Behavioral Effects of Calcium Channel Blockers: Acute Exposures and Neuroprotection Against Methylmercury Neurotoxicity

by

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Abstract

The maintenance of intracellular calcium homeostasis is among the most important homeostatic functions of the nervous system as it allows the calcium ion to function as a chemical messenger. Often damage to the central nervous system (CNS) disrupts intracellular calcium homeostasis, causing increased intracellular concentrations of calcium ions, which result in cellular dysfunction and/or death and manifests as changes in the organism's behavior. Calcium channel blockers (CCBs) have been touted as "cognitive enhancers" but result in divergent effects on behavior that seem to depend upon the presence or absence of nervous system insult. When administered in the absence of CNS injury CCBs often have deleterious effects, are beneficial or no effect at all on behavior. However, in the presence of a variety of CNS injuries resulting in increased intracellular calcium, CCBs reliably offer protection. Here, CCBs were administered acutely to healthy, adult mice to determine their effects in the absence of CNS injury. Following that, one dihydropyridine CCB (nimodipine) was administered chronically to mice with and without chronic co-exposure to 15 ppm Methylmercury (MeHg), a toxicant known to elevate intracellular calcium concentration and cause cognitive and motor impairments. When administered acutely to healthy animals CCBs either had no effect or reduced responding and accuracy on an incremental repeated acquisition (IRA) procedure, depending on dose. When administered chronically to MeHg exposed animals, nimodipine prevented the declines in performance and responding on IRA. Importantly, nimodipine had no effect on any of these endpoints when administered to the non-MeHg control animals, suggesting

a MeHg-dependent effect of nimodipine. The series of experiments presented here support the hypothesis that CCBs are exclusively beneficial when CNS injury is present. Additionally, the beneficial effects of nimodipine + MeHg exposure support the hypothesis that MeHg-induced changes in calcium homeostasis mediate the behavioral toxicity seen with MeHg exposure.

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$[Ca^{++}]_e$	Extracellular Calcium Ion
$[Ca^{++}]_i$	Intracellular Calcium Ion
5-HT	5-hydroxytryptamine
ACh	Acetylcholine
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
ATP	Adenosine-5'-triphosphate
Balb/c	Bagg's Albino Strain
Ca^{++}	Calcium Ion
CCB	Calcium Channel Blocker
CD-1	Caesarian Derived-1 Strain
CNS	Central Nervous System
DA	Dopamine
DRL	Differential Reinforcement of Low Rate
DBA	Dilute Brown Non-Agouti Strain
EAB	Experimental Analysis of Behavior
FAA	Free Arachidonic Acid
FI	Fixed Interval
FR	Fixed Ratio
HIV	Human Immunodeficiency Virus

IRA	Incremental Repeated Acquisition
K_d	Dissociation Constant
LD_{50}	Lethal dose in 50% of population
LGCC	Ligand-gated Calcium Channel
LTP	Long-term Potentiation
MeHg	Methyl Mercury
NMDA	*N*-methyl *D*-aspartate
PQ	Progress Quotient
ROS	Reactive Oxygen Species
SER	Smooth Endoplasmic Reticulum
SHR	Spontaneously Hypertensive
VGCC	Voltage-gated Calcium Channel
Zn^{++}	Zinc Ion

Chapter 1: Calcium Signaling in the Central Nervous System and the Role of Calcium Antagonists in Behavior

Intracellular calcium movement

Movement of the calcium (Ca^{++}) ion into and out of the cytoplasm functions as a signal for many cellular processes including neurotransmitter release, neurite outgrowth and synaptogenisis, as well as cell survival and plasticity in the nervous system. In the adult nervous system, calcium influx into the presynaptic dendrites is a critical event in long-lasting synaptic changes associated with learning and memory processes (e.g. long-term potentiation (LTP)). But the functional role of calcium is not restricted to synaptic events as mechanisms that rely on Ca^{++} movement are also involved with gene transcription and expression (Hu et al., 1993; Hu & Wieloch, 1993).

Ca^{++} ions enter the cell via a variety of calcium-permeable ion channels located on the plasma membranes of the cell and of internal organelles (e.g. mitochondria). Voltage-gated calcium channels (VGCCs) are a group of ion channels that are sensitive to changes in membrane potential, and are found on the plasma membrane of excitable cells (e.g., muscle, glial cells, neurons, etc.). At physiologic membrane potential VGCCs are closed, but are activated (i.e., opened) at depolarizing membrane potentials. VGCCs are complex proteins composed of $\alpha 1$, $\alpha 2$, β, γ and δ subunits. The $\alpha 1$ subunit forms the calcium-conducting pore, while the rest of the subunits modulate pore function during the process of calcium ion influx in excitable cells (Catterall, 2000; Ertel et al., 2000; Horn, 2000). VGCCs are classified into high-voltage activated channels including the L, N, P/Q and R-type channels and low-voltage activated channels

represented by T-type channels. A heterogeneous group of compounds, calcium channel blockers (CCBs), antagonize VGCCs.

Ca^{++} ions also enter the intracellular space via ligand-gated calcium channels (LGCCs), which open or close in response to the binding of a chemical messenger, such as a neurotransmitter. These too are found on the plasma membrane, but are also present on various intracellular membranes. Structurally LGCCs are complex and some, like those that make up the NMDA receptor, are involved with synaptic plasticity. The release of Ca^{++} from internal pools of sequestered calcium like those contained in the mitochondria and the smooth endoplasmic reticulum (SER) occurs via LGCCs located on the membrane of these organelles.
Although transient increases in $[Ca^{++}]_i$ are necessary for neuronal signaling, uncontrolled increases or decreases in $[Ca^{++}]_i$ can result in neuronal dysfunction and death. In normal, healthy cells any change in intracellular calcium concentration that occurs via voltage-gated calcium influx, ligand-gated calcium influx or intracellular calcium release always decays back to baseline levels (Conner et al., 1988; Brown & Jaffe, 1995). This decay back to baseline is achieved primarily by endogenous calcium buffers, calcium extrusion and calcium sequestration but is also accomplished via the activity of various calcium ion pumps and Na+/Ca^{++} ion exchangers coupled to the plasma membrane (Carafoli, 2002).

Although delicately and precisely regulated when young and in good health, failures in these processes of calcium regulation have been shown to occur following a variety of CNS insults (e.g. brain trauma and cerebral ischemia, environmental toxicant exposure, HIV/Aids, and certain diseases of aging like Parkinson's, Huntington's and Alzheimer's as well as the normal process of brain aging). Destabilizing the highly regulated mechanisms of Ca^{++} homeostasis initiates cellular dysfunctions including protein phosphorylation abnormalities, impairment of

neurotransmission, membrane-function damage, the production of toxic glutamate levels and alterations in neuroimmune and neuroendocrine systems (Mattson et al., 1992). Many of these processes can then lead to neural degeneration via apoptotic or necrotic cell-death pathways (Brown & Jaffe, 1995). Additionally, excess intracellular calcium depletes the metabolic resources that regulate intracellular calcium levels, leading to even further dysregulation and accumulation of calcium ions.

The consequence of such cellular dysfunction and death manifests as behavioral impairment. During aging this appears as slowing motor speed (Marriott & Abelson, 1980; Medin et al., 1974; Bartus et al, 1974; Burwell & Gallagher, 1993; Houx & Jolles, 1993; Wilkinson & Allison, 1989) but also as reduced accuracy on procedures that are thought to measure learning- or memorial-processes like impaired acquisition of conditioned eye-blink response (Graves & Solomon, 1985; Woodruff-Pak et al., 1987), poor maze performance (Doyere et al., 2000), perseveration on discrimination reversal procedures (Means & Holsten, 1992) and impairment on timing tasks (Coelho et al., 2004; Vennesta & Pouthas, 1999). Also, intra-individual variability increases with age on many tasks (Smith et al., 1998; Balota et al., 2004; West, 1996; West et al., 2002), including some of those mentioned above. Interestingly, a few reports have indicated that aged animals are less sensitive to reinforcer frequency than younger animals (Tripp & Alsop, 1999; Sanford, 1978) and this reduction may have contributed to some of the apparent learning deficits described above.

As with aging, MeHg exposure results in increased intracellular concentrations of calcium (Landfeild, 1987; Verkhratsky & Toescu, 1998; Atchison & Hare, 1994) and corresponds to much of the same behavioral impairment, including sensory disturbances (Merigan, 1980; Rice, 1996; 1998; Rice & Gilbert, 1982; 1990; 1992) motor dysfunction (Cox et

al., 1989; Harada, 1995) and learning or memory deficits (Newland et al., 1994; Newland et al., 1996; Newland et al., 2004; Sakamoto et al., 2004; Cuomo et al., 1984; Sakamoto et al., 2002; Zanoli et al., 1994) including perseveration on discrimination reversal procedures (Reed et al., 2006; Paletz et al., 2007; Widholm et al., 2004; Dore et al., 2001). Similar too are the behavioral impairments that arise following cerebral hypoxia Nyakas et al., 1990; Yanpallewar et al., 2004), bilateral hippocampal lesion (Finger et al., 1990), and in spontaneously hypertensive rats (Meneses et al., 1997), all of which are conditions associated with increased intracellular concentrations of calcium ions.

Calcium Channel Blocking Drugs

Clearly, calcium regulation is important for proper cellular function and a dysregulation in intracellular calcium homeostasis results in deleterious effects on the cell with resulting impairments in with behavior. Because the majority of the dysfunctions associated with alterations in calcium homeostasis occur following an *elevation* in intracellular calcium concentrations, the use of compounds that block the influx of calcium ions from the extracellular space have been hypothesized to prevent elevations in cytosolic-free calcium concentrations and thus prevent the cellular and behavioral impairments associated with such calcium dysregulation. The hypothesis that CCBs may protect or prevent abnormal increases in cytosolic calcium is consistent with the experimental evidence that CCBs are beneficial in treating and preventing cellular and behavioral impairments that follow CNS injuries associated with elevated cytosolic calcium. This hypothesis would not, however, suggest that CCBs should be viewed as general cognitive enhancers (as has been done in the literature), rather it suggests that CCBs should only be helpful when calcium levels are disrupted, (such that CCBs act to normalize the increased cytosolic calcium levels induced by CNS injury) and that the administration of CCBs to an

uninjured CNS (one in which proper calcium homeostasis is maintained) should act to disrupt that homeostasis by preventing the necessary influx of extracellular calcium ions through voltage-gated calcium channels that is necessary to maintain proper calcium homeostasis. In fact, this prediction is consistent with some of the CCB literature (reviewed below).

The group of compounds that are most often used to reduce elevations in intracellular calcium are CCBs, which represent a heterogeneous group of drugs that are subdivided into four classes based largely on their therapeutic usage, chemical structure and pharmacokinetic profile. These include the dihydropyridines, phenylalkyamines, benzothiazapines and difluorinated piparazines. Drugs in all four classes block calcium entry at specific L-type channels on neuronal cell bodies and cerebral vasculature as well as on cardiac smooth muscle and peripheral vasculature (Bean, 1989; Miller, 1987). As such, the CCBs are potent vasodilators and are used clinically to treat a wide range of cardiovascular and neurological conditions including angina, hypertension, cerebrovascular disease and migraine. The dihydropyridine CCBs in particular have been investigated as possible treatments for age-associated impairments in behavior and it is a drug from this subclass, nimodipine, which will be highlighted in the present review. While nimodipine is one of the most commonly used CCBs in the literature, verapamil, isradipine, nifedipine, diltiazem, amlodipine and flunarizine are also investigated.

Nimodipine is a 1,4-dihydropyridine compound that readily passes the blood-brain-barrier (Van den Kerckhoff & Drewes, 1985) and binds, with high affinity (Kd = 1.1 nMolar in displacement studies), and specificity to the dihydropyridine receptors on L-type calcium channels (Belleman et al., 1982; Dompert & Traber, 1984; Peroutka & Allen, 1983). Functionally, this blocks the calcium current through these channels. Through this calcium antagonism, nimodipine interferes with many cellular processes including neuronal excitability,

neurotransmitter release, axonal transport and the activity of enzymes that are calcium-dependent. However, it is though this same antagonism that nimodipine attenuates the impairment associated with various CNS insults. Despite this highly replicated attenuation following insult, the effects of these compounds in young, healthy animals is less clear. In fact, their effects may depend upon the cellular environment into which they are administered such that the beneficial properties of CCBs may only appear when damage to the CNS is present. Their administration may cause harm or be of no consequence in the absence of CNS injury. To this end, the present review will focus on the effects of CCB administration following injury to the CNS and in the absence of CNS injury.

CCB administration in the presence of CNS injury is beneficial

A number of nervous system injuries result in cell dysfunction and cell death, which corresponds to behavioral impairment. A large subset of these injuries involves uncontrolled increases in intracellular calcium concentration, which is thought to underlie the cellular dysfunction and destruction that accompanies such injury. In cases for which increases intracellular calcium concentration is the mechanism from which dysfunction arises, CCBs have proven to be valuable drug of treatment. CCB protection has been shown experimentally in MeHg toxicity (Sakamoto et al., 1996), glutamate toxicity (Sucher et al, 1991), AMPA+Zn^{++} toxicity (Freund & Reddig, 1994), HIV infection (Lipton, 1994; Dreyer et al., 1990), brain ischemia (Uematsu et al., 1989), medial septal (Schuurman et al., 1986) and bilateral hippocampal lesion (Finger et al., 1990), normal aging and models of age-related disease (Schuurman & Traber, 1989; Deyo et al. 1989; Disterhoft et al., 1989, Sandin et al., 1990) among others. Recently, nimodipine has also been classified as a ‘cognitive enhancer’ by some

researchers, who have found that it improves the cognitive deficits associated with a wide range of brain traumas (Finger et al., 1990; Izquierdo, 1990; Sansone et al., 1999).

With respect to MeHg toxicity, there is substantial evidence that nimodipine protects against numerous endpoints of MeHg toxicity but, with a single exception, this evidence comes from *in vitro* studies. The relevance of this protection to behavior is not well understood. In the single exception, Sakamoto et al. (1996) administered various CCBs to rats exposed to MeHg and applied CCBs to rat cerebellar cells concurrently exposed to MeHg. In vitro, several L- and T-type CCBs were administered, including flunarizine (T-type), nifedipine (L-Type), nicardipine (L-Type) and verapamil (L-Type). All CCBs tested conferred some protection against MeHg's neurotoxicity (i.e. higher LD50 of MeHg in cerebellar granular cells), although flunarizine was most effective (Sakamoto et al., 1996). When rats were orally administered MeHg and CCBs concurrently, CCB-treated rats (particularly those receiving flunarizine) showed significantly fewer signs of toxicity. They had higher body weights, fewer instances of neurological disorders (like hind-limb cross) and lived much longer than their counterparts who did not receive CCBs (Sakamoto et al, 1996). Figure 1, taken from Sakamoto et al. (1996) shows the dose-dependent protection offered by flunarizine from MeHg toxicity in terms of body weight loss, a sign of severe toxicity. Fahey et al. (1989) showed that when cells incubated with nimodipine were exposed to methylmercury, the cells did not deteriorate and cell death did not occur to the same extent as it did in the cells not treated with nimodipine. By preventing the influx of calcium into the cell, which would lead to mitochondrial dysfunction, followed by a decrease in the production of ATP, nimodipine increased the survival of these MeHg-exposed cells (Fahey et al., 1989).

Similarly, nimodipine prevents age-related motor deficits in rats (Schuurman & Traber, 1989), enhances acquisition of conditioned eye-blink in aging rabbits (Deyo et al., 1989; Disterhoft et al., 1989), improves working memory in aging primates (Sandin et al., 1990) and improves water-maze performance in aged rats (Schuurman & Traber, 1994). Figure 2, taken from Schuurman et al. (1987), shows protection by nimodipine from age-related gait disturbances in rats. Note the striking improvement in gait from the 27-month old rat receiving nimodipine treatment.

CNS insults associated with increased intracellular calcium, aside from those that characterize aging and MeHg-toxicity, also benefit from CCB administration. Patients with HIV/Aids related dementia have experienced some modest cognitive improvement following the administration of nimodipine (Turchan et al., 2003) while in other patients dementia stabilizes following nimodipine administration (Navia et al., 1998). Nimodipine and nifedipine application reduced the increase in intracellular calcium that followed HIV-1 infection in a variety of cell types, including cerebellar granular cells, and as a result they sustained less cell damage from HIV-1 than those cells not treated with a CCB (Savio & Livi, 1993).

Cognitive impairment following hypoxic brain damage is similarly attenuated by CCB administration. Prenatal exposure to nitrates (a situation that induces hypoxia) is associated with deficits in auditory and visual discrimination and impaired retention of a passive avoidance response in young rats. Concurrent prenatal exposure to nitrites and nimodipine, however, attenuates these deficits (Nyakas et al., 1993). Similar benefits of nimodipine are seen when hypoxia is induced during adulthood via brief asphyxiation and reperfusion. Without nimodipine, hypoxia induces poor Morris water maze performance and diminished movement in an open field but nimodipine-treated hypoxic animals had shorter latencies to find the submerged

platform in Morris water maze and were scored as having less “anxious” and “listless” behavior in the open field than the non-treated hypoxic animals (Yanpallewar et al., 2004). At the cellular level, hypoxia causes an increase in brain free arachidonic acid (FAA) level, which is correlated with decreased performance on a number of tasks, including a passive avoidance retention task (Mršić et al., 1996). Doses of nimodipine that prevent FAA accumulation also improved performance on a retention test of passive avoidance (Mršić et al., 1996).

