). Ca^{2+} compartmentalization requires energy. Several mechanisms regulate $[Ca^{2+}]_i$. Rapid sequestration of Ca^{2+} into intracellular organelles or active transport of Ca^{2+} out of the

cell occurs following influxes. Sustained high concentrations lead to cell death (Lee et al., 1999) via rundowns of major energy reserves and/or activation of catabolic function.

Another mechanism by which MeHg affects all cerebellar granule cells is by altering function of the $GABA_A$ receptor. Muscimol stimulation of the $GABA_A$ receptor increases $[Ca^{2+}]_i$ in granule cells found in the external germinal cell layer presumably before the "switch" of Cl^- flow reversal occurs. The $GABA_A$ receptor antagonist, bicuculline, exacerbated MeHg-induced increase in $[Ca^{2+}]_i$ in the internal granule cell layer possibly due to disinhibition of the granule cells following GABAergic stimulation. However, bicuculline did not significantly alter the MeHg-induced increase of Fluo-4 in the external germinal cell layer. Therefore, any effect of MeHg on the $GABA_A$ receptor in the first 15 min of exposure was not altered by bicuculline. Applying muscimol during MeHg exposure initially increased $[Ca^{2+}]_i$ to a greater extent than did MeHg alone. However, the level drops during subsequent pulses suggesting that the $GABA_A$ receptor was blocked. A characteristic stimulation and then block of the $GABA_A$ receptor channel was previously found in mature $GABA_A$ receptors as well (Yuan and Atchison, 2003b), suggesting that the effect may not be dependent on subunit composition, at least at high MeHg concentrations.

Stimulating the receptor with muscimol may have altered the time-to-block by MeHg. Both MeHg and the sulfhydryl alkylating agent N-ethylmaleimide enhanced flunitrazepam binding (EC_{50}=15.24 μM MeHg) in a concentration -dependent manner in cultures of granule cells. This suggests that MeHg's effects on $GABA_A$ receptor physiology may, at least, include alkyation of -SH groups of its cysteine residues at the benzodiazepine site (Fronfria et al., 2001). In the present study, binding of muscimol

may have caused a conformational change that exposes more thiol groups to which MeHg can bind and block the receptor-associated channel.

The $GABA_A$ receptor appears to play a role in MeHg's effects on $[Ca^{2+}]_i$ levels in immature granule cells as well as during the final stages of granule cell development. At the given concentrations of MeHg, it is likely that the VDCCs are affected as well. The combined effect of Ca^{2+} disruption may interfere with the intrinsic Ca^{2+} -oscillation rhythm and extrinsic signal transduction regulating Ca^{2+} -oscillations during granule cell development. Thus, MeHg's ability to disrupt Ca^{2+} regulation in immature granule cells appears to involve intracellular Ca^{2+} stores in Phase 1 as well as increased $GABA_A$ receptor function and influx of Ca^{2+} through VDCC in Phase 2 followed by block of both.

CHAPTER SIX

GENERAL DISCUSSION

Summary

The objectives of this study were to investigate particular mechanisms (See Fig. 1.1) in cerebellar granule cell migration that, when perturbed, lead to impairment of migration. The results herein indicate that: 1) inhibition of the NR2B subunit-containing NMDA receptor both impaired migration of and decreased basal levels of $[Ca^{2+}]_i$ in immature cerebellar granule cells, 2) N- and L-type VDCCs, but not the NR2B subunit-containing NMDA receptor, are activated by the $GABA_A$ receptor in immature cerebellar granule cells, 3) continous exposure to submicromolar levels of MeHg significantly impairs cerebellar granule cell migration, and 4) MeHg-induced stimulation and then block of the $GABA_A$ receptor may be one mechanism by which MeHg disrupts Ca^{2+} homeostasis in immature cerebellar granule cells.

NR2B-NMDA receptor actions

The results in Chapter Two defined the NR2B subtype of NMDA receptor as being critical in facilitating cerebellar granule cell migration from the external germinal cell layer to the internal granule cell layer. The NR2B subunit is the predominant subunit subtype expressed in migrating neurons (Farrant et al., 1994), and it is most likely the subtype contributing to the Ca^{2+} -oscillations that mediate migration. This receptor subtype is tonically active as suggested by the reduced $[Ca^{2+}]_i$ following ifenprodil application (See Chapter Three) and is probably the NMDA receptor subtype that was tonically active in Rossi and Slater's studies (Rossi and Slater, 1993), though it was not identified.