While hypoxia induces widespread damage to all brain regions, damage to specific brain regions has also been treated with CCBs. Specifically, the brain damage following bilateral hippocampal lesion has been attenuated by nimodipine when administered following the lesion surgery. Rats not receiving nimodipine after lesion surgery earn fewer reinforcers and are less “efficient” on both DRL 20s and 40s schedules than are nimodipine-treated and sham-lesioned animals (Finger et al., 1990). In fact, nimodipine treatment for the 14 days following lesion surgery resulted in performance indistinguishable from that of the sham-lesion rats (Finger et al., 1990). This effect is demonstrated in Figure 3, which shows the improvement offered by nimodipine during the DRL 20s schedule in hippocampus lesioned rats. Relatedly, patch voltage-clamp studies have demonstrated a high affinity of nimodipine for voltage-gated L-type calcium channels in the hippocampus, where it appears nimodipine potently blocks the depolarization-induced increase in free calcium in the cell body of hippocampal neurons (Scriabine et al., 1989), thus preventing cell dysfunction or death and the corresponding behavioral impairments. Taken together, the clinical uses of nimodipine in CNS injured humans and animals are overwhelming and span a range of CNS injuries and offer protection at both the behavioral and cellular level. Table 1 summarizes this literature.

CCBs have no effect or are deleterious in the absence of CNS injury

Despite the consistent finding that CCBs are beneficial when administered in the presence of a wide variety of CNS injuries, which is attributable to a correction in Ca^{++} homeostasis, a coherent theoretical or mechanistic explanation is missing for the divergent effects seen when the administration of CCBs occurs in the absence of CNS injury. In fact, conflicting evidence suggests that CCB administration to young, intact animals has no effect, is beneficial or is deleterious on cognitive and/or motor endpoints. While only a few studies to date have explicitly assessed the effects of CCBs on healthy animals, the control and untreated groups from many of the studies of protection against impairment discussed above provide useful for addressing this question. The group of 3 month old rabbits from Deyo et al. (1989) served as the control group in an aging study in which both the 3 month old and 37 month old rabbits were administered nimodipine via micro-infusion. While the nimodipine-treated aged rabbits had significantly improved acquisition of conditioned eye blink response, the young rabbits who received the same administration of nimodipine were indistinguishable from the young animals not receiving nimodipine. See Figure 4, taken from Deyo et al. (1989). Similarly, Nomura (1988) reported that young intact Wistar Kyoto rats performed equally as well with and without acute nimodipine treatment on a brightness discrimination task. However, SHR (spontaneously hypertensive rats) benefited significantly from the administration of nimodipine. Finally, Vetulani et al. (1997) reported no effect of acute nimodipine administration on young intact C57BL/6 or CD-1 mice on a shuttle-box avoidance task. They did, however, report that one dose (1.0 mg/kg) of nimodipine improved the performance of DBA mice on this task, but all other doses had no effect on this strain (Vetulani et at., 1997).

Perhaps of greater clinical importance are the studies indicating that CCB administration in the absence of CNS injury actually has deleterious effects on the organism. Maurice et al. (1995a) demonstrated that when young, intact male Swiss mice were given nimodipine before a variety of simple learning tasks, they performed more poorly than control mice. Specifically, the nimodipine-treated mice had impaired performance on a spontaneous alternation Y-maze task at doses of 0.3-1.0 mg/kg, had decreased step-down latency in a retention test of passive avoidance at 0.3-3.0 mg/kg and impaired place-learning in a water maze during both acquisition and retention of the task (Maurice et al., 1995a). Data from water maze performance for both groups are shown in Figure 5. These results were replicated in another study from this lab, in which it was shown that PRE-084, a phencyclidine derivative, attenuated the learning deficits induced by nimodipine (Maurice et al., 1995b).

Similarly, Cain et al. (2002) found that nimodipine and nifedipine administration significantly increased resistance to extinction of a conditioned fear response (i.e. freezing) in young intact C57BL/6 mice. Despite this impairment, nimodipine treated mice did not have deficits in the acquisition or expression of the conditioned fear response. Finally, visual discrimination has also been shown to suffer when nimodipine is administered to healthy animals. Deyo (1990) administered 0.5-5.0 mg/kg of nimodipine to 5 day old chicks. In a dose-dependent manner, nimodipine treated chicks took significantly more trials to reach the preset acquisition criterion than controls. During a retest (following 24 hr delay) these deficits were heightened. See Table 2 for a summary of the literature on CCB administration to young, healthy animals when it is of no effect or causes impairment.

CCBs may be beneficial in the absence of CNS injury

When administered to young, healthy animals some researchers have shown CCBs to accelerate the acquisition of conditioned eye blink response (Deyo et al., 1998), improve the retention of passive avoidance response (Quartermain et al., 2001; Vetulani et al., 1997), improve acquisition and retention of linear maze (Quartermain et al., 2001), 8-arm radial maze (Levy et al., 1991), Morris water maze (McMonagle & Fanelli, 1993) and Barnes circular platform (Kane & Robinson, 1999) task, and improve accuracy on a sequential automated arm-maze task (Martin et al., 2004). However, these effects may be transient and few studies tested the animals after the cessation of CCB administration.

Quartermain et al. (2001) has shown dose-dependent facilitation in young animals by a variety of CCBs on retention of passive avoidance response and retention of linear maze. Figure 6 was taken from this study and plots latency to enter a shock-component during a retention test (24 hr after a one-trial training session). Every CCB, except verapamil, produces a dose-dependent increase in latency to enter the shock compartment. Similar improvement was demonstrated when retention of a linear maze task was measured, therein representing two distinct types of learning that benefited from this administration: a appetitive spatial discrimination (linear maze) and shock-avoidance behavior.

Table 3 summarizes the studies demonstrating CCB-associated enhancement in young healthy animals, which are used as evidence for the hypothesis that CCBs are general cognitive enhancers. However, even those who advocate this hypothesis do not know the mechanism by which this enhancement might occur. Presumably CCBs act to correct a dysfunctional process of calcium regulation that leads to excess intracellular calcium, but the young, intact organism presumably does not have a dysfunction in calcium regulation and therefore CCB administration

should act to *disrupt* calcium homeostasis in these organisms. For this reason it has been suggested that the benefits of CCBs are restricted to the case of CNS injury (e.g. Maurice et al., 1995a; Cain et al., 2002). Others (e.g. Deyo et al., 1998; Kane & Robinson, 1999; Martin et al., 2004), however, contend that via intracellular calcium reduction, CCBs promote healthy cell function and optimize cellular performance even in the absence of any dysregulation. One hypothesis is that by lowering Ca^{++} entry through VGCC's an improvement in the signal to noise ratio is improved from the Ca^{++} that enters through LGCC's.

It seems a reasonable starting point, when looking for an explanation for why inconsistencies in the literature occurs, is an analysis of the methodological differences between the studies that show enhancement and those that show no effect or impairment (e.g. dosing regimen, species, behavioral task, etc.). To facilitate this comparison Tables 1, 2 and 3 are similarly structured and include all relevant methodological information. A plausible hypothesis may be that dose and duration of exposure (i.e. acute vs. chronic exposure) to CCB treatment may be a relevant factor in determining the effects of CCBs in young healthy animals. A number of studies that report enhancement used a chronic dosing regimen (Kane & Robinson, 1999; Levy et al., 1991; Martin et al., 2004; McMonagle & Fanelli, 1993) while the studies that report no effect or impairment used an acute dosing regimen (Normura 1998; Vetulani et al., 1997; Deyo, 1990; Cain et al., 2002; Maurice et al., 1995a; Maurice et al., 1995b). Why chronic exposure would yield benefits over acute administration is not known, however it seems plausible that acute administration may disrupt cellular calcium homeostasis repeatedly whereas chronic administration may allow for a stable reduction of intracellular calcium.

Mechanism of action of Nimodipine

Nimodipine has numerous clinical uses, primarily for the treatment of hypertension and cerebral ischemia but it is not currently prescribed therapeutically for the treatment of age- or disease-related learning or memory impairment. Further, there is considerable evidence that dihydropyridines (like nimodipine) have beneficial physiological consequences in the treatment of both hypertension and stroke (Betz et al., 1985; Scriabine et al., 1989) however, the mechanisms by which these physiological effects occur are probably different. For example, it is likely that reduced tissue contraction benefits hypertension but it is the reduction of cell death by inhibiting calcium overload which benefits stoke victims if, that is, the drug is administered within ~12 hours of the ischemic episode. However, dihydropyridines also have considerable behavioral effects, but these are often transient in nature and depend upon the systemic presence of the drug in the central nervous system, and include learning- or memory- effects.

It has been suggested that the CCBs may enhance learning or memorial processes via a direct blockade of L-type calcium channels on neurons. Disterhoft and his associates have shown that concentrations of nimodipine which facilitate eye-blink conditioning in rabbits reduce the after-hyperpolarization which follows bursts of action potentials, thereby increasing neuronal firing rate (Thompson, Deyo, & Disterhoft, 1990; Disterhoft, Moyer, & Thompson, 1994; Disterhoft, Thompson, Weiss, Moyer, Van der Zee, Carrillo, Kronforst- Collins, & Power, 1995). These data raise the possibility that the facilitation of retention (or, memory) induced by CCBs in these studies could be the result of their enhancement of neuronal excitability.

A number of studies, including Levere & Walker (1991) (described above) have shown that the effects of nimodipine in aging animals are transient and only occur while the drug is being administered. Specifically, the behavioral benefit of nimodipine in these cases is probably

not due to the drug's ability to prevent cell death (like it is following some forms of traumatic brain insult, like a stroke) because if this were the case then one would expect to see continued improvement after the drug administration was terminated. However, in these studies (Levere & Walker, 1991; Sandin et al., 1990) that was not the case, rather, improvement only occurred during the weeks of nimodipine treatment in the aged animals (rodents and primates, respectively) and not the weeks following drug cessation (in these studies a CCB was administered for 2 weeks at each dose with a one week drug-free period in between each dose; performance during the off-weeks did not differ from control performance). Therefore, in the case of aging, it is more likely that the CCB temporarily changes how neurons function and this operational change benefits the memorial-process of aged animals when the drug is active. What exactly that operational change is, is still not clear – but the work of some electrophyisologists and cellular biologists has suggested that alterations in neurotransmitter release (a calcium mediated change) may be an important component to this improvement. In this way, nimodipine isn't halting cell death per se, but it is improving neuronal function in malfunctioning neurons. This hypothesis is supported by the study from Kabuto et al (1995) showing that nimodipine attenuated the age-related decline in neurotransmitter (DA, ACh, and 5-HT) release in mouse brain tissue.

There is also evidence of a certain degree of brain-region specificity with respect to the beneficial effects of nimodipine to the injured CNS. Age-related decline in learning and memory may be due at least in part to changes in the responsivity of hippocampal neurons to calcium. The integrity of the hippocampus is correlated with some types of learning (Lynch et al., 1983) and hippocampal cells appear to be very sensitive to changes in calcium homeostasis associated

with aging and dementias (Khachaturian, 1984; Landfeild, 1988; Landfeild & Pytler, 1984) and some hippocampal neurons are especially sensitive to acute ischemia (Simon et al., 1984).

However, CCBs also have profound effects on the vascular system and this is what underlies their clinical use and may also contribute to some of the behavioral changes seen following their administration. Yanpallwar et al. (2004) concluded that the enhanced cerebral blood flow and pronounced vasodilation in cerebral vessels that follows nimodipine administration improved brain metabolism in the early post-ischemic period. They offered this as the primary mechanism by which CCBs treat the behavioral impairment that follows cerebral ischemia. In fact nimodipine has been shown to have cerebrovasodilatory and anti-ischemic properties at doses that have little or no effect on peripheral circulation (Scriabine et al., 1990) this is because nimodipine is especially potent in dilating blood vessels in injured parts of the brain (Gaab et al., 1985; Kaxta et al., 1985). Also, as previously noted, nimodipine penetrates the blood-brain-barrier better than other dihydropyridines.

The potent vasodilating potential of CCBs has led to the hypothesis that they may be influencing learning or memory processes by enhancing blood flow to the brain (Deyo & Hittner, 1995). Some support for this explanation was provided by studies reported by Deyo and colleagues (Deyo & Hittner 1993, 1995) which showed that memory facilitation occurred when CCAs were administered peripherally but not when the vascular system was bypassed by injecting the drugs directly into the brain (via administration into to the cerebrospinal fluid).

The Importance of Behavioral Endpoints in Animal Models

Behavior is the primary means by which an organism interacts with its environment (Weiss & Cory-Slechta, 1994). To survive, organisms must be sensitive to events occurring in their environments and respond appropriately. To this end, the whole animal should be the

ultimate concern, rather than a particular brain region or specific neurotransmitter system. As Weiss (1978, pg 52) described it, the final criterion in any study of CNS insult is the whole animals' "…development, longevity, functional integrity, quality of performance, and its feelings." And because the whole animal is our interest, it is necessary that behavior is our subject matter as only it reflects the summed and integrated capacity of an organism to handle the environment in which it exists (Weiss, 1978).

While some laboratory assessments of behavior are designed to yield qualitative measures and to emphasize the broad scope of possible adverse effects in humans, other, more specific assays can target a specific function e.g. a specific impairment of learning (Weiss & Cory-Slechta, 1994). These, tasks are particularly useful in animal models of human behavior, as they *can be* (but are not always) highly controlled and reliable tasks. It seems reasonable to classify these 'specific' tasks on grounds of their methodology. When doing so, two groups seem to emerge: tasks grounded in the principals of operant conditioning (referred to here as studies from the experimental analysis of behavior (EAB) tradition), and those that are not. The latter are characterized largely, though not exclusively, by aversive control, one-trial learning and maze-learning. Such procedures, coming from other traditions, tend to be less refined or sensitive, and have utility when the rapidity of assessment is more important than identify behavioral determinants of action. Further, procedures using aversive control (e.g. water mazes) necessarily increase stress (and levels of corticosterone), which can influence the behavior seen. Reinforcement procedures bypass this process.

The former, however, is better characterized by appetitive control, steady-state performance and schedule controlled behavior. Operant tasks tend to offer the benefit of many (sometimes hundreds) of trials, yielding highly reliable data. They also emphasize 'free operant'

behavior which is more akin to human behavior than the 'discrete trials' used in other traditions. However, the discrete-trials employed by the other traditions are more akin to the psychological testing protocols used currently in neuropsychology. Finally, within the EAB tradition, highly quantitative procedures and fine-grained analyses are preferred and response units (e.g. a lever-press or hole-poke) are more readily definable, unlike that used in other studies of learning (e.g. climbing onto a platform, reaching a maze goal-box, etc.). The nature of EAB procedures allows for these response units to be used with great versatility, across many different types of procedures and these response units are also useful because their frequency or intensity (or 'strength') has room to change. Response rate, for example, of lever-pressing can increase with procedural variations unlike swimming behavior in a water maze, which occurs at maximal strength during baseline and cannot be altered with procedural manipulations. To this end, vigor or strength of response cannot be subtracted from the demonstration of memory or learning in maze (and similar) tasks. This is not problematic with rate measures in EAB procedures. Moreover, EAB tends to control variance experimentally not statistically by, for example, employing numerous trials, which enhances the reliability of these studies.

A significant problem facing any approach to measuring learning or memory is the role of motor function, which is intrinsically intertwined in tasks from both EAB and other disciplines. Not surprisingly, functional impairments in motor ability are a significant hurdle when attempting to draw conclusions about learning or memory from tasks that require motoric responses from the organism. Within EAB and other approaches, this is dealt with via a number of interventions. For EAB, the use of interval rather than ratio schedules can diminish the impact of motor function and employing specific experimental controls for motor function (for instance, by having certain control conditions that would detect motor impairment) and by plotting both

rate and accuracy-type measures so that motor changes could be detected are all frequently used tactics. Outside of EAB similar controls are often used to measure sensory and/or motor function separate from the learning task itself (for instance, stimulating paw-pads with shock to ensure tactile sensitivity before a shock-avoidance behavior is measured).

Finally, a distinction between EAB and other traditions may be in made with respect to *what* is actually measured during learning or memory tasks. The EAB approach has been successful in distinguishing motor function from learning and Nevin (1967) demonstrated rate-independent effects on accuracy during extinction trials. There, accuracy remained constant but the probability of responding steadily decreased over the course of 10 extinction trials. Figure 7 shows the differential effects of extinction on accuracy and response probability from this study.

These and other similar data have led to discussions regarding the appropriate endpoints for memory or learning tasks. It has been suggested that learning or memory tasks from EAB are much better suited for measuring directly the mechanisms involved in memory (e.g. the peak procedure, King et al. (2001)) than others that measure the 'performance' of memory (e.g. water maze). The factors that contribute to performance are numerous and are not restricted to just motor function, and include things like motivation, attention and health status (King et al., 2001). These variables may affect behavioral indices of memory but they are in fact functionally independent of memory (King et al., 2001). This may be a relevant criticism of non-EAB approaches, particularly maze-learning, whereas many timing procedures and other complex learning tasks are better suited for measuring memorial or learning behavior per se. With that said, motor function itself is often of interest as any reduction in the capacity for coordinated movement reduces an organism's ability to cope with the demands of its environment, such that even subtle defects will influence how effectively it functions. As is well known, learned motor

skills play an especially salient role in many human activities. Therefore, while it is requisite to have a measure of learning or memory that is independent of motor function for appropriate interpretations of the data, motor behavior itself is a useful indicator of CNS.