The NR2B-NMDA receptor is both ligand- and voltage- dependent. The source of factors activating the receptor is not clear. The ligand, glutamate, has previously been reported to be released in a paracrine manner from parallel fibers in the molecular layer, but whether parallel fibers are the only source of glutamate and whether this glutamate reaches NR2B-NMDA receptors on the inward moving soma and/or leading process is not certain. The NR2B subtype has a low voltage-threshold to activation, suggesting that it responds to low-levels of neurotrophic factor or regulators. However, the source of membrane depolarization to remove the Mg^{2+}-block is also unknown. In Chapter Three, the hypothesis that the source of membrane depolarization is $GABA_A$ receptor stimulation was tested. The results indicate that application of ifenprodil did not inhibit a muscimol-induced increase of $[Ca^{2+}]_i$. Partial inhibition of the NR2B-NMDA receptor alone decreased $[Ca^{2+}]_i$ by 31.8% ± 9, and subsequent $GABA_A$ receptor membrane depolarization increased $[Ca^{2+}]_i$ to 2.11% ± 2 above controls, suggesting that the $GABA_A$ and NMDA receptors act independently. Of course, ion flow through both receptor channels affects $[Ca^{2+}]_i$, and the sum of the receptors' actions was represented by the mean percent change in $[Ca^{2+}]_i$. These results taken together with the finding that bicuculline application did not prevent an ephrinB2/Fc-potentiated, glutamate-mediated rise in $[Ca^{2+}]_i$ suggest that the $GABA_A$ receptor did not mediate NR2B-NMDA receptor activation. The results leave the question "What is removing the voltage-dependent block by Mg^{2+}?".

The intracellular tail of the NR2B subunit is subject to regulation by multiple factors (See Chapter One), and these factors may be a part of an activating pathway. Intracellular regulating factors or local neurotrophic factors could modify the NR2B

receptor subunit and thereby activate it or, at least, lower the threshold to activation. For example, one study suggested that PKC could reduce voltage-dependent block (Chen and Huang, 1992) by an unknown mechanism. However, other studies suggest that cAMP-dpk (PKA) (Leonard and Hell, 1997) and PKC enhance NR2B-NMDA receptor response in spinal cord dorsal horn (Gerber et al., 1989) and hippocampus (Aniksztejn et al., 1992; Markram and Segal, 1992) without decreasing the affinity of the receptor for Mg^{2+} (Leonard and Hell, 1997; Xiong et al., 1998). Tyrosine phosphorylation may also be a mechanism by which the threshold to activation is reduced.

One function of the NR2B-NMDA receptor may be mediation of cytoskeletal movement. The NMDA receptor is well known to facilitate structural changes in maturing synapses by interaction with co-localized enzymes such as cupidin, the actin binding partners F-actin and drebin, or other involved proteins (Shiraishi et al., 2003). NMDA receptor involvement in cytoskeletal dynamics has been studied previously in other neuron types. NMDA receptor-mediated Ca^{2+} influx in hippocampal synapses blocks dendritic spine motility (Ackermann and Matus, 2003) and induced spine swelling or increased spine volume (Fifkova and Van Herreveld, 1977) that is dependent on NMDA receptor activation and calmodulin, suggesting a Ca^{2+}-triggered calmodulin-dependent actin rearrangement (Matsuzaki et al., 2004). Ca^{2+} could activate gelsolin to cap and sever f-actin (Star et al., 2002) or prolifin (Ackermann and Matus, 2003). The ability of the mature receptor to mediate the cytoskeleton could also be inherent in the immature NR2B-NMDA receptor, and serve to modulate transposition of the migrating soma or actin-based motion in the lamellapodium of the leading process. TheNR2B-NMDA receptor is co-localized with factor(s) that regulate the cytoskeleton (Oertner and

Matus, 2005). Kinases such as cdk-5 (Gao et al., 2002; Bock and Herz, 2003) that phosphorylate the NR2B subunit, also interact with regulators of the cytoskeleton such as reelin and doublecortin (Couillard-Despres et al., 2005).

$GABA_A$ receptor –mediated activation of N- and L-Type VDCCs

Although NR2B-NMDA receptors in immature cerebellar granule cells are not activated by $GABA_A$ receptor stimulation as Ben-Ari (2002) proposed to occur in developing hippocampal neurons, N-type VDCCs are activated (Ben-Ari, 2002). The present finding suggests that the role of the NR2B-NMDA receptor and $GABA_A$ receptor-mediated activation of the N-type VDCC may serve separate functions within the cell aside from Ca^{2+}-oscillations.