One operant task in particular is perhaps well-suited for assessing both motoric change and for measuring learning: incremental repeated acquisition of behavior chains (IRA). With this task both motor function and learning or memory can be assessed following the presence or absence of CNS injury. IRA is not, however, without its limitations as the use of an appropriate measure of learning can be difficult due to the complex nature of the response requirements.

Incremental Repeated Acquisition as a Means for Assessing the Behavioral Effects of Calcium Channel Blocking Drugs

Learning refers to behavior in transition from one state of behavior to another (Cohn & Paule, 1995; Newland & Reile, 1999; Sidman, 1960). This is an important distinction because learning is a transient event and this creates the need for a specialized task that can measure behavior in transition accurately. The incremental repeated acquisition of behavioral chain (IRA) procedures were developed to do just that. By directly measuring the acquisition of novel response sequences within a session these procedures repeatedly measure behavior that is in some degree of transition.

Specifically, IRA requires the subject to learn a different set of behavioral responses during each experimental session. This is accomplished by employing behavioral chains, which are essentially sequences of responses or response units. Any chained schedule of reinforcement has a different external stimulus associated with each component and requires the completion of a sequence of components for reinforcer delivery. This more closely resembles the complexities of the natural environment in which we are routinely faced with many contingent relationships

simultaneously (Weiss & Cory-Slechta, 1994). With IRA, this behavior chain is built within a session, so that chain-length becomes a measure of acquisition. This also allows for within-session changes in task difficulty, as the chain is made progressively longer throughout the experimental session.

IRA is typically composed of two components, learning and performance. It is during the learning component that the acquisition of a novel response chain is required for reinforcement delivery. Once in the performance component the animal is required to generate the same response sequence during each iteration of the performance component, i.e., the chain is the same every session. This procedure creates a steady-state of responding across sessions as both the pattern of responding and the accuracy of acquisition stabilizes. An important strength of IRA is that it generates the acquisition of a complex response sequence in a single session, so that acute drug effects can be examined.

With respect to studies of drug action, the IRA procedure has a number of advantages over other commonly used procedures (e.g. mazes). For instance, IRA allows researchers to detect subtle changes in the acquisition and response patterns for progressively more complex behavioral responses. The IRA procedure generates acquisition curves for a complex response sequence in a single session and can generate stable baselines of acquisition, for short response sequences, in relatively few sessions (Cohn & Paule, 1993; Paule & McMillan, 1984; Pieper, 1976). In addition to the creation of a steady state of acquisition, IRA has a number of other advantages over traditional learning tasks. Specifically, positive reinforcement, rather than aversive or stressful stimuli are used during IRA. This is advantageous for human-application but also because avoidance and reinforcement may tap different behavioral and neural processes (Everitt & Trevor, 2005; Mowrer, 1947). Also, a within-subject design is possible, so each

subject serves as its own control comparison when drugs are administered and a repeated measure of learning for the same subject over an extended period of time is possible. This is advantageous because (as with most repeated measures designs) numerous extraneous variables are avoided as individual differences account for less variability. Other advantages to a within-subject design include: an exact definition of learning can be obtained (i.e. the particular response sequence) through specific experimental manipulations; the experimenter can precisely control baseline levels of accuracy (through stimuli and schedule manipulations); and a microanalysis of response patterns is possible (for example, the experimenter can analyze in great detail whether a decrease in accuracy, or increase in errors, is due to an increase in random responding, or preservative responding) (Boren & Devine, 1968; Cohn & Paule, 1995; Evans & Wenger, 1990, 1992; Howard & Pollard, 1983; Pieper, 1976; Thompson, 1978; Wenger, Schmidt & Davisson, 2004).

In addition to the aforementioned advantages for the learning component of the IRA procedure, the performance condition also provides distinct advantages. The performance condition acts as a control for non-specific motor or sedative effects when drugs are administered. This is a major advantage of IRA (and other complex schedules) because there is great flexibility for schedule and response requirement combinations (like the performance and learning components or, in other procedures the combination of FI and FR schedules). So that more than one baseline can be studied concurrently in the same animal.

While compared to other simple memory tasks, IRA greatly increases the total number of dependent measures available to the researcher, the most appropriate dependent measure is debatable. Some of the measures available with IRA include: maximum chain length reached, overall accuracy and accuracy in each link of a multi-link chain (e.g. accuracy in the 1st link in a

4-link chain, or in the 4th link of a 4-link chain), response rate, total errors and total corrects for each link, a weighted sum of correct responses (e.g. a progress quotient, "PQ", Bailey et al., 2010; Johnson et al., 2010) and multiple learning curves that can be generated from a single session.

One of the methodological influences over the appropriateness of a dependent measure in IRA is the type of chaining procedure chosen to train the response chains. Both forward and backward chain training has been used in the literature (Bailey et al., 2010) but these yield different obstacles for the analysis of learning. An accuracy measure that divides total correct by total incorrect responses can be artificially lowered when backward chain training is used, because backward training adds the newest link in the chain to the start of the sequence and therefore can result in an accumulation of incorrect responses, whereas forward training adds the newest link to the end of chain allowing correct responses to accumulate. In this way, an accuracy score can be overly influenced by the chain training procedure chosen. Another complication with interpreting IRA data is the degree to which the novel learning chains are allowed to differ from the performance chain, as well as other characteristics of the chain definitions used (e.g. consecutively repeating links in the chain). Chain definition can greatly influence accuracy and maximum chain-length measures, but these measures are problematic during IRA in their own right. With respect to maximum chain-length reached, failing to distinguish a maximum chain length that was produced once from one that is produced reliably (e.g. earning one reinforcer in the 6-link chain, versus earning 50 reinforcers in the 6-link chain) and result in the situation in which a single chain-length score represents two very different performances. With accuracy, using an incremental procedure (particularly one that progresses with the animals own performance) can result in very high accuracy even when the chain length

is low (e.g. an animal that made it to the 6-link chain, a relatively difficult task, could have a lower accuracy than an animal remaining in a 3-link chain, an easier task, and only making periodic errors).

In an attempt to resolve some of the difficulties associated with accuracy measures Bailey et al. (2010) developed a progress quotient in which corrects earned during each chain length were weighted according to the chain length in which they were earned. The formula for PQ is given in Equation 1. Under a variety of conditions PQ was more sensitive to disruptors (e.g. d-amphetamine administration) and more descriptive of the data than accuracy.

Using an amphetamine challenge (Bailey et al., 2010) PQ was more sensitive and more descriptive than accuracy in determining drug effects procedural differences. Specifically, accuracy failed to detected significant dose-dependent differences between the two training procedures used (backward and forward chaining) and failed to capture an improvement in performance at low doses under some conditions. PQ successfully detected these changes. Also, PQ changes were independent of changes in response rate in this study. As previously mentioned the importance of measuring rate-independent changes in memory or learning ability is highly desirable for the assessment of compounds like the CCBs that have been described as cognitive enhancers. IRA is particularly well-suited for detecting rate-independent effects on accuracy or PQ because response allocation information is collected. Responding across three response devices for response-chains up to 6-links in length is collected and analyzed. This provides better information regarding response allocation (than for example a maze would), which in turn allows for the detection of rate-independent effects on learning. Additionally, as previously mentioned, the performance component of this procedure also serves as a control for motor impairment.

In fact, during IRA accuracy or PQ has been shown to change in the absence of changes in rate (Bailey et al., 2010). Unpublished data from our laboratory has shown that errors will increase during longer-chains, late in the experimental session, while corrects remain relatively constant. This results in a constant response-rate but decreases in accuracy as the session progresses and chain length increases. Bailey et al. (2010) demonstrated that low to moderate doses of d-amphetamine improved PQ score on chains containing a repeated response within the chain (e.g. L*BB*R) while leaving response-rate unchanged. Figure 8, from Bailey et al. (2010) shows this effect during the learning component of IRA. However, subsequent but not yet published, work has yielded mixed results with respect to the rate-independence of PQ (these data are presented in chapters 2 and 3 of the present document). As such, the appropriate measure for IRA may remain an issue. Despite this obstacle, the IRA task has many advantages over the tasks previously used in the CCB literature, and should be a sensitive and reliable indicator of learning changes that follow CCB administration.

Proposed Study

To investigate the role of CNS insult in mediating the benefits of CCB administration I propose a series of studies. First, to determine the effect of acute CCB administration on healthy animals, a variety of CCBs from both the dihydropyridine and Phenylalkylamine classes will be administered. Behavior under an IRA procedure will be the measure of learning. Second, to determine the role of CNS insult, and potential neuroprotection by a CCB, mice exposed chronically to MeHg alone or in combination with chronic nimodipine will also be evaluated using the IRA procedure. Methylmercury (MeHg) is a nearly ubiquitous environmental contaminant with well-documented behavioral effects. Evidence has accumulated suggesting MeHg disrupts that intracellular calcium homeostasis, such that intracellular concentrations of

calcium are elevated. Such a disruption to calcium regulation results in numerous deleterious effects to normal cellular function and can result in cell death. These cellular consequences are thought to underlie the cognitive and motor disturbances seen in individuals with MeHg-toxicity. Between these two studies two primary comparisons will be made 1) the effect of CNS insult (MeHg exposure vs. healthy) and 2) the effect of duration of exposure to nimodipine (acute vs. chronic). Tables 4 and 5 depict the experimental designs for the acute and chronic studies, respectively.

Most of the studies previously described from the literature have investigated a single CCB (most frequently nimodipine). Little attempt to design studies where representatives of the different classes of CCBs are compared directly using behavioral procedures which permit separation of drug effects on learning from those on other processes such as motor function has been made. Further, no study to my knowledge has employed a relatively complex operant task, like IRA, that allows for the distinction between steady-state performance and acquisition while also controlling for rate effects. Therefore, the purpose of the present study is to determine the effectiveness of several CCBs representing different drug classes as memory enhancers in a multi-trial appetitively-motivated operant procedure (IRA) comprising two important components: performance and learning. An additional aim was to determine if CCBs would facilitate acquisition in young, healthy animals as they have been reported to do in senescent subjects (e.g., Thompson, Deyo & Disterhoft, 1990) and to examine the importance of duration of exposure to one CCB, nimodipine. A final aim was to determine if one CCB, nimodipine, would offer protection on this sophisticated task from MeHg exposure.

Tables

Beneficial Properties of CCBs Administered in the Presence of CNS Injury

Reference	CCB	Dose	RoA	Acute/Chronic	Species	Task	Experimental Design/Effect of CCB
Kabuto et al., (1995)	Nimodipine	375 ppm (~75 mg/kg)	PO	Chronic	SAM (senescence accelerated mouse) aged 11 mo.	*in vitro*	Measured levels of DA ACh and 5-HT. These NTs are known to decrease with age. Nimodipine administration attenuated this decrease.
Lipton (1994)	Nimodipine and Nifedipine	4 n*M* free drug	PO	unknown	AIDS patients with dementia	*in vitro*	HIV causes a rise in intracellular Calcium via NMDA and VGCC channels. Nimodipine and nifedipine prevented the rise in Ca and the subsequent neuronal damage associated with application of HIV.
Thompson et al. (1990)	Nimodipine, nifedipine and fluarizine	.01, .1, 1.0 and 10.0 ug/kg/min	IV	Acute	Female albino rabbits aged 47 months (and young ones aged 4 mo)	Single-cell firing	Spontaneous single-unit firing rates of pyramidal neurons and theta interneurons from the dorsal hippocampi (of 9 intact but aged rabbits). Nimodipine (ONLY) caused an increase in the firing rates of the pyramidal neurons with greater increases in firing in aged, compared to young, animals.
Straube et al. (1990)	Nimodipine	860 ppm	PO	chronic	Aged female new zealand albino rabbits	Trace conditioning	Nimodipine improved acquisition of the conditioned eye blink response in aging rabbits without altering the size and latency of the CR, the amplitude of the UR, food consumption or body weight
Deyo et al. (1989)	Nimodipine	1 ug/kg/min	IV	Acute	New zealand albino rabbit aged 37 months and young control (3 months)	Trace conditioning	Nimodipine caused no change in the young group but it improved acquisition of task in the aged group.
Kowalska & Disterhoft (1994)	Nimodipine	.1, .5, 1.0, 3.0 and 5.0 ug/kg/min	IV	Acute	Female albino rabbits aged 38 months	Trace conditioning	Nimodipine improved acquisition of the conditioned eye blink response in aging rabbits.
Levere & Walker (1992)	Nimodipine	0.3, 1.0, 3.0, 9.0 mg/kg	PO	Sub-chronic	Aged hooded long-evans rats (retired breeders, began testing at 25 mo of age)	Spatial learning (8-arm radial maze)	Measured 'correct entries', which means number of alley entries before reentering a previously chosen alley. At 1.0 and 3.0 mg/kg nimodipine, this number increased significantly from control (from 3.5 during control to ~5.5 alley entries following nimodipine administration).

Yanpallewar et al., (2004)	Nimodipine	4 mg/kg	IP	Acute	Charles-Foster rats with induced ischemia and post-ischemia reperfusion injury	Spatial learning (Morris water maze)	ROS generation associated with reperfusion following ischemia is attenuated following nimodipine administration. Nimodipine also attenuated the 'listlessness', anxiety and poor Morris water maze performance that followed ischemia (and reperfusion).
Sandin et al. (1990)	Nimodipine	1, 3, 9 mg/kg	PO	acute	Female rhesus monkeys aged >28 years	Spatial learning	Monkey's chose the well that had been baited following either no or 15 sec delay. Nimodipine-dose dependent improvement on task (more correct responses) during the 15 sec delay condition.
Nyakas et al., (1990)	Nimodipine	10 mg/kg	PO	Chronic (during gestation only)	Wistar rats exposed prenatally to 2 g/l nitrite (induces hypoxia)	auditory & visual discrimination, passive avoidance	Auditory and visual discrimination performance and the long term retention of a passive avoidance response was decreased in nitrite exposed animals. Prenatal nimodipine administration attenuated these effects.
Norman et al. (2002)	Nimodipine	1 and 10 mg/kg	IP	Acute	8 mo old lister hooded-rats with memory impairing dose of scopolamine at the same time as Nim - before sessions	visual discrimination	Object recognition task in open field, if the animal "remembers" the old object he will explore the new one more. Nimodipine prevented the effects of scopolamine on this task. Scopolamine made animals explore the old object as much as the new object.
Mršić et al., (1997)	Nimodipine	.03, .1 .3, 1.0 mg/kg	IP	Acute	Female Hannover-Wistar rats with induced hypoxia	Passive avoidance	Hypoxia alone caused an increase in brain free arachidonic acid (FAA) levels and decreased performance on passive avoidance retention task (hypoxia was induced after learning trial response was acquired). Nimodipine at doses of 0.3 and 1.0 prevented FAA accumulation and improved performance on retention test of passive avoidance.
Finger et al. (1990)	Nimodipine	15 mg/kg	PO	Sub-chronic	Female albino rats given bilateral hippocampal lesions.	DRL 20-s, DRL 40-s	Lesioned animals performed very poorly on DRL task but nimodipine treated animals performed as well as sham operation controls, during both the DRL 20 and 40 s condition.
Meneses et al. (1997)	Nimodipine	.4 mg/kg/day for 28 days	SC	Sub-chronic	Fistar-kyoto rats (WKY) & spontaneously hypertensive rats (SHR) - both aged to 12 months	Acquisition of reinforced lever pressing	Described as an autoshaping procedure, in which lever pressing resulted in the delivery of a sucrose pellet. Nimodipine treated animals acquired lever pressing after fewer trails than non-nimodipine treated animals.
Nyakas et al., (1990)	Nimodipine	3 and 10 mg/kg	SC	Acute	Wistar rats were asphyxiated postnatally to induce anoxia/ischemia	Open field, pole-jumping, conditioned avoidance, hole-board	Nimodipine attenuated the orientation motility deficits and 'behavioral depression' in open field and the learning deficits in the hole-board test, and to a lesser degree improved performance of the conditioned avoidance task

Turchan et al. (2003), Navia et al. (1998)	Nimodipine	"moderate"	PO	Chronic	AIDS patients with dementia	ratings of forgetfulness	Helped slightly on some cognitive endpoints. In other cases the dementia stopped worsening, nimodipine was said to 'stabilize' dementia progression.

Table 1. Table summarizes the beneficial effects of CCB administration in the presence of CNS injury.