In the effort to determine whether the $GABA_A$ receptor activates VDCCs and/or NR2B-NMDA receptors in immature cerebellar granule cells, it was found that the N-type VDCC are, at least in part, activated by $GABA_A$ receptor stimulation. Bicuculline alone did not significantly decrease $[Ca^{2+}]_i$, and ω -conotoxin GVIA did; both results suggest that there are other factors that depolarize the membrane and activate N-type VDCCs under control conditions. The results agree with Komuro's finding in which inhibiting either N-type VDCC or NMDA receptor impaired migration, but bicuculline did not (Komuro and Rakic, 1993). The $GABA_A$ receptor-mediated activation of N-type VDCC and activation of the NMDA receptor appear to be separate mechanisms and the two may function in different pathways. Simultaneous stimulation of both receptors could cause a large increase in $[Ca^{2+}]_i$ necessary for forward movement of the cell because, as observed by Komuro and Rakic, inhibition of either protein channel significantly impairs the Ca^{2+}-oscillations of migration.

Another possible source of activation for the NR2B and/or N-type VDCC could be the IP_3 receptor stimulation. Previous studies suggest that IP_3 receptor stimulation releases Ca^{2+} that activates other Ca^{2+} -permeable channels such as the NMDA receptor, VDCCs, or Ca^{2+}-induced- Ca^{2+}-release receptors (Putney, 1999). L-type VDCCs were not active under control conditions, and they appeared to account for less than half of the $GABA_A$ receptor-mediated increase in $[Ca^{2+}]_i$. The results differ from those of Rego et al. (2001) in which $GABA_A$ receptor-mediated increases in $[Ca^{2+}]_i$ in primary cultures of cerebellar granule cells were inhibited by bicuculline and nifedipine suggesting that the $GABA_A$ receptor only activated L-type VDCCs (Rego et al., 2001). The difference in N-type VDCC activity could be due to the difference in model system used. The present studies were observed in acute preparations of cerebellar slices which maintains interactions among the cells, where as, Rego et al. (2001) used primary cultures of cerebellar granule cells. $GABA_A$ receptor-mediated activation of L-type VDCC has previously been implicated in the differentiation of cerebellar granule cells. $GABA_A$ receptor-mediated activation of L-type VDCCs may play a role in growth cone motility in the non-synaptic granule cells (Borodinsky, 2003).

The muscimol-induced increase in $[Ca^{2+}]_i$ did not appear to involve release of Ca^{2+} from intracellular stores. A small release of Ca^{2+} from the SER was seen by application of thapsigargin alone suggesting that if muscimol application induced a release of Ca^{2+} from intracellular stores, it would be noticeable and quantified during Ca^{2+} -imaging. However, none was apparent.

The role of $GABA_A$ receptor –mediated activation of N-type VDCCs has not been defined in the migration of cerebellar granule cells. However, the increased $[Ca^{2+}]_i$ could

potentially activate any number of Ca^{2+}-dependent proteins. In hypothalamic neurons, the $GABA_A$ receptor-mediated pathway activates the MAPK cascade, which leads to phosphorylation of CREB and a subsequent increase in BDNF expression (Obrietan et al., 2002). The increased BDNF increases the expression of the $GABA_A$ receptor forming an excitatory feedback loop that was discovered in developing hypothalamic neurons. High levels of $GABA_A$ receptor stimulation and BDNF induce expression of the mature receptor subunit subtype α_6 (Bao et al., 1999; Obrietan et al., 2002). BDNF also modulates mature $GABA_A$ receptor activity through interactions with the TrkB receptor in cultures of mature cerebellar granule cells (Cheng and Yeh, 2003).

Despite the results in Chapter Three showing that the NR2B- NMDA receptor is not activated by the $GABA_A$ receptor, the two receptors still have indirect, intracellular interactions (Brandoli et al., 1998). BDNF down regulates NMDA receptor function in cerebellar granule cells, particularly NR2A and C subunit expression (Brandoli et al., 1998). Repetitive NMDA receptor stimulation in immature cerebellar granule cells decreased NR2A levels by 80% at the level of translation and/or post-translational modifications (Resink et al., 1995). In addition, both BDNF and ephrin could also potentiate protein synthesis via eukaryotic translation initiation factors (Miyata et al., 2005).