CCBs Administered in the Absence of CNS Injury Causes Impairment Or Has No Effect

Reference	CCB	Dose	RoA	Acute/ Chronic	Species	Task	Experimental Design/Effect of CCB
Maurice, Bayle, &Privat (1995)	Nimodipine	0.3 to 3.0 mg/kg	IP	Acute & subchronic (10 days)	Young, adult male swiss mice	Y-maze; passive avoidance; water maze	Acute: Impaired spontaneous alternation in Y-maze at doses .3-1; decreased step-down latency in passive avoidance at 0.3-3.0; decreases place learning in water maze during acquisition and retention. Subchronic: nimodipine at 0.3 and 1.0 mg/kg IP for 10 days had no effect on perfromance in the Y maze task or passive avoidance test.
Maurice, Su, Parish & Privat (1995)	Nimodipine	0.3 to 3.0 mg/kg	IP	Acute	Young, adult male swiss mice	Y-maze; passive avoidance; water maze	Impaired spontaneous alternation in Y-maze at doses .3-1; decreased step-down latency in passive avoidance at 0.3-3.0; decreases place learning in water maze during acquisition and retention
Cain et al. (2002)	Nimodipine and Nidefipine	Nim: 4, 8, 16 mg/kg; Nifed: 1-40 mg/kg	SC	Acute	Young, adult male C57bl/6 mice	Conditioned fear response	Both CCBs prevented extinction of conditioned fear response (in which tone - foot shock was trained then tone alone was tested). Freezing was the DM. CCBs had no effect on the acquisition or expression of the conditioned fear response.
Deyo (1992)	Nimodipine	0.5, 1.0, 5.0 mg/kg	IP	Acute	5 day old female cornish rock broiler chicks	Visual discrimination	Visual discrimination task in which they scored number of pecks to pebbels instead of to food pellets that were glued to a board (peck to pebble is error). During the first 20 acquistion trials there was no effect of 0.5 mg/kg but 1.0 and 5.0 mg/kg groups had increased errors compared to control. For the 40-60 trials of acquisition no effect of 0.5 but the 1.0 mg/kg dose group had fewer errors and the 5.0 had many more errors. Upon a 24 h later retest all groups were the same except the 5.0 mg/kg group still made sig. more errors.
Deyo et al. (1989)	Nimodipine	1 ug/kg/min	IV	Acute	New zealand albino rabbit aged 37 mo & young control (3 mo)	Trace conditioning	Trace-conditioning eye blink task. Nimodipine caused no change in the young group (but it improved acquisition of aged group).
Nomura (1988)	Nimodipine	9 mg/kg	PO	Acute	Young adult male Wistar Kyoto (and SHR) rats	Visual discrimination	A brightness discrimination learning task is used. No difference in accuracy between the non-nimodipine and the nimodipine groups of Wistar rats. They did however show that the SHR's treated with nimdipine had higher accuracy on this task than the SHR's not treated.
Vetulani et al. (1997)	Nimodipine	.25, .5, 1.0, 2.5, 5.0 mg/kg	IP	Acute	Young CD-1, C57bl/6, and DBA male mice	Avoidance learning	Shuttle-box avoidance learning. One dose (1.0 mg/kg) improved performance slightly in one strain (DBA) on this task. All other doses and strains there was no effect.

Table 2. Table summarizing the deleterious or null effect of CCB administration in the absence of CNS injury.

Beneficial Properties of CCBs Administered in the Absence of CNS Injury

Reference	CCB	Dose	RoA	Acute/Chronic	Species	Task	Experimental Design/Effect of CCB
Deyo Straube and Disterhoft (1989)	Nimodipine	1.0 ug/kg/min	IV	unknown	New Zealand albino rabbits (3 mo and 37.7 mo)	Trace conditioning	Nimodipine infusion accelerated acquisition of conditioned eye blink in both young and old rabbits (but it did not alter the amplitude of responses).
Quartermain, deSoria and Kwan (2001)	Nimodipine, Nifedipine, Flunarazine, Diltiazem and Verapamil	1.0-20 mg/kg	SC	Acute	Young male Swiss Webster mice	Passive avoidance, linear maze	All the drugs except verapamil facilitated retention of passive avoidance in dose-dependent manner. All the drugs facilitated retention of the linear maze task.
Kane & Robinson (1998)	Nimodipine	30 mg pellet	SC	Chronic	Young male Long-Evans rats	Barnes circular platform memory test	A circle platform with 18 holes along the periphery one of which has an escape tunnel (position of tunnel randomly changed btw animals but remained constant for experiment).Memory for tunnel location was evaluated 15 days after the acquisition criteria was met. Nimodipine decreased the number of trails required to reach both the acquisition criteria and retention criteria.
Levy et al. (1991)	Nimodipine	20 mg pellet	SC	Chronic	Young male Fischer-344 rats	8-arm radial maze	8-arm radial maze - required to learn a win-shift strategy. Nimodipine increased 'choices per unit time' on this task as well as increasing correct choices.
Martin et al. (2004)	Nimodipine	5 mg.kg	SC	Chronic	Young male Wistar rats	Sequential radial arm task	Complex arm sequence task in automated radial maze with 4-arms each with a lever and pellet dispenser at the end. Levers had to be pressed in specific order. Each lever press resulted in reinforcement. Nimodipine treated animals acquired task (to criterion) more quickly.
McMonagle and Fanelli (1993)	Nimodipine	10, 20, 40 mg pellet	SC	Chronic	Young Wistar rats	Morris water maze	Nimodipine improved performance of all age groups, in terms of latency to reach platform.
Vetulani et al. (1997)	Nimodipine	.25, .5, 1.0, 2.5, 5.0 mg/kg	IP	Acute	Young CD-1, C57bl/6, and DBA male mice	Avoidance learning	Shuttle-box avoidance learning. One dose (1.0 mg/kg) improved performance slightly in one strain (DBA) on this task. All other doses and strains there was no effect.

Table 3. Table summarizing the beneficial effects of CCB administration in the absence of CNS injury.

Acute CCB Administration Experimental Design

Drug Classification	Drug Name	RoA	Dose Range	N
Dihydropyridine Calcium Channel Blocker	Nimodipine	IP	1.0 -10.0 mg/kg	11
Dihydropyridine Calcium Channel Blocker	Nifedipine	IP	1.0 -10.0 mg/kg	9
Dihydropyridine Calcium Channel Blocker	Cinnarizine	IP	1.0 -10.0 mg/kg	8
Phenylalkylamine Calcium Channel Blocker	Verapamil	IP	0.3 -10.0 mg/kg	10
NMDA Receptor and Calcium Channel Antagonist	Ketamine	IP	0.3 -60.0 mg/kg	10

Table 4. Table summarizing the specific CCB's to be used in proposed study #1.

Chronic CCB Administration Experimental Design		
	MeHg Dose	
Nimodipine Dose	0 ppm	15 ppm
0 mg/kg/day	N = 12	N = 12
20 mg/kg/day	N = 12	N = 12
200 mg/kg/day	N = 12	N = 13

Table 5. Table outlining the experimental design and exposures for proposed experiment #2.

Figures

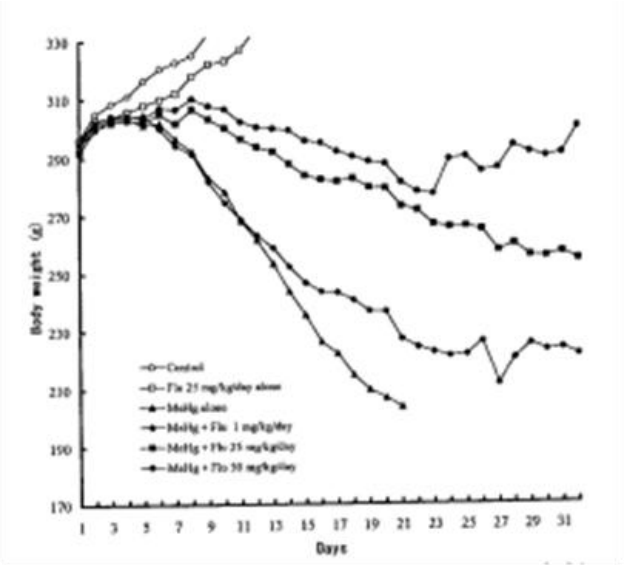

Figure 1. The effect of flunarizine on body weight in rats administered 5 mg/kg/day MeHg for 12 days. From Sakamoto et al., (1996).

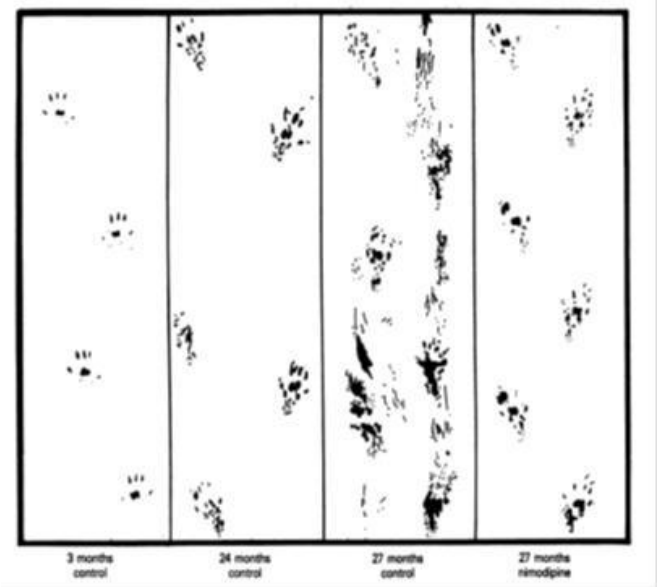

Figure 2. Prints of the hind paws of a young, old and very old rat without nimodipine (3 left-most panels) and a very old rat with nimodipine (right-most panel) administration (860 ppm nimodipine in the food). From Schuuman et al. (1989).

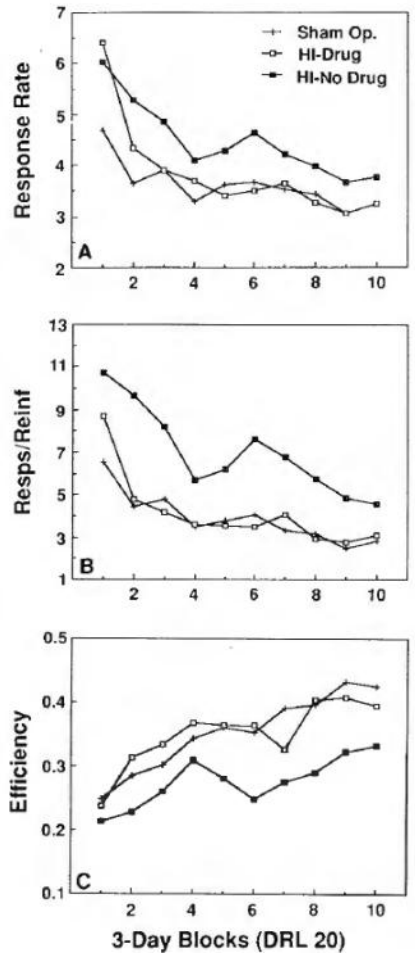

Figure 3. From Finger et al. (1990). Data from rats performing under a DRL 20s schedule of reinforcement. The 'Sham Op.' group did not experience the hippocampal lesion, the 'Hl-Drug' received hippocampal lesion plus nimodipine PO for 14 days following lesion and the 'Hl-No Drug' group received the lesion but not nimodipine treatment.

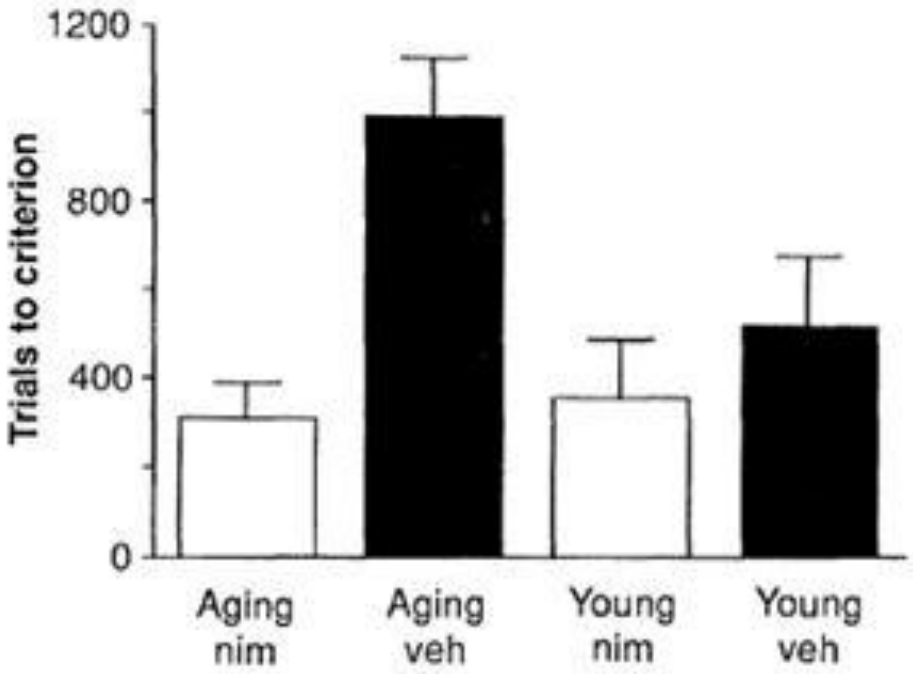

Figure 4. From Deyo et al. (1989). Trials to criterion on trace conditioning eye blink are shown for aged and young rabbits. No significant statistical difference was reported between the young nimodipine and young vehicle groups on this task.

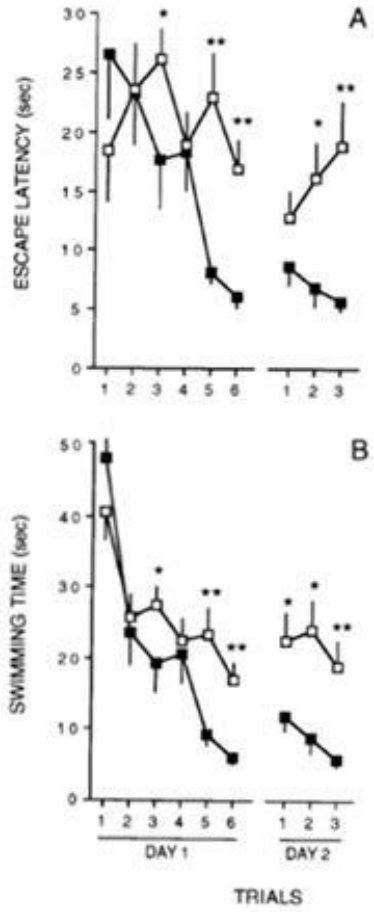

Figure 5. From Maurice et al. (1995a). Data from young intact Swiss mice on water maze task. Open symbols are from nimodipine (0.3 mg/kg) treated animals and closed symbols are from the vehicle group. Panel A plots time to arrive at the submerged platform and B plots total time spent swimming.

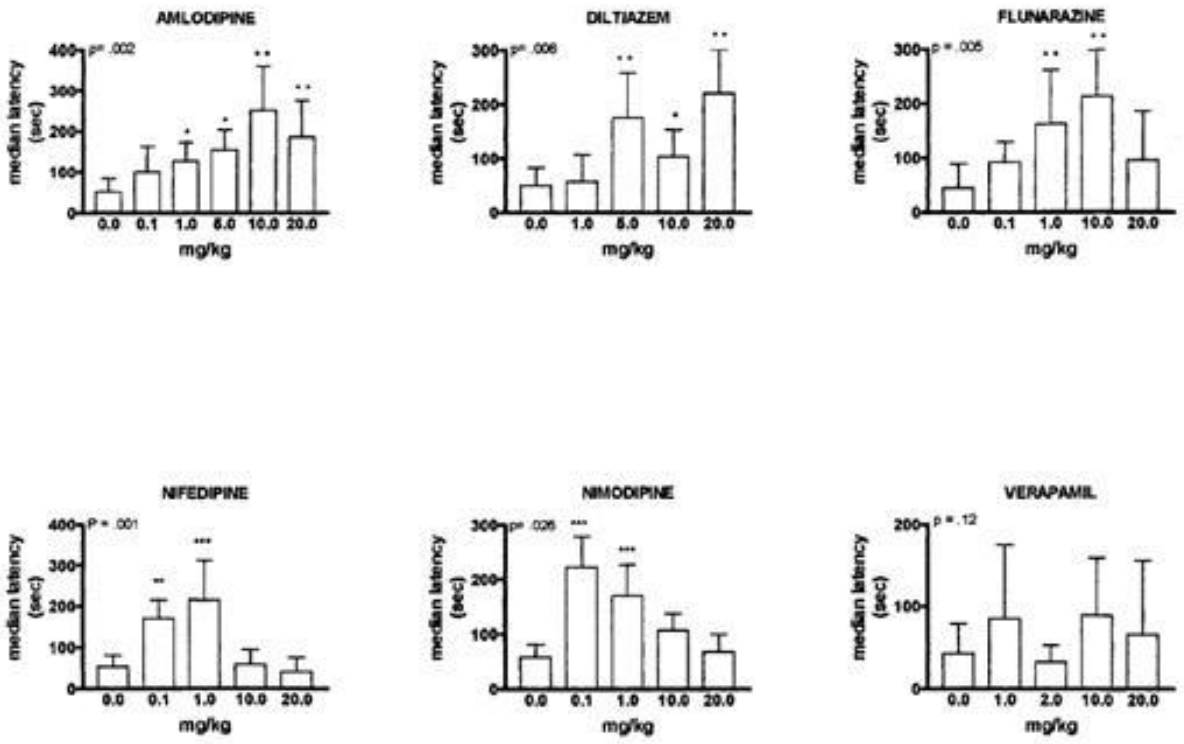

Figure 6. From Quartermain et al. (2001). Median latency scores on a passive avoidance task are shown for a variety of doses of CCBs. Not the dose-dependent improvement in performance (i.e. longer latency to end shock compartment).

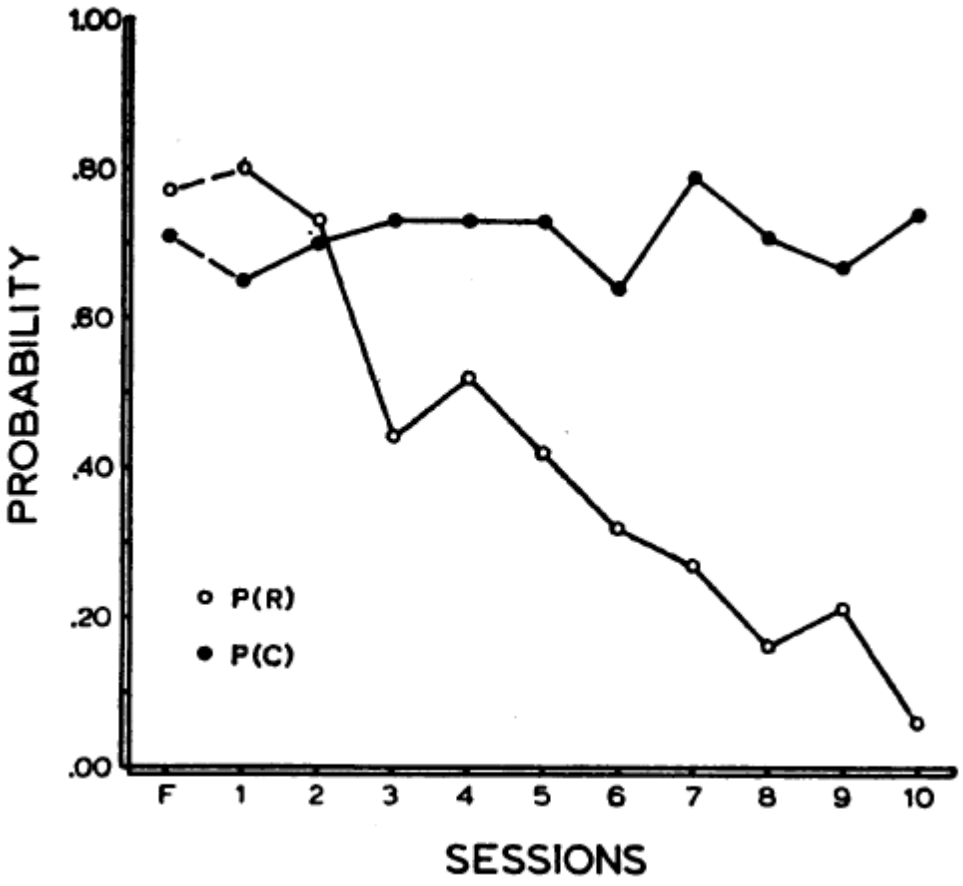

Figure 7. From Nevin (1967). Probability of a correct response (filled circle) or any response (open circle) during extinction sessions.

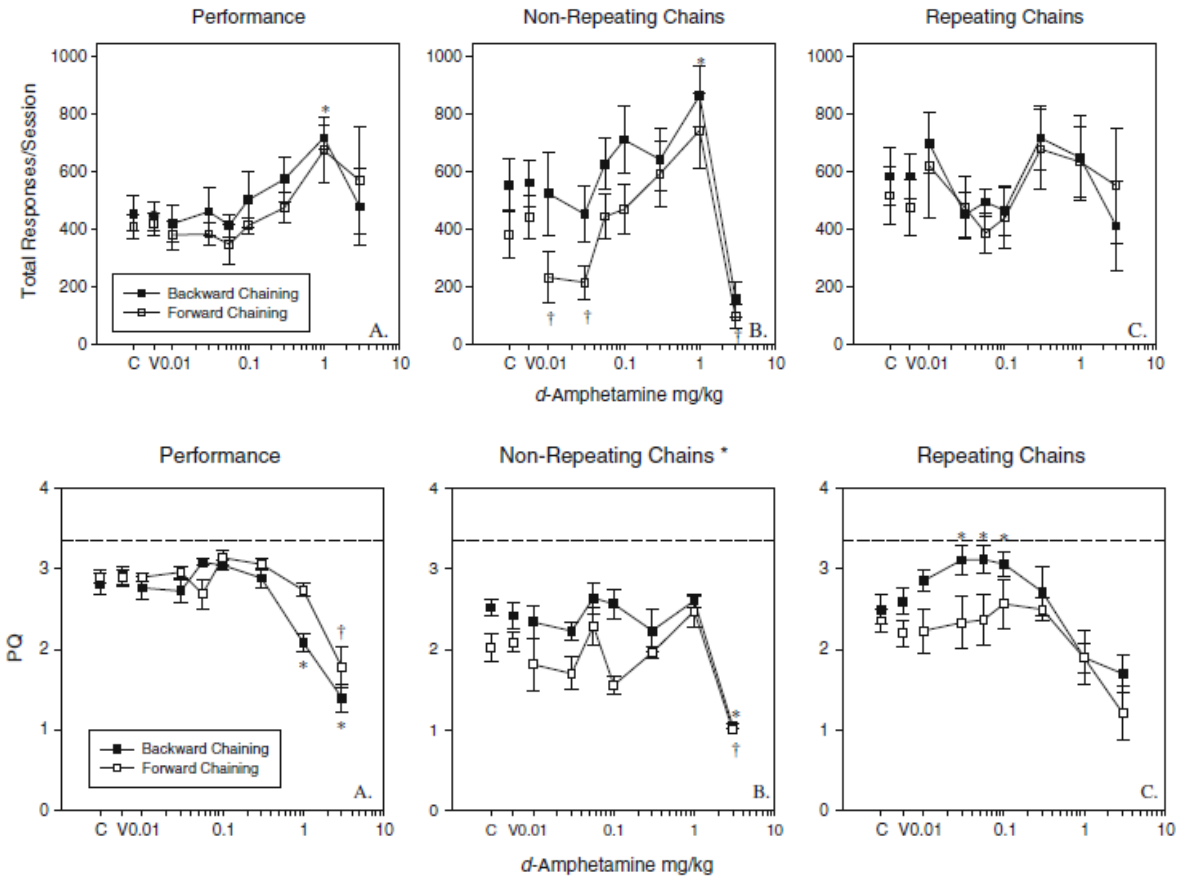

Figure 8. From Bailey et al. (2010). Responding and PQ score as a function of d-amphetamine dose. Comparing the right-most panels, note the PQ improvement across doses in the absence of rate changes.

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Chapter 2: Acute Behavioral Effects of Calcium Antagonism in Healthy Mice

Abstract

Calcium regulation has long been implicated in learning processes and compounds that affect this regulation may have detrimental consequences to normal cognitive functioning. Most studies have used drugs that act on ligand-gated calcium channels to investigate this issue. Here we emphasize L-type calcium-channel blockers (CCBs), which act on voltage-gated channels and are used clinically for the treatment of cerebral ischemia and hypertension (among other uses). CCBs have been used experimentally to treat and attenuate various CNS injuries, however their impact on the healthy organism is unclear. Multiple doses on the L-type Ca^{++} channel blockers nifedipine, verapamil, nimodipine, cinnarizine and the NMDA antagonist, ketamine, were administered to healthy mice performing an incremental repeated acquisition (IRA) procedure. This procedure requires that a mouse acquire a different response chain (e.g., left-right-back-left nosepoke) every day. A control procedure required that they perform the same chain every day. We hypothesized that the learning task would be more sensitive than the performance task and that effects would occur at doses lower than those that produce non-specific decreases in response rate. Response-rate decrements and learning and performance deficits were seen with nimodipine at 3 mg/kg during both IRA components. Verapamil disrupted response rate during one component of IRA. Nifedipine had no effect on behavior. Ketamine selectively impaired learning at 3 mg/kg and higher doses, in the absence of response rate changes. Although the CCBs have similar mechanisms of action, they differ from each other in their behavioral effects and differ as a group from ketamine.

Introduction

The maintenance of intracellular calcium homeostasis is necessary for normal nervous system function since the calcium ion functions as a messenger for many important processes, including the mediation of neurotransmitter release and synaptic vesicle function (DeLorenzo & Freedman, 1977) as well as influencing gene transcription and expression (Hu et al., 1993; Hu & Wieloch, 1993) and synaptic plasticity (Kumar & Foster, 2002). As such, calcium homeostasis is relevant not only to normal cell functioning but also to behavioral processes of learning and even motor function.

*Dys*regulation of intracellular calcium is a common mechanism underlying both cell and behavior impairment associated with a variety of CNS insults. Specifically, increases in intracellular calcium ($[Ca^{++}]_i$) concentrations result in cellular dysfunction and/or death (Mattson et al., 1992; Brown & Jaffe, 1995), with consequent changes in the organism's behavior. The behavioral impairment associated with advanced age (Khachaturian, 1984, 1989; Landfeild, 1987), exposure to the neurotoxicant MeHg (Landfeild, 1987; Verkhratsky & Toescu, 1998; Atchison & Hare, 1994), and cerebral ischemia (Uematsu et al., 1989) is thought to be mediated, at least in part, by elevated $[Ca^{++}]_i$.

A group of drugs, calcium channel blockers (CCBs), have shown promise in treating and preventing the behavioral impairment that results from a range of CNS insults, all of which are associated with increased intracellular calcium concentration. The CCBs are a heterogeneous group of drugs that can be subdivided into four classes and differ on therapeutic usage, chemical structure and pharmacokinetic profile. These include the dihydropyridines, phenylalkylamines,

benzothiazepines and the difluorinated piperazine derivatives. Drugs in all four classes block calcium entry at specific L-type voltage-gated calcium channels on neuronal cell bodies and cerebral vasculature as well as on cardiac smooth muscle and peripheral vasculature, although with varying degrees of specificity (Bean, 1989; Miller, 1987). In the brain, L-type calcium channels have been implicated in mediating long term changes in neuronal activity (Wickens & Abraham, 1991; Kapur et al., 1998; Weisskopf et al., 1999) and in the modulation of behavior such as spatial memory (Borroni et al., 2000), fear conditioning (Shinnick-Gallagher et al., 2003) and the extinction of conditioned fear (Cain et al., 2002) and they have altered the behavioral response to some drugs (Licata et al., 2004; Zhang et al., 2003). Clinically L-type CCBs are used to treat a wide range of cardiovascular and neurological conditions including angina, hypertension, cerebrovascular disease and migraine, as they are potent vasodilators.

The dihydropyridines, nimodipine in particular, have been investigated as a potential therapy for dementia. While experimental evidence from human trials of their efficacy has been mixed, this characterization finds support on both theoretical grounds and from animal studies (e.g. Scriabine, 1993). However, the mechanism by which CCBs are beneficial in treating dementia appears to depend upon the origin of the dementia. As the dihydropridines are potent vasodilators, their rational for use in vascular dementia is easily understood (Scriabine, 1993), while their utility in the treatment of AIDS-related dementia is, probably related to its ability to reduce the calcium-mediated damage that is caused by the virus (Dreyer et al., 1990). The rational for CCB treatment of Alzheimer's' dementia is more indirect, and based on general concerns regarding the altered $[Ca^{++}]_i$ homeostasis that is associated with this disorder (e.g. Verkhratsky & Toescu, 1998). As such, increased blood flow and reductions in intracellular calcium concentrations are often reported as rationale for the use of CCBs in Alzheimer's

dementia (for a review of nimodipine's efficacy in treating dementia see Birks & Lopez-Arrieta, 2002).

However, the experimental utility of CCBs is not specific to dementia; their administration has also been beneficial in treating various motor and cognitive deficits that follow a variety of CNS insults, all of which are associated with increased intracellular calcium homeostasis. Thus, CCBs are beneficial when treating MeHg toxicity (Sakamoto et al., 1996), cerebral ischemia (Mršić et al., 1996; Yanpallewar et al., 2004), normal aging (Kabuto et al., 1995; Deyo et al., 1989; Kowalsha & Disterhoft, 1994), bilateral hippocampal lesion (Finger et al., 1990), hypertension (Meneses et al., 1997) and memory-impairing doses of scopolamine (Norman et al., 2002). While the preceding studies examined behavioral endpoints, similar benefits of CCB administration following CNS insult have been seen at the molecular level (i.e. Sakamoto, 1996).

It is likely *because* of these benefits that the use of CCBs in the experimental literature has been nearly exclusively for treatment or prevention of CNS insult. Little emphasis has been placed on the effects that CCBs may have in the *absence* of CNS insult. It is important to determine the extent to which the compounds used to treat CNS insult have behavioral effects in their own right. In fact, very few reports exist in the literature explicitly examining this issue. In fact, the majority of reports concerning the effects of CCBs administered to healthy animals come from studies of CNS insult in which a control group is administered the CCB in the absence of CNS insult. However, an examination of these CCB-administered control groups suggests that there may be some beneficial or deleterious consequences of these drugs when administered alone.

Specifically, studies using trace-conditioning of eye-blink response (Deyo et al., 1998), passive avoidance behavior (Quartermain et al., 2001; Vetulani et al., 1997), and performance on a variety of maze-tasks (Quartermain et al., 2001; Kane & Robinson, 1999; Levy et al., 1991; Martin et al., 2004; McMonagle & Fanelli, 1993) have indicated that CCB administration improved performance in uninjured animals. In contrast, others have reported that CCB administration impaired Y- maze and water-maze performance, passive avoidance behavior (Maurice et al., 1995a; Maurice et al., 1995b) and simple tactile/visual discrimination (Deyo, 1999) in uninjured animals. In other reports, CCB administration has no effect on tasks of brightness discrimination (Nomura, 1988), passive avoidance (Venulani et al., 1997) and trace-conditioning of eye-blink response (Deyo et al., 1998).

When trying to reconcile these data some obvious considerations must be taken, including behavioral endpoint, CCB subclass, dose and route of administration. However, despite the inconsistent effects, no study to our knowledge has systematically evaluated the effects of CCB administration in the absence of CNS insult nor has any study addressed the aforementioned considerations. Most of the studies described previously have investigated a single CCB, typically a dihydropyridine, with few attempts to directly compare several CCBs within a study. Further, no study to our knowledge has employed a relatively complex operant task that allows for the distinction between steady-state performance and acquisition while also controlling for rate effects.

Incremental repeated acquisition procedures (IRA) have a number of benefits over other learning tasks and are well-suited to measure the changes in learning that may follow CCB administration. IRA procedures generate a steady state of acquisition by requiring the acquisition of novel pattern of responses, usually a response chain in one (the “learning”) condition and by

requiring the performance of a previously learned response pattern in the "performance" condition. Because this procedure generates is a stable baseline of learning, a wealth of data can be collected and acute dose-effect curves can be generated that permit a separation of learning from performance measures utilizing a powerful within-subject experimental design. This approach offers some advantages over other learning tasks that use very few trials (e.g. single-trial learning tasks, maze learning, avoidance procedures, etc.) or that measure exclusively a steady state of performance (e.g. object recognition, place learning, discrimination procedures, etc.). Also, IRA procedures that employ a criterion-based approach to increasing chain length allow the animal to continue collecting reinforcers, even in the case of impairment. Repeated acquisition procedures have been used in studies of drug (Paule & McMillan, 1984) and of toxicant (Cohn et al., 1993) effects as a sensitive indicator of subtle changes in learning; further, IRA has been described as an analog to human IQ testing (Paule et al., 1999).

The present study was designed to evaluate CCBs in unimpaired animals using a sophisticated operant procedure and multiple CCBs. Also of interest is a comparison between voltage-gated calcium antagonism (i.e. CCB administration) and antagonism at the hybrid (i.e. ligand+voltage gated) NMDA channel. The NMDA antagonist ketamine is well known to disrupt performance on learning and memory procedures in humans and animals (e.g. Lmre et al., 2006; Krystal et al., 1994). Therefore, the goal here is to determine the effectiveness of CCBs representing different subclasses plus an NMDA receptor antagonist in an appetitively-motivated operant procedure (IRA) comprising two components, performance and learning. This will facilitate an evaluation of CCBs on complex learning in healthy animals and allow for a comparison between CCB administration and calcium antagonism (via ketamine) that is known to disrupt behavior.

Methods

Animals. Adult male Balb/c mice, maintained at approximately 85% free feeding weight (i.e. 25g) and housed individually in a temperature- and humidity-controlled AAALAC-accredited colony room that operated on a 12-hour light-dark cycle (lights on at 7:00 a.m.), were used. Mice had free access to water in their home cages for the duration of the experiment. These animals were previously used as vehicle-control mice in a study of chronic nimodipine release where they never received nimodipine. During that study, mice were group-housed and no behavioral endpoints were collected. Naïve to operant chambers at the start of the present experiment, mice were autoshaped to nose-poke in standard operant chambers before training on IRA.

Apparatus. Standard rodent operant chambers (Med Associates Inc. St. Albans, VT, model # Med ENV 007) fit to accommodate mice and surrounded by a sound-attenuating shell were used. Three nose pokes devices, one to the left (L) and right (R) of a 20 mg sucrose dispenser and one in the center of the back wall (B) opposite the sucrose dispenser, were active during all sessions. A 28-volt house light could illuminate the chamber and a Sonalert tone (2900 Hz) and white noise generator could produce two distinct auditory stimuli. A black and white checkerboard pattern could be placed on the clear, plastic side-wall/door of the chamber to serve as a visual stimulus. Programs for experimental procedures and data collection were written using MED-PC IV (Med-Associates, St. Albans, VT), and all session events were recorded with 0.01" resolution. All programming equipment was located in a room adjacent to the testing room.

Procedure. All experimental sessions were conducted at approximately the same time each day. An incremental repeated acquisition of response-chain procedure was used in which a chain incremented from a one- to a six-link chain within an experimental session. Sessions ended

after 50 reinforcers in the six-link chain were obtained or one hour passed. Nose-poke response-chains were built using backward chaining such that a new link was added in front of the previously learned link(s). For example, the response chain LBLRBR was trained as follows: R → sucrose, B – R → sucrose, R – B – R → sucrose, L – R – B – R → sucrose, etc. until a 6-link chain was formed. Chain-length was increased using a pre-set mastery-based criterion: six consecutive correct responses (no errors) advanced the chain from a one- to a two-link chain and three consecutive correct responses advanced the 2^{nd} through 5^{th} chain lengths. During a trial the house light was off and lights inside the nose-poke devices provided illumination. Nose-poking an incorrect nose-hole at any point in the chain resulted in a 2 sec timeout during which the house light was illuminated and no response was reinforced. Any response during the timeout period prolonged the timeout by 2". Following this timeout period, the current chain length reset to the beginning. Each link in the chain was paired with a discrete auditory stimulus.