Another example of how the $GABA_A$ and NMDA receptor-mediated pathways may converge is in their regulation of GAP-43, which is found within granule cells of the immature and mature rat cerebellum. Higher levels of GAP-43 are detected in the neonate compared to the adult. Studies using cultures of cerebellar granule cells suggest that levels of GAP-43 messenger RNA, *in vivo*, are modulated by input from both

NMDA-mediated excitatory and $GABA_A$ receptor-mediated input, and have a resultant influence on granule cell maturation during development in the neonate and neuroplasticity in the adult (Console-Bram et al., 1998).

In summary, the $GABA_A$ receptor activated the N- and L-type VDCCs, but did not activate the NR2B-NMDA receptors in non-synaptic, immature cerebellar granule cells. However, the two receptor-mediated pathways interact through intracellular proteins and transcription factors (Fig. 6.1). Impairment of migration can occur by inhibition, deletion, or alteration of one or more of the mechanisms discussed. MeHg poisoning affects these mechanisms, which could lead to impaired development.

Mechanisms of MeHg-induced impairment of migration

In Chapter Four, 10 and 20 μM MeHg exposure for 30 min elevated $[Ca^{2+}]_i$ levels in cerebellar granule cells at all stages of development. This was expected based on previous research in Dr. Atchison's lab showing a MeHg-induced multiphasic increase in $[Ca^{2+}]_i$ in neurons (See Chapter One). The most immature granule cells experienced the most dramatic increase in $[Ca^{2+}]_i$, possibly due to a low resting $[Ca^{2+}]_i$ as well as low expression of Ca^{2+} –sequestering molecules. Imaging for longer than 30-45 min was not possible due to loss of membrane integrity as visualized by non-definable membranes on the cells imaged. The 0.5 and 1.0 μM MeHg-induced increase in $[Ca^{2+}]_i$ kills cerebellar granule cells of primary cultures in a concentration-dependent manner within hrs following acute exposure for 45 min (Marty and Atchison, 1998). Slices of cerebellum contain other neurons, glia, and extracellular matrix that can absorb some of the MeHg applied in the experiments of Chapter Four and application was for 30 min (15 min less than in Marty and Atchison 1998). The granule cells in the acute slice preparations may

Figure 6.1.

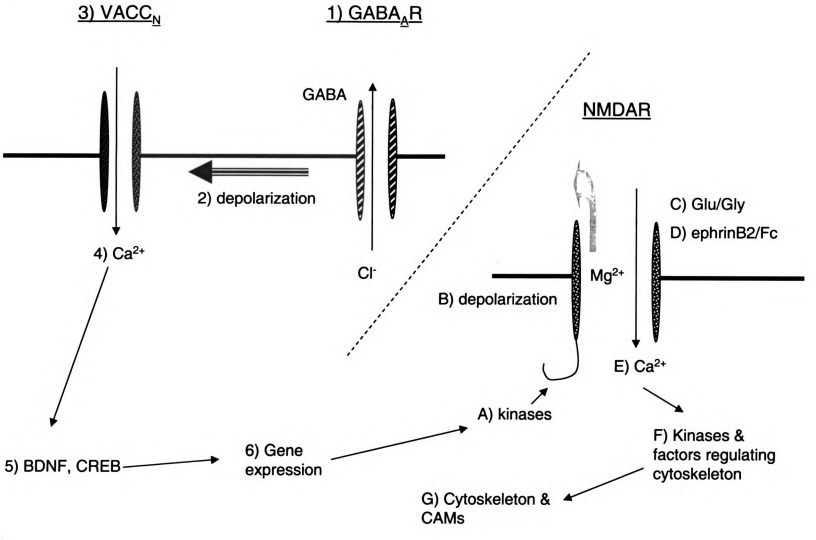

3) VACC$_N$
1) GABA$_A$R
GABA
NMDAR
2) depolarization
C) Glu/Gly
D) ephrinB2/Fc
4) Ca^{2+}
Cl^-
Mg^{2+}
B) depolarization
E) Ca^{2+}
A) kinases
5) BDNF, CREB
6) Gene expression
F) Kinases & factors regulating cytoskeleton
G) Cytoskeleton & CAMs