Animals were exposed first to the performance condition in which the same six-link sequence was correct each session. Learning sequences (in which the animals were required to acquire a different response sequence within the experimental session) were introduced after 10 performance sessions, at which point the animals were reliably reaching a 4-link chain. A total of 17 learning sequences were cycled through the course of the experiment with learning and performance sessions alternated daily. All chains sampled each response location (R,B,L) but response locations never consequently repeated within a chain (e.g. RBBLCR would not qualify but RBLBLR would). All efforts were made to ensure the learning chains were equivalent and any obvious pattern (e.g. clockwise rotation) was avoided. A checkerboard pattern was placed on the front door of the experimental chamber before each learning session, no pattern was present on the chamber door before performance sessions.

Drug administration. Drug administration commenced after behavior in both performance and learning conditions showed no systematic trend in overall response rate or progress quotient (PQ) for ten consecutive sessions. All drugs were supplied by Sigma-Aldrich (St. Louise, MO, USA). Ketamine (0.3-60 mg/kg), verapamil (0.3-10.0 mg/kg), nimodipine (1.0-10.0 mg/kg), cinnarizine (1.0-10.0 mg/kg) and nifedipine (1.0-10.0 mg/kg) were administered ip in an ascending fashion on Tuesdays and Fridays, vehicle was administered on Thursdays and Saturdays and non-injection control days were on Mondays and Wednesdays. Ketamine was dissolved in physiological saline. Verapamil, nimodipine and nifedipine were dissolved in a NaCl solution containing MeOH (50% NaCl , 50% MeOH). Cinnarizine was dissolved in a NaCl solution containing DMSO (10% NaCl, 90% DMSO). The appropriate vehicle was used for each dose-effect curve. An injection volume of 0.025 ml was used for all drugs and vehicles. Dose-effect curves were generated within subject, meaning each animal received all doses of each drug. A 5-day wash-out period separated the generation of each dose-effect curve determination.

Data Analysis

Three dependent measures were analyzed in the present study: Response rate (number of responses per time available in which to respond), maximum chain length (MCL) reached and a progress quotient (PQ) previously presented by Bailey et al. (2010):

Equation 1.

$$PQ = \frac{\left(\sum_{i=1}^{6} w_i R_i \right)}{R_t}$$

where R_i = number of reinforcers earned on a chain length of length i, w_i = the weight given to chain length of length i (where the weight is equal to the chain length, e.g. weight of chain length 4 = 4) and R_t = total reinforcers earned in the session. This index serves as a measure of progress and avoids many problems that are associated with using a measure of accuracy or maximum

sequence length reached during a mastery-based criterion IRA procedure, as the one used here. PQ weights each reinforcer earned by the chain length during which it was earned. Therefore, a reinforcer earned in a 4-link chain is favored over one earned in a 1-link chain. Characterized differently, PQ is also a count of the total criterion responses during a session, or responses that comprise criterion/reinforced chains, divided by earned reinforcers.

All animals are included in each analysis, RMANOVA's were used to analyze the effects of dose. The Greenhouse-Geisser correction was used when necessary (i.e. GG epsilon < 0.6). Planned pair-wise comparisons between saline and an active dose were conducted if there was a statistical main effect of dose. To correct for multiple comparisons and retain a p-value of 0.05, this value was divided by N (N= the number of comparisons for any dose-effect relationship) therefore planned comparisons required a p value of 0.007 to be significant if 7 comparisons were made. Post-hoc's conducted on baseline data were corrected for multiple comparisons using the Bonferroni correction. Conclusions about group differences were addressed by examining graphs. All error bars represent the S.E.M. and $p < .05$ was the criterion for statistical significance unless otherwise stated. Statistical analyses were conducted using SYStat (San Jose, CA).

Results

Nimodipine reduced responding and PQ at the highest two doses. During the performance component both PQ ($F(4,20)= 5.507$, $p = .004$) and rate ($F(4,20)= 11.168$, $p= .000$) decreased as a function of dose, see Figure 1. MCL ($F(4,20)= 5.367$, $p=.004$) also significantly decreased as a function of dose (not shown). During the learning component, PQ ($F(4,16) = 4.671$, $p=.011$) and rate ($F(4,16)= 6.819$, $p= .002$) decreased with increasing dose, see Figure 1.

Verapamil did not produce any changes in PQ or MCL (all p's > .150), even at 10 mg/kg, during either component. Rate, however, did change as a function of dose in the learning component only ($F(4,28)= 4.529$, $p=.006$), driven by a single dose, see Figure 2. Note, two mice died following a dose of 20 mg/kg of nimodipine and of verapamil (data from that dose are not included here). Nifedipine administration did not produce any behavioral effects even at 10 mg/kg, Figure 3. Cinnarizine administration significantly affected rate ($F(5,35)=12.926$, $p <.000$) and PQ ($F(5,35)=2.701$, $p =.036$) during the performance component as well as rate ($F(5,35)=11.362$, $p <.000$) and PQ ($F(5,35)= 4.831$, $p=.002$) during the learning component. Post-hoc analyses revealed a significant effect of vehicle (NaCl + DMSO solution) for cinnarizine, Figure 4. Ketamine administration selectively impaired PQ during both the performance ($F(7,49= 5.111$, $p<.000$) and learning ($F(7,49)= 5.264$, $p<.000$) condition, however this decrease occurred at a lower dose during learning than it did during performance. Rate, however, was only reduced during the performance component ($F(7,49)=5.679$, $p<.000$). See Figure 5.

Figures 6 and 7 show the relationships between response rate, PQ score and maximum chain length reached (MCL). Data includes all active doses from all drugs from the performance IRA component. All relationships were significantly and positively correlated (all p's< .000).

Discussion

Summary of Results. Four voltage-gated CCBs and ketamine were administered to healthy, adult mice behaving under an incremental repeated acquisition procedure. The CCBs administered each had behavioral effects divergent from one another and from the glutamate antagonist, ketamine. We found no evidence that calcium antagonism was beneficial for the acquisition or performance of an IRA response chain. In fact, verapamil and nimodipine

decreased PQ, response rate and MCL at the higher doses. Nifedipine had no effect on any measure. The only possible exception is the improvement over vehicle offered by 1.0 mg/kg cinnarizine in PQ during the learning component only, an improvement that was accompanied by a corresponding increase in response rate at the 1.0 mg/kg dose. Therefore, we hesitate to draw meaningful a strong conclusions about the apparent learning improvement seen here.

Impairment or No Effect Following CCB administration. Specifically, the most consistent effects seen here were the deleterious effects of CCB administration on measures of response rate and PQ as moderate to high doses of some CCBs (verapamil and nimodipine), but not nifedipine, impaired IRA performance relative to control. Specifically, when verapamil was administered to healthy adult mice no change in PQ was detected, despite a response rate increase at 3.0 mg/kg that occurred during the learning component only. Here, an increase in response rate did not correspond to a change in PQ, and response rate only changed during one IRA component, which is difficult to explain, as differences in response rate between components is difficult to understand. Further, this effect seems to be driven by an effect at a single dose.

Unlike the other drugs administered, nifedipine had no detectable effect on response rate or on PQ in the present study, although these data, PQ in particular, are considerably variable (i.e. large SEM). This dose-effect occurred despite evidence from the literature that the doses used are behaviorally active. Viveros et al. (2007) reported that nifedipine administration, at similar doses as those used here, resulted in a reduction of general motor behavior while Blake et al. (1996) reported that nifedipine suppressed self-injurious behavior.

With respect to nimodipine, doses of 3.0 and 10.0 decreased response rate and PQ similarly during both components of IRA. Therefore, it seems reasonable to conclude that the

decreases seen in PQ may be driven by changes in rate, and therefore do not reflect a true "learning impairment". There is some evidence in the literature that nimodipine, like nifedipine, results in a reduction in general motor activity (Vivaros et at., 2007). This may have manifested as learning impairments in some of the reports previously mentioned (in which nimodipine was administered during a learning task that relies heavily on motor function). For example, Maurice et al. (1995) reported impaired Y-maze, water-maze and passive-avoidance performance in adult mice following acute i.p. nimodipine administration in doses similar to those used here (i.e. 0.3 – 3.0 mg/kg). It is not clear, however, the extent to which general motor decline may have accounted for the effects reported by Maurice and colleagues.

It is worth noting that the dose-ranges used here were chosen to span the behaviorally active range for each drug and were selected based on dose-ranges published in the literature. However, these drugs are known to differ in potency such that nimodipine is more potent than the other dihydropyridine CCBs investigated here as well as verapamil in rat cerebral cortex (Scraibine et al., 1989). Nimodipine binds, with high affinity and specificity to the dihydropyridine receptors on L-type calcium channels (Belleman et al., 1982; Dompert & Traber, 1984; Peroutka & Allen, 1983) and is highly lipophilic, readily passing the blood-brain-barrier (Van den Kerckhoff & Drewes, 1985). These characteristics may contribute to the greater disruptive properties of nimodipine than of the other CCBs in the present study.

Conclusions from the cinnarizine dose-effect function must be tempered somewhat due to the significant effect of vehicle administration; because of which, all conclusions about dose-effects are based on a comparison from vehicle (for all other drugs, vehicle and control were indistinguishable). Cinnarizine is not readily water-soluble and therefore DMSO was added to the saline solution to facilitate the creation of a cinnarizine solution suitable for injection. While

DMSO is used frequently in veterinary practices and is generally considered safe (at the very low concentration used here), its use nonetheless disrupted performance during both components of the IRA procedure. Despite this vehicle-effect, cinnarizine did appear to cause an increase from vehicle at a dose of 1.0 mg/kg during the learning component, with a corresponding increase in rate at that dose. A decrease in PQ at 10.0 mg/kg during the performance component occurred in the absence of decreasing rate.

Ketamine, the glutamate antagonist that blocks intracellular calcium entry via calcium channels on NMDA receptors, resulted in a different pattern of PQ, MCL and response rate changes than the CCBs. While PQ decreased significantly as a function of dose, it did so at a much lower dose during the learning component than during the performance component. This effect occurred in the absence of any rate-changes during the learning component. Ketamine has been shown in the literature to impair performance on learning or memory tasks, but its effects under the IRA procedure were previously unknown. The ability of ketamine to selectively affect learning behavior (at a dose of 3.0 mg/kg), while leaving performance behavior unaffected, supports the well-established hypothesis that glutamate is involved in processes of learning and memory (for a review see Reidel et al., 2003). Glutamate plays a critical role in processes of synaptic plasticity, like long-term potentiation (for a recent review see Peng et al., 2011).

Rate-Independent Learning Effects. Rate-independent changes in a measure of learning, such as PQ, are particularly interesting as they may help elucidate specific calcium-mediated learning processes. The ability to detect such a change is one of the key benefits to using a procedure like IRA, as many other more traditional learning or memory tasks make it virtually impossible to dissociate rate from learning effects. The factors that contribute to performance on any learning task are numerous and not restricted just to motor function, they also must include

motivation, attention and health status (King et al., 2001). These variables may affect behavioral indices of memory but they are in fact functionally *independent* of memory (King et al., 2001). This is likely a relevant criticism of some of the studies cited from the CCB literature, particularly maze-learning. This criticism is applicable to a much lesser extent, if at all, to an IRA procedure. The performance component of an IRA procedure is conceptualized as a control for motor or sedative effects when drugs are administered. In this way, any effects that occur in the learning component alone, i.e. in the absence of performance component effects, likely point to the involvement of learning processes per se. Such specificity was especially evident following ketamine administration, as doses of 3.0 and 10.0 mg/kg significantly reduced learning PQ, but not performance PQ.

Here, some CCBs produced what appear to be rate-independent changes in PQ. The learning component was strikingly more sensitive to ketamine administration than was the performance component Changes in PQ in the absence of rate-changes and learning changes in the absence of performance-changes support the hypotheses that 1) IRA is sensitive enough to detect even subtle changes in learning and 2) that voltage- and ligand-gated calcium antagonism may specifically influence learning or memorial processes, before affecting motor function. With that said, motor function itself is often of interest as any reduction in the capacity for coordinated movement reduces an organism's ability to cope with the demands of its environment, such that even subtle defects will influence how effectively it functions. Clearly, learned motor skills play a salient role in many human activities. Therefore, while it is requisite to have a measure of learning or memory that is independent of motor function for appropriate interpretations of the data, motor behavior itself is a useful indicator of CNS function. It is this

consideration that drives the present emphasis on both a measure of response rate and a measure of learning (PQ) for determining drug effects.

Calcium Regulation and "Cognitive Enhancers". Most examples of Ca^{++} dysfunction represent excessive intra-neuronal Ca^{++}, and CCBs are thought to act by helping to reinstate homeostatic levels. This relationship does not, however, suggest that CCBs should be viewed as generic cognitive enhancers, as has been suggested in the literature (see Herrmann & Stephan, 1991; and Shubhada et al., 2008 for a review). Rather, it can be rationalized that CCBs should only be helpful when calcium levels are disrupted, (in a since the CCBs act to normalize the increased cytosolic calcium levels induced by CNS injury). The administration of CCBs to an uninjured CNS (one in which proper calcium homeostasis is maintained) should act to disrupt that homeostasis by preventing the necessary influx of extracellular calcium ions through voltage-gated calcium channels that is necessary to maintain proper calcium homeostasis. Such a hypothesis has been offered, although not fully evaluated, in the literature (Maurice et al., 1995a; Cain et al., 2002).

Summary. The present experiment evaluated the effects of CCB administration to adult, healthy animals with the goal of determining what, if any, impact calcium antagonism has on cognitive and motoric endpoints. A sensitive behavioral procedure (IRA) was used to measure these endpoints. Response rate and cognitive endpoints (PQ, MCL) revealed divergent effects between each CCB and for ketamine. In general, CCB administration had no effect or was deleterious to performance on either component of the IRA procedure. Often, alterations in PQ occurred in the absence of response rate changes, indicating rate-independent cognitive effects. Ketamine administration was deleterious to PQ at a lower dose during the learning component

than during the performance component. Based on these data acute CCBs administration should not be considered ‘cognitive enhancing’.

Tables

Acute CCB Administration Experimental Design

Drug Classification	Drug Name	RoA and Vehicle	Dose Range	N
Dihydropyridine Calcium Channel Blocker	Nimodipine	IP; NaCL + MeOH	1.0 -10.0 mg/kg	11
Dihydropyridine Calcium Channel Blocker	Nifedipine	IP; NaCL + MeOH	1.0 -10.0 mg/kg	9
Dihydropyridine Calcium Channel Blocker	Cinnarizine	IP; NaCL + DMSO	1.0 -10.0 mg/kg	8
Phenylalkylamine Calcium Channel Blocker	Verapamil	IP; NaCL + MeOH	0.3 -10.0 mg/kg	10
NMDA Receptor and Calcium Channel Antagonist	Ketamine	IP; NaCL	0.3 -60.0 mg/kg	10

Table 1. Summary of experimental design including CCBs that were administered, route of administration and CCB channel-subclass.

Figures

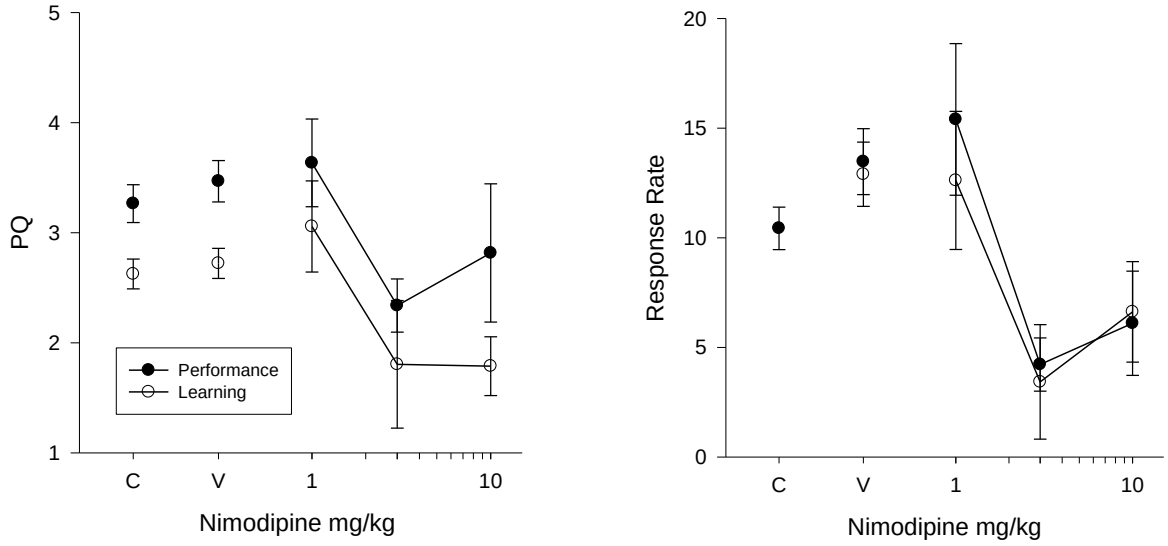

Figure 1. Nimodipine dose effect function for PQ score (left panel) and response rate (right panel). Filled circles represent performance data and open circles represent learning data.