Figure 6.1. Pictoral representation of dissertation results and putative interactions between the $GABA_A$ and NMDA receptor. Stimulation of the $GABA_A$ receptor (1) depolarizes the plasma membrane (2), which activates VDCCs (3). The influx of Ca^{2+} (4) through the VDCCs activates numerous kinases and transcription factors (5), which influence gene expression (6). Kinases and newly expressed proteins modify the NMDA receptor (A). The NMDA receptor is activated and opened by membrane depolarization induced by an unknown source (B) as well as glutamate and glycine binding (C). The NMDA receptor is potentiated by neurotrophic factors such as ephrinB2/Fc (D). The open NMDA receptor channel allows Ca^{2+} (E) to enter the cell, which activates co-localized factors (F) that regulate the cytoskeleton. Modified organization of the cytoskeleton and cell adhesion molecules (CAMs) (G) moves the cerebellar granule cell leading process and/ or soma.

have died or been dying if kept for imaging longer than 45 min. Loss of plasma membrane integrity is a sign of cell death, which could explain the loss of definable membranes on granule cells imaged after 30 min of MeHg exposure.

The $GABA_A$ receptor in non-synaptic, immature cerebellar granule cells is affected by MeHg. Submicromolar levels of MeHg may alter $GABA_A$ receptor functon (See Chapter One). In Chapter Four, 20 μM MeHg was used in oerder to quantitate $[Ca^{2+}]_i$ changes before the granule cells leaked Ca^{2+} -indicator dye. The results suggest that MeHg alters $GABA_A$ receptor function and thereby prevents $GABA_A$ receptor-mediated activation of VDCCs. In the external germinal cell layer, a characteristic MeHg-induced stimulation of the $GABA_A$ receptor prior to block was identifiable. Application of muscimol in the presence of MeHg initally induced an increase in $[Ca^{2+}]_i$ that was greater than that caused by either chemical alone. This phenomenon could result from $GABA_A$-mediated opening of VDCCs in addition to MeHg-induced increases in $[Ca^{2+}]_i$. Subsequent applications of muscimol appear to lower the $[Ca^{2+}]_i$ below the levels induced by MeHg alone, suggesting that muscimol somehow amplified or facilitated block of the receptor by MeHg. The stimulation and subsequent block of the $GABA_A$ receptor at low concentrations could be one mechnism by MeHg disrupts Ca^{2+} regulation, particularly in the external germinal cell layer (Fig. 4.1). Because MeHg alters $GABA_A$ receptor-mediated opening of VDCCs and Ca^{2+} influx through VDCCs facilitates migration, MeHg effects on the GABA could be one way in which MeHg impairs cerebellar granule cell migration.

Another mechanism by which MeHg may impair cerebellar granule cell migration is by antagonism of the NMDA receptor by Zn^{2+}. As mentioned previously, MeHg

displaces Zn^{2+} from proteins leading to an increased $[Zn^{2+}]_i$ (Denny and Atchison, 1995). Zn^{2+} is an allosteric inhibitor of of polyamine-sensitive sites on NMDA receptors other than the site of action of ifenprodil (Berger and Rebernik, 1999). The MeHg-induced increase in $[Zn^{2+}]_i$ could inhibit the NMDA receptor and, thereby, impair migration.

Subchronic, low-level MeHg exposure during development

It was important to show that subchronic MeHg poisoning causes more severe impairment of cerebellar granule cell migration at lower concentration than following more acute conditions because of the chronic pattern of poisoning is more common today than in the past. The most prominent reactions of immature neurons to acute submicromolar concentrations of MeHg are an increase in $[Ca^{2+}]_i$ (Limke et al., 2003), stimulation and then irreversible block of the $GABA_A$ receptor (Yuan and Atchison, 2003a), and reversible neurite retraction (Heidemann et al., 2001). The human brain is adaptable in many ways to a low level, acute assault by MeHg. Cerebellar granule cells can regain control of $[Ca^{2+}]_i$ levels and repair themselves. Although the effect of MeHg on $GABA_A$ receptors is irreversible, the cell could be able to repair itself and express more $GABA_A$ receptors. MeHg induced the retraction of neurites in developing forebrain neurons at MeHg levels (0.5 and 0.25 μM) that did not induce cell death (Heidemann et al., 2001). Following an acute exposure, cerebellar granule cells re-extend neurites and continue normal development. If unable to prevent cell death, new granule cells can be generated from surviving progenitor cells of the outer external germinal cell layer. These newly formed cells can migrate and mature in a fairly normal manner. However, if the MeHg exposure is continuous throughout cerebellar granule cell migration (P7-15 in rats and 6 months gestation up to 4 years of age in humans), the existing immature granule

cells may not be able to recover from MeHg's neurotoxic effects, and newly formed granule cells would be unable to migrate.

The results of Chapter Four show that MeHg impaired migration in all cortical layers. Previous studies showed that the frequency of Ca^{2+} elevations and the rate of cell movement was significantly reduced at each boundary between cerebellar cortical layers. Moreover, it appears to result from specific neurotrophic factors such as somatostatin, to which migration responds in a stage-specific manner (Yacubova and Komuro, 2002; Kumada and Komuro, 2004; Komuro and Kumada, 2005). Somatostatin increased the amplitude and rate of Ca^{2+} fluctuatutions and migration in the external germinal cell layer and molecular layer, but slowed migration in the inner region of the internal granule cell layer (Yacubova and Komuro, 2002). The uniform impairment induced by MeHg is unlike manipulation of specific neurotrophic factors, and probably occurs as a result of toxic effects on an intracellular mechanism that mediates migration in all layers. The disrupted mechanism is most likely Ca^{2+} -oscillations and/or the factors mediating Ca^{2+}-oscillations.

The effect of exposure to 0.2 μM MeHg on cerebellar granule cell migration for 3 d may shed light on mechanisms of MeHg neurotoxicity. There was a slight, though insignificant, increase in migration into the internal granule cell layer following 0.2 μM MeHg treatment for 3 d (Fig. 4.2). This increase could have occurred as a result of MeHg-induced release of Ca^{2+} from intracellular stores. This resultant increase in $[Ca^{2+}]_i$ may then have signaled the cell to migrate despite extrinsic factors because continuous increase in $[Ca^{2+}]_i$ from intracellular stores by caffeine or thimerosal treatment have previously been shown to alter migration of granule cells in a variety of ways. The

exogenously-induced release of Ca^{2+} from intracellular stores caused accelerated cell movement at the bottom of the internal granule cell layer, changed the direction of migration and induced backward movement of the cells (toward the Purkinje cell layer–internal granule cell border), and/or significantly delayed the completion of migration (Kumada and Komuro, 2004; Komuro and Kumada, 2005). Neither caffeine nor thimerosal changed the Ca^{2+} transient frequency or the cell motility at the top of the internal granule cell layer. Therefore, a sustained increase in $[Ca^{2+}]_i$ does not necessarily lead to a normal completion of migration and maturation of cerebellar granule cell, which could explain why treatment with 0.2 μM MeHg for 7 d did not incease migration. Transduction of the extrinsic signal may well be impaired by a sustained MeHg-induced increase in $[Ca^{2+}]_i$.

Another mechanism by which MeHg could cause a temporary increase in migration is by metallic ion substitution. MeHg could be demethylated within neurons yielding Hg^{2+} (Chang and Verity, 1995; Klaasen, 1996), which in turn could substitute for other metals (Klaasen, 1996) or MeHg itself may bind cysteine resisidues and displace previously bound metallic ions. Mn^{2+} has been shown to increase migration rate of neurons at 0.2 μM Mn^{2+}, but can kill the cells at slightly higher concentrations (Vallar et al., 1999). If MeHg displaced Mn^{2+} or reacted with the Mn^{2+}-dependent proteins, MeHg could mimic a Mn^{2+}- induced increased migration rate. In conclusion, developing cerebellum exposed to 0.2 μM MeHg for 3 or more days may not have a well developed adult granule cell layer.

Our results support the hypothesis that repeated exposure to submicromolar levels of MeHg throughout the normal developmental time period for migration significantly

impairs cerebellar granule cell migration. Migration out of the external germinal cell layer was impaired by continuous exposure to 0.5 μM MeHg, which is approximately 40 times less than the concentration that produced acute symptoms in Iraqi people in 1971-2 (Bakir et al., 1973; Limke et al., 2004a). At 8 DIV, the organotypic cultures were representative of *in vivo* cerebellum at P16 in rat or 30 weeks gestation in humans (Altman and Bayer, 1997; Miyamoto et al., 2001). According to the present and previous studies, the developing nervous system is more susceptible to MeHg-induced subtle behavioral abnormalities than the adult central nervous system (Miyamoto et al., 2001; Baraldi et al., 2002). Continuous or repeated exposure of the brain to submicromolar levels of MeHg as a result of ingestion of seafood by pregnant mothers or breast-milk by children could result in a similar impairment.