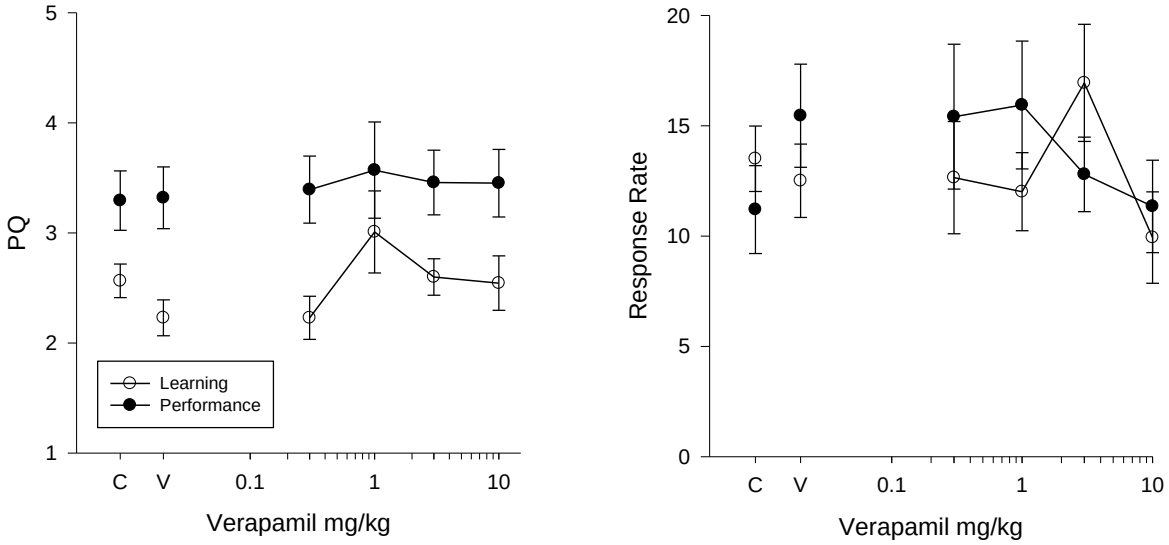

Figure 2. Verapamil dose effect function for PQ score (left panel) and response rate (right panel). Filled circles represent performance data and open circles represent learning data.

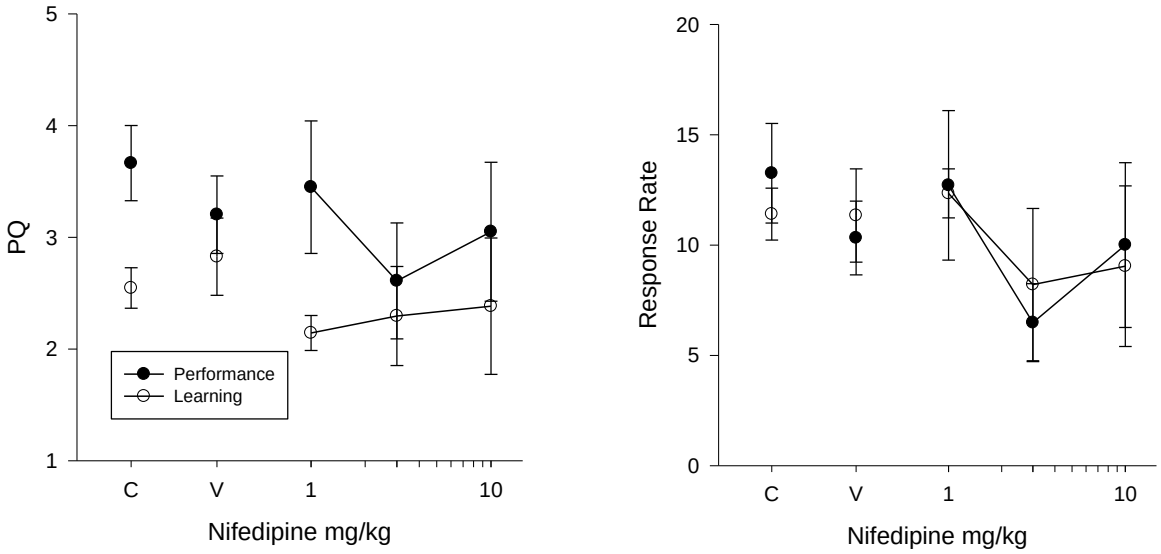

Figure 3. Nifedipine dose effect function for PQ score (left panel) and response rate (right panel). Filled circles represent performance data and open circles represent learning data.

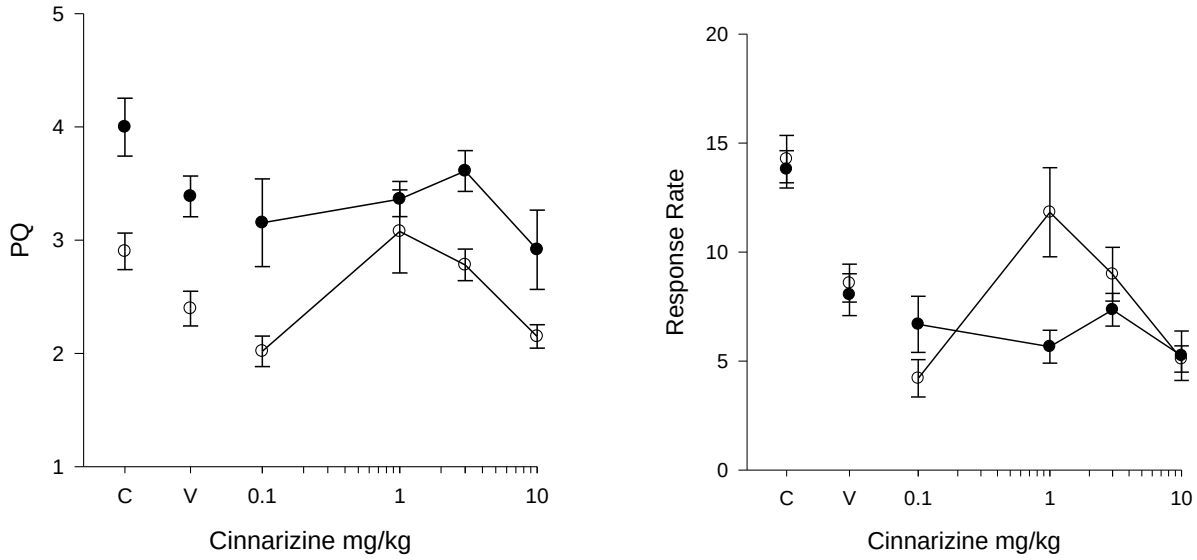

Figure 4. Cinnarizine dose effect function for PQ score (left panel) and response rate (right panel). Filled circles represent performance data and open circles represent learning data.

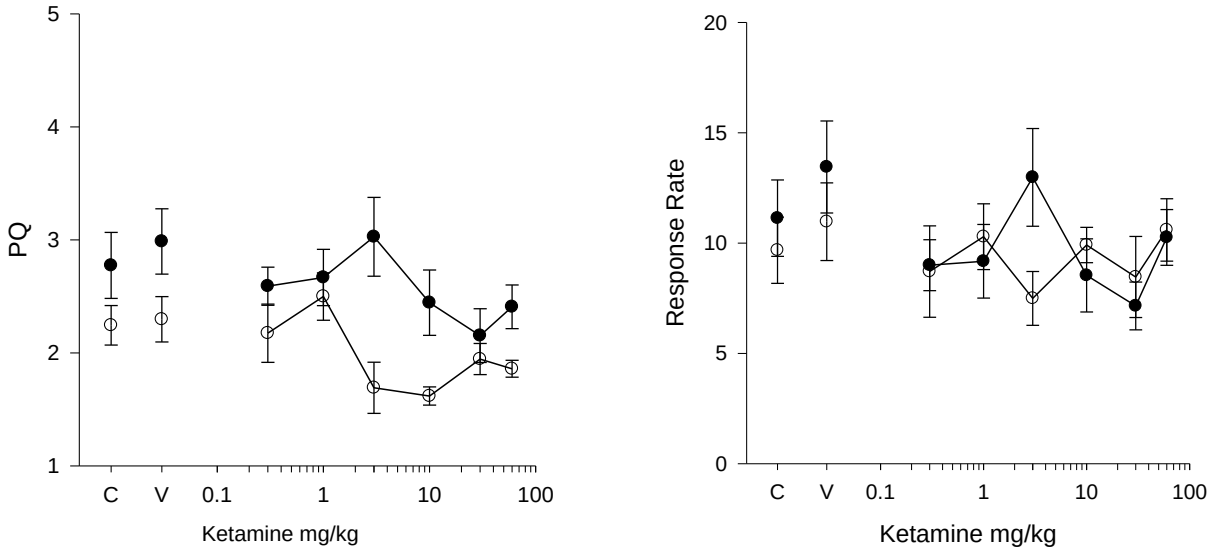

Figure 5. Ketamine dose effect function for PQ score (left panel) and response rate (right panel). Filled circles represent performance data and open circles represent learning data.

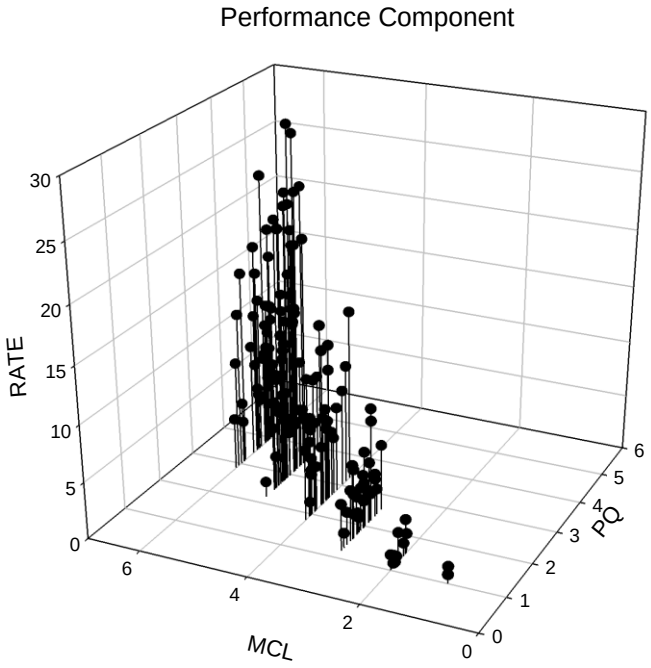

Figure 6. Scatterplot of MCL, PQ and response rate date from the performance component, across all active drug doses.

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Chapter 3: Dietary Nimodipine Delays the Onset of Methyl Mercury Neurotoxicity in Adult Mice

Abstract

Methylmercury is a ubiquitous environmental toxicant to which people can be exposed chronically, via the consumption of contaminated oceanic and fresh-water fish, shellfish and marine mammals. The consequences of gestational, but not adult-onset, MeHg exposure for behavior are well characterized and include cognitive and sensorimotor deficits (that differ depending upon characteristics of exposure). Adult-onset exposure has been described as resulting in primarily sensory and motor deficits. One mechanism by which chronic MeHg may exert its neurotoxicity is by disrupting intracellular calcium homeostasis, with a consequent increase of intracellular Ca^{++} ions in vulnerable neurons. A heterogeneous group of compounds, calcium channel blockers, have been shown *in vitro* to attenuate MeHg's toxicity. To evaluate the role of calcium antagonism in MeHg toxicity functionally adult BALB/c mice were exposed chronically to 0 or 15 ppm of Hg (as MeHg) via drinking water and to nimodipine, a dihydropryidine, L-type Ca^{++} channel blocker with action in the CNS. Nimodipine was administered orally in diets (0, 20, or 200 ppm of nimodipine, producing approximately 0, 2, or 20 mg/kg/day of nimodipine). An incremental repeated acquisition (IRA) of behavioral chains procedure was used to test cognitive function. Here, different response-chains were presented to test the animals' ability to re-acquire a novel response sequence. A "performance" condition in which the same chain was produced every session served as control. MeHg impaired

performance on the IRA task, and this was partially or completely blocked by dietary nimodipine, depending on dose. [Supported by NIH ES003299.]

Introduction

Methyl mercury (MeHg) is an environmental contaminant with well-documented consequences to the nervous system that manifest as behavioral toxicity. Sensitivity to MeHg depends critically upon the age at which exposure occurs and the dosing regimen. A consistent finding from the animal literature is that gestational exposure results in cognitive, motor and sensory impairment (e.g. Rice, 1983; Rice, 1996a; Rice, 1996b; Newland et al., 2004), which is consistent with its widespread accumulation in the brain, including the entire neocortex (Eto, 1997). In contrast, adult-onset exposure results in sensory and motor decline (e.g. Evans et al., 1975; Heath et al., 2010), which is consistent with its more limited accumulation in the cerebellum and sensory and motor cortical regions (Castoldi et al., 2003; Harada, 1995; Health et al., 2010). Less is known about the cognitive impact of chronic, adult-onset exposures but it is possible that learning or memory deficits could occur either directly or secondary to sensory and motor toxicity.

Substantial evidence has accumulated from *in vitro* studies that MeHg disrupts intracellular calcium regulation, leading to a hazardous increase in intracellular calcium concentration (Bearrs et al., 2001; Limke et al., 2003; Marty & Atchison, 1997). Such disruptions in calcium signaling alter neuronal excitability and neurotransmitter release, among other effects (Atchison, 2003; Atchison & Hare, 1994; Yuan & Atchison 2007). Some MeHg-induced neural dysfunction is likely linked to these disruptions in synaptic function (Yuan & Atchison, 2007).

A biochemically heterogeneous group of drugs referred to collectively as calcium channel blockers (CCBs) appears to protect against CNS insults that are associated with increased intracellular calcium concentration. This includes MeHg toxicity (Hare et al., 1993;

Hare & Atchison, 1995; Marty & Atchison, 1998), aging (Thompson et al., 1990; Straube et al., 1990; Levere & Walker, 1991; Kowalska & Disterhoft, 1994), brain trauma (Finger et al., 1990; Mršić et al., 1996) and brain ischemia (Yanpallewar et al., 2004). The effects of CCB administration in the absence of CNS insult is much less clear, however, as some report improvement (e.g. Quartermain et al., 2001) others impairment (e.g. Maurice et al., 1995a) and still others no effect at all (e.g. Deyo et al., 1989). With respect to MeHg toxicity, the evidence for CCBs' protective effects comes largely from *in vitro* studies where they ameliorated MeHg's effects on cell survival, neurotransmitter release, and elevations in intracellular calcium (Hare et al., 1993; Hare & Atchison, 1995a; Hare & Atchison, 1995b; Marty & Atchison, 1998; Sakamoto et al., 1996). In the single *in vivo* case of CCB administration to MeHg exposed animals L- and T-type CCBs, decreased mortality and improved body weight in rats (Sakamoto et al., 1996). More sensitive behavioral endpoints, however, have not yet been evaluated and it is not clear if CCBs might prevent more subtle effects of MeHg exposure including declining sensorimotor function or learning deficits.

Nimodipine is a 1,4-dihydropyridine compound that, like other CCBs, can be used to treat cardiovascular condition, including angina, hypertension, cerebrovascular disease and migraine, as it is a potent vasodilator. Nimodipine passes the blood-brain-barrier more readily than other CCBs (Van den Kerckhoff & Drewes, 1985) and binds with high affinity and specificity to the dihydropyridine receptors on L-type calcium channels in the brain (Belleman et al., 1982; Dompert & Traber, 1984; Peroutka & Allen, 1983), functionally blocking the calcium current through these voltage-dependent channels. Because of its ability to penetrate the blood-brain-barrier and the demonstration *in vitro* of its efficacy, the CCB nimodipine is an excellent choice for studies of CNS injury *in vivo*.

The present study was designed to examine the impact of *adult onset* MeHg exposure on learning deficits, potential neuroprotection by a CCB, and potential benefits conferred by the CCB alone, in the absence of neurotoxicant exposure. To accomplish this we used an incremental repeated acquisition procedure (IRA), which has a number of benefits over other learning tasks. Specifically, IRA procedures generate a steady state of acquisition by repeatedly requiring the acquisition of novel patterns of responses (the 'learning' component), and also by repeatedly requiring the performance of a previously learned response pattern (the 'performance' component). Via these two components, a wealth of behavioral data is collected. This is quite dissimilar to more traditional learning tasks which use very few trails (e.g. single-trial learning tasks, maze learning, avoidance procedures, etc.) or which measure a steady state of performance (e.g. object recognition, place learning, discrimination procedures, etc.). Repeated acquisition procedures have been used in studies of drug action (Paule & McMillan, 1984) and toxicant exposure (Cohn et al., 1993) as a sensitive indicator of subtle changes in learning following such exposure and IRA has been described as an analog to human IQ testing (Paule et al., 1999).

Methods

Subjects. Adult male Balb/c mice purchased from Harlan laboratories (Indianapolis, IN) were housed (2 per cage) in clear polycarbonate cages with wire tops and woodchip bedding. A diagonal Plexiglas® barrier separated cage mates, who were always in the same exposure group. The vivarium was temperature- and humidity-controlled and maintained on a 12-h light-dark cycle (lights on at 6:00 a.m.). All animals were maintained at approximately 24g by feeding a measured quantity of food daily. Two MeHg exposures and three nimodipine diets produced a 2 (MeHg) x 3 (nimodipine) full factorial design with 12-14 animals in each of the six exposure groups. Both MeHg- and nimodipine-group assignments were accomplished so that groups were

indistinguishable on pre-exposure evaluation of free-feeding body mass, performance on rotorod, and autoshaping of operant nose-poking. Animals were approximately 11 weeks old at the start of the experiment and 36 weeks old at the end.