Implications in humans

The current US EPA reference dose of MeHg in the developing nervous system is 0.1g/kg (Rice et al., 2000). Reports have shown that in 2000, > 300,000 newborns in the United States may have been exposed *in utero* to MeHg concentrations higher than those considered to be without increased risk of adverse neurodevelopmental effects associated with MeHg exposure (Mahaffey et al., 1999 and 2000). Women can eat more than two fish or shellfish meals per week and have lower maternal blood-MeHg levels than that which would cause symtoms (EPA). However, these same MeHg levels in pregnant women may be high enough to impair cerebellar granule cell migration during the third trimester of gestation and during breast feeding. Even at these submicromolar levels, delayed or permanently impaired developmental milestones could occur due to delayed synaptogenesis and maturation of cerebellar granule cells in the internal granule cell

layer. The impaired development of the cerebellar granule cells reduces the excitatory input into the relatively MeHg-resistant Purkinje cells. The Purkinje cells, the sole output of the cerebellar cortex, then have less inhibitory firing onto neurons in the cerebellar deep nuclei such as the fastigial nucleus and dentate nucleus. Neurons from the fastigeal nucleus run in the vestibulocerebellar tract for coordination of head, neck and eye movements and spinal tracts for regulation of muscle tone and execution and coordination of limb movements. Neurons from the dentate nucleus run in the dentatorurothalamic tract to cerebral cortex for regulation of the planning, initiation, timing, and coordination of discrete movements of the limbs, eyes, and vocal aparatus and in the olivocerebellar tract through the central tegmental tract, which may be involved in motor learning (Watson, 1995). Impairment of cerebellar output to these tracts could account for the symptoms often attributed to MeHg poisoning.

Submicromolar levels of MeHg disrupt Ca^{2+} homeostasis and microtubule stability, both of which are critically involved in neuronal migration. Ca^{2+} regulates many intracellular protein kinases, phosphatases, and molecular machinery. MeHg also affects membrane-associated receptors. Further research is needed to understand the consequences of chronic, submicromolar exposure to MeHg with respect to the molecular mechanisms involved in neuronal migration.

The results presented here suggest that blockade of $GABA_A$ receptor-mediated activation of N-type VDCC is one pathway by which submicromolar levels of MeHg can begin to impair cerebellar granule cell migration. MeHg could impair the NR2B-NMDA receptor-mediated pathway to Ca^{2+} - oscillations if IP_3 receptor is involved because MeHg affects the IP_3 receptor (Limke et al., 2003).

Limitations

One limitation of the experiments in Chapter Four was the high level of MeHg used. The high concentrations were used in order to obtain a clear response to MeHg before Ca^{2+}-indicator dye leaked out of the cell and/or the cell began to die. 10 μM MeHg is 20 times higher than the level at which impaired migration following continuous exposure was observed in Chapter Four. Also at this [MeHg], the $GABA_A$ receptor is not the only protein or even one of a few proteins affected. VDCCs would be highly effected at this concentration, which confound the results of $[Ca^{2+}]_i$ analysis.

Other limitations of the experiment in Chapter Four were the use of a low magnification (40x), and the averaging of a large number of optical frames. Averaging more than 50 optical frames in a series of frames through the depth of the slice (the z-series) may have reduced the sensitivity of the analysis. This aspect was improved in the experiments of Chapter Two by using a 63x objective and taking no more than 40 optical frames per z-series.

Future studies

One study that would be an appropriate follow-up would be to use an inverted laser confocal microscope with an incubation chamber mounted on the stage to conduct Ca^{2+}-imaging studies on live, healthy organotypic slice cultures of developing cerebellum. Investigations of Ca^{2+}-oscillations in the presence of MeHg and/or the pharmacological probes/manipulations used in Chapter Three in actively migrating granule cells would provide a plethora of information regarding mechanisms in cerebellar granule cell development. Experiments could elucidate which factors lead to activation

of the NR2B-NMDA receptor, BDNF's role in regulating $GABA_A$ and NMDA receptor expression, and NR2B-NMDA receptor-cytoskeletal interactions during active migration. The effects of low-level MeHg exposure on $GABA_A$ receptor-mediated Ca^{2+} changes could be explored further. In addition, the functions of glia that are affected by MeHg during migration could be explored.

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