Nimodipine and MeHg exposure. Mice were exposed chronically to 0 or 15 ppm of mercury as methyl mercuric chloride, dissolved in their only source of drinking water, corresponding to 0 and approximately 2600 µg/kg/day based on calculations of consumption undertaken during exposure. Within each mercury exposure group, mice were exposed chronically to 0, 20 or 200 ppm of the dihydropyridine L-type calcium channel blocker nimodipine in their chow. Chow was manufactured by Harlan Teklad custom research diets and based on the Global 18% protein rodent diet (TD.00588) formula. These doses correspond to approximately 0, 2 and 20 mg/kg/day of nimodipine, based on daily consumption. Exposure to both MeHg and nimodipine began three weeks after the mice arrived to the laboratory when the mice were approximately 11 weeks of age.

Apparatus. Experiments were conducted in sixteen operant chambers (Med Associates Inc. St. Albans, VT, model # Med ENV 007) purchased from Med Associates that were enclosed in sound-attenuating cabinets and fit to accommodate mice. Each chamber contained two nose-poke holes on the front panel (right "R" and left "L"), separated by a food tray (connected to a 20 mg pellet dispenser), and one nose-poke hole in the center of the back panel (back "B"). Inserting the nose (or paw) into the nose-hole interrupted an infrared beam located inside the hole, which registered as a response. A single 2.8-W house light was located near the ceiling of the chamber on the back panel. A Sonalert tone generator was located in the top left and a white-noise generator was located in the top right of the back panel.

Procedure. An IRA procedure was used in which a response chain incremented from a one- to a six-link chain within an experimental session. Nose-poke response-chains were built using backward chaining such that a new link was added before (or, in front of) the previously learned link(s). For example, the 6-link response chain "LBLRBR" was trained as follows: R → sucrose, B – R → sucrose, R – B – R → sucrose, L – R – B – R → sucrose, etc. until a 6-link chain was formed. Chain-length was increased using a pre-set mastery-based criterion. Six consecutive correct responses (no errors) increased the chain from a one- to a two-link chain. All subsequent increases in chain length (from two to three link, then to four, and so on) required three consecutive correct response-chains. Each link in the chain was paired with a discrete auditory stimulus. Nose-poking an incorrect nose-hole at any point in the chain resulted in a 2" timeout period in which the house light was illuminated and no response was reinforced. Following this timeout period, the current chain length reset to the beginning. Sessions ended after 1 hour passed or 50 consecutive correct 6-link response-chains occurred. All experimental sessions were conducted at approximately the same time each day, in the same testing room.

Animals were first exposed to the performance component. Here, the same six-link sequence was required during every session. "Learning" sequences, in which the animals were required to acquire a different response sequence within the experimental session, were introduced after 10 performance sessions, at which point the animals were reliably reaching a 4-link performance chain. A total of 17 learning sequences were cycled through the course of the experiment with learning and performance sessions alternating daily. Learning sequences were selected according to the following criteria: all three response locations (R,B,L) were used but response locations never consecutively repeated (e.g. RBBLCR would not qualify) and chains very similar to the performance chain or that had any obvious pattern (e.g. clockwise rotation)

were excluded. A checkerboard pattern covered the door of the experimental chamber during learning sessions, but not during performance sessions. With an IRA procedure, the "performance" component provides a more typical measure of learning, while the "learning" component has the added benefit of allowing for an assessment of a steady state of acquisition.

Criteria for euthanasia. Animals were inspected daily and if an animal appeared ill or moribund (e.g. weight loss, failure to eat, failure to explore or locomote when placed on an open surface, etc.) the attending veterinarian was consulted. Every effort was undertaken to keep an animal alive, providing it did not prolong distress. Animals meeting predefined criteria for euthanasia were euthanized. The Auburn University Institutional Animal Care and Use Committee approved all euthanasia procedures.

Data analysis. Three dependent measures are presented: response rate (total responses divided by time available to make a response, i.e., omitting timeouts), maximum chain length (MCL) reached and a progress quotient (PQ) previously presented by Bailey et al. (2010):

$$PQ = \frac{\left(\sum_{i=1}^{6} w_i R_i \right)}{R_t}$$

Equation 1.

where R_i = number of reinforcers earned on a chain length of length i, w_i = the required chain length and R_t = total reinforcers earned in the session. This index serves as a measure of progress and avoids many problems that are associated with using a measure of accuracy or maximum sequence length reached during a mastery-based criterion IRA procedure, such as the one used here. The numerator weights each reinforcer earned by the chain length during which it was earned so reinforcers following long chains are weighted heavily. Characterized differently, the numerator is a count of all the responses comprising criterion chains. The denominator normalizes PQ by the number of criterion chains that occur. A higher PQ score corresponds to

better IRA performance. This measure is favored over accuracy, because high accuracy can occur even if the mastery criterion (6 or 3 consecutive criterion chains) keeps a mouse at a short chain. Maximum chain length, which provides some guide of progress, does not distinguish between a mouse reaching, say, a six length chain but producing it only once from another that produced that chain length reliably after reaching it (see Bailey et al. 2010 for details).

A LOESS smoothing algorithm was applied to the raw group PQ, MCL and response rate data. The LOESS was used only for graphical display. Data from the performance and learning components are always presented separately. Tests for main effects and interactions between MeHg, nimodipine and session were carried out using a linear mixed-effects (hierarchical) model (or, LME) using the statistical package SYStat 11®. LME was chosen because these models have a number of advantages over traditional repeated-measures analyses of variance (ANOVA) including, among others, that they are able to model unbalanced and incomplete repeated-measures data.

The effects reported below represent the best model for all dependent measures, which we arrived at via a series of model comparisons. Likelihood ratio tests were used to evaluate goodness of fit. The degrees of freedom for the test is given by difference in degrees of freedom for the full and restricted models. The full model included the following fixed effects: session, MeHg, nimodipine, session x nimodipine, session x MeHg and session x nimodipine x MeHg and the random intercept term. Rather than testing all possible restricted models, we were interested in main effects of MeHg and nimodipine exposure and their interactions with each other and with session. Therefore, we tested these restricted models against the full model. Across all dependent measures, the best fit was achieved with session, MeHg dose x session, nimodipine dose x session and nimodipine dose x MeHg dose x session as fixed factors, and a

random intercept. Note that main effects of nimodipine and MeHg are not included. This is reasonable since their effects would likely to be delayed, i.e., would interact with session. Other comparisons revealed that a square root transformed session term to be most appropriate, as it accommodates the downward inflection in the dependent measures that occurred. Therefore all analyses reported below used this transformed session term. Analyses were conducted separately for performance and learning components.

To determine if learning (i.e. PQ or MCL) effects were driven by motor impairment, we tested the hypothesis that MeHg affected PQ differently than rate over the course of the experiment. To do this we compared PQ and response rate of the non-nimodipine, MeHg-exposed animals to that of the non-MeHg exposed animals (filled black symbols in the figures). A two-sample t-test conducted on rate and PQ separately from the performance component, at various time points throughout the experiment was used to evaluate this question.

Initially, a t-test was conducted every 20^{th} session from MeHg exposure day 1 to day 82 (corresponding to MeHg days 26, 54 and 82). In an effort to pinpoint the time at which performance began to deteriorate more precisely, we conducted a t-test every 5^{th} performance session from day 92 (corresponding to MeHg days 92, 106 and 116). To correct for multiple comparisons, the p-value considered significant for all t-test's was adjusted to <0.017 (only comparisons from day 92 on are reported). All graphs were made in SigmaPlot and statistical analyses were conducted using SYStat (San Jose, CA).

Results

PQ. During performance sessions, there was a main effect of session on PQ score, reflecting acquisition of this task ($\beta = 0.472$, SE $\beta = 0.033$, $z = 14.099$). Also, effects of nimodipine dose ($\beta = -0.142$, SE $\beta = 0.022$, $z = -6.525$) and of MeHg dose ($\beta = -0.057$, SE $\beta =$

0.015, z = -3.786) were more pronounced in later sessions (i.e. with prolonged exposure duration), resulting in significant interaction terms (nimodipine by session and MeHg by session, respectively). Finally, the effect of nimodipine was more pronounced for the MeHg exposed group during later sessions, resulting in a significant three-way interaction between among nimodipine, MeHg and session ($\beta = 0.059$, SE $\beta = 0.010$, $z = 6.087$). Here, all p's < 0.000. During learning sessions a main effect of session, nimodipine by session interaction and three-way, nimodipine by MeHg by session, interaction term reached significance (all p's < 0.041). Figure 1 illustrates these results.

Maximum Chain Length Reached (MCL). As with PQ, there was a main effect of session on MCL during performance sessions, again, reflecting acquisition of this task ($\beta = 0.615$, SE $\beta = 0.041$, $z = 14.915$). Also, the effects of nimodipine dose ($\beta = -0.160$, SE $\beta = 0.027$, $z = -6.007$) and of MeHg dose ($\beta = -0.076$, SE $\beta = 0.019$, $z = -4.081$) were more pronounced in later sessions, resulting in significant interaction terms. Finally, the effect of nimodipine was more pronounced for the MeHg exposed group during later sessions, resulting in a significant three-way interaction among nimodipine, MeHg and session ($\beta = 0.064$, SE $\beta = 0.012$, $z = 5.320$). Here, all p's < 0.000. During learning sessions a main effect of session, nimodipine by session interaction and three-way, nimodipine by MeHg by session, interaction term reached significance (all p's < 0.004). Figure 2 illustrates these results.

Response Rate. During performance sessions, there was a main effect of session on rate ($\beta = 2.242$, SE $\beta = 0.334$, $z = 6.706$). Also, the nimodipine by session ($\beta = -0.494$, SE $\beta = 0.216$, $z = -2.284$) but not MeHg by session interaction term reached significance. As with PQ and MCL, the effect of nimodipine on rate was more pronounced for the MeHg exposed group during later sessions, resulting in a significant three-way interaction among nimodipine, MeHg

and session ($\beta = 0.282$, SE $\beta = 0.097$, $z = 2.890$). Here, all p's < 0.022. Figure 3 illustrates these results. During learning sessions, however, only the main effect of session reached criteria for significance ($p < 0.000$) while the three-way interaction term was marginally significant ($p = 0.054$).

MeHg effects on response rate and PQ as a function of session. MeHg exposure was associated with a significant decline in PQ score starting at MeHg day 94 $t(18) = 2.881$, p=.010. However, MeHg exposure was not, at any point, associated with a decline in rate (all p's > 0.273).

Discussion

It has been shown *in vitro* that MeHg disrupts intracellular calcium homeostasis, but the relevance of this to the behaving organism has not been clear. Here, mice exposed to MeHg in the absence of nimodipine, had markedly impaired acquisition and performance of the repeated acquisition of response chains. Dietary nimodipine blocked this learning impairment in a dose-related fashion. In fact, the MeHg exposed animals receiving the higher dose of nimodipine (200 ppm) were indistinguishable from those not exposed to MeHg. This is consistent with reports from the molecular literature showing CCBs prevent cell death and diminish alterations in neurotransmitter release following MeHg application (Hare et al., 1993; Hare & Atchison, 1995a; Hare & Atchison, 1995b; Marty & Atchison, 1998; Sakamoto et al., 1996). Further, evidence that MeHg-induced impairment in PQ preceded response-rate decreases suggests decreasing rate does not solely drive PQ deficits.

With respect to a behavioral mechanism, PQ deficits that do not appear to be directly resulting from rate changes, suggests that, while indeed necessary for the demonstration of learning, a rate measure (or, motoric contribution) is not sufficient for learning or memorial

processes to operate. This provides support for the conclusion that IRA is a sensitive behavioral task capable of detecting even subtle changes in cognition, well before changes in response rate occur. In fact, repeated acquisition procedures, such as the one used here, generate complex behavior for which even subtle changes in cognitive function may be detected (Bailey et al., 2010) and have proven useful in both studies of drug (Paule & McMillan, 1984) and toxicant (Cohn et al., 1993) effects. In children, measures of accuracy on IRA tasks are correlated (r=0.6) with score on IQ tests, so the two measures may tap similar higher-order functions. (Paule et al., 1999). Further, IRA progress may be considered apical, requiring attention, working memory as well as intact sensory and motor systems. Finally, IRA procedures have the added benefit of including a performance component, wherein stimulus control is presumably high, which captures the execution of an already learned task. The learning component may be conceptualized as having weaker stimulus control and likely captures the acquisition of a novel requirement.

The experimental design employed here not only facilitated an evaluation of nimodipine's neuroprotection from MeHg, but also allowed for a determination of the effects of nimodipine on the intact, unexposed animal. This is particularly relevant because disparate effects of CCBs have been demonstrated in the animal literature when administered in the absence of CNS insult. Reports from discrimination procedures (Deyo & Hittner, 1995; Quartermain et al., 1993), passive and active avoidance (Quartermain et al., 1993), and spatial learning (McMonagle-Strucko & Fanelli, 1993) studies have indicated CCB administration was beneficial in the absence of CNS injury. However, other studies, including those using spontaneous alternation (Maurice et al., 1995), passive avoidance (Maurice et al., 1995;

Clements et al., 1995) and simultaneous discrimination (Clements et al., 1995) procedures, have shown either impairment or no effect of CCB administration in the absence of CNS injury.

Here, when nimodipine was administered chronically to healthy animals there was no detectable change in learning or performance of the IRA response chain. Conceptually, these data stand on relatively solid ground, as a CCB should act to correct an already present dysregulation of calcium; however, in the absence of such dysregulation a CCB would have no effect or, in the extreme case, may be thought to have detrimental consequences to the uninjured organism. In that case, the administration of a CCB may act to actually disrupt normal calcium homeostasis. Despite the absence of an effect on learning, nimodipine administration did correspond to changes in response rate, however these rate-changes did not manifest in PQ or MCL score. Motoric effects of acute nimodipine have been reported in the literature, however, rate reductions, not increases, are noted (Viveros et al., 2007).

The data presented here that indicate neuroprotection from MeHg by nimodipine, combined with the *in vitro* work, may implicate the cerebellum as a relevant brain area involved with the behavioral effects described. The cerebellar granular cells of the cerebellum are known to be especially susceptible to MeHg (Takeuchi et al., 1962; Atchison & Hare 1994a; Yuan & Atchison; 2007) and recently the cerebellum has received renewed attention as evidence has accumulated indicating the role of the cerebellum in executive function (for a review see Strick et al., 2009). Underlying this role in cognition is the evidence that cerebellar projections arrive at non-motor cortical areas. As such, increased intracellular calcium in the cerebellum, leading to cell dysfunction or death, may correspond to both the motor and cognitive effects associated with MeHg exposure. This is an important consideration when discussing changes in learning or

memorial processes following exposure to a toxicant that is known to have high selectivity for cells of the cerebellum, as opposed to the prefrontal cortex.

Finally, it is likely not the case that CCBs and MeHg act on identical mechanisms of cytosolic calcium entry. While it is known that CCBs work by effectively blocking calcium ion entry via voltage-gated calcium channels on the cell membrane, this is likely not the sole mechanism by which MeHg results in elevated intracellular calcium levels. MeHg has relatively widespread effects on the nervous system, many of which are calcium mediated. One way in which MeHg causes increased intracellular calcium concentrations is via mitochondrial alterations. Specifically, following chronic exposure, MeHg appears to inhibit various mitochondrial enzymes and can depolarize the mitochondria membrane. In brief, this reduces ATP production and calcium buffering capacity. A reduction in calcium buffering alters intracellular calcium homeostasis and results in excess concentrations of calcium inside the cell. Levesque and Atchison (1991) showed that mitochondria isolated from rat forebrain, when exposed to MeHg, released mitochondrial-associated calcium ions, and at the same time prevented uptake of calcium ions by the mitochondria. MeHg was also shown to open the mitochondrial permeability transition pore (MPTP) leading to apoptotic cell death (Limke & Atchison, 2002; Limke et al., 2003, 2004). This could result in dissipation of mitochondrial membrane potential (MMP), which eventually could result in efflux of mitochondrial calcium ions and inhibition of mitochondrial calcium ion uptake (Denny et al., 1993; Hare et al., 1993; Komulainen & Bondy, 1987). Effects on the SER have also been implicated with MeHg toxicity. Limke et al. (2004) described the effect of MeHg on a M3 muscarinic receptor-linked pathway that increased intracellular calcium. They found that MeHg activates M3 receptors, which subsequently generate $Ip_{3,}$ which in turn activates Ip_3 receptors on the SER causing a release of

calcium from the SER to the cytosol, thereby increasing intracellular calcium. It may be reasonable to hypothesize, however, that CCB administration results in a net decrease in intracellular calcium, therein halting the negative consequences of elevated intracellular calcium concentrations caused by MeHg.

In summary, the behavioral data collected here correspond to reports by Atchison and colleagues and Sakamoto and colleagues that showed CCB treatment to attenuate the negative consequences (e.g. synaptic alterations, cell survivability, etc.) of MeHg exposure at the cellular level. These behavioral data, in combination with data from *in vitro* designs, provide support for the hypothesis that a key mechanism by which MeHg exerts its cellular and behavioral effects is through disrupted intracellular calcium homeostasis. Therapeutically this information is of paramount importance as it implicates CCBs in the prevention or treatment of MeHg toxicity. Also, cognitive decline has been demonstrated following MeHg exposure occurring exclusively during adulthood. Finally, these data speak to the nature of CCB effects in the absence of CNS injury. Here, there was no impact, in terms of cognitive function, of any dose of nimodipine in groups not exposed to MeHg. This is inconsistent with some other reports in the literature that have suggested CCBs may have cognitive enhancing properties even in the absence of obvious CNS insult.

Figures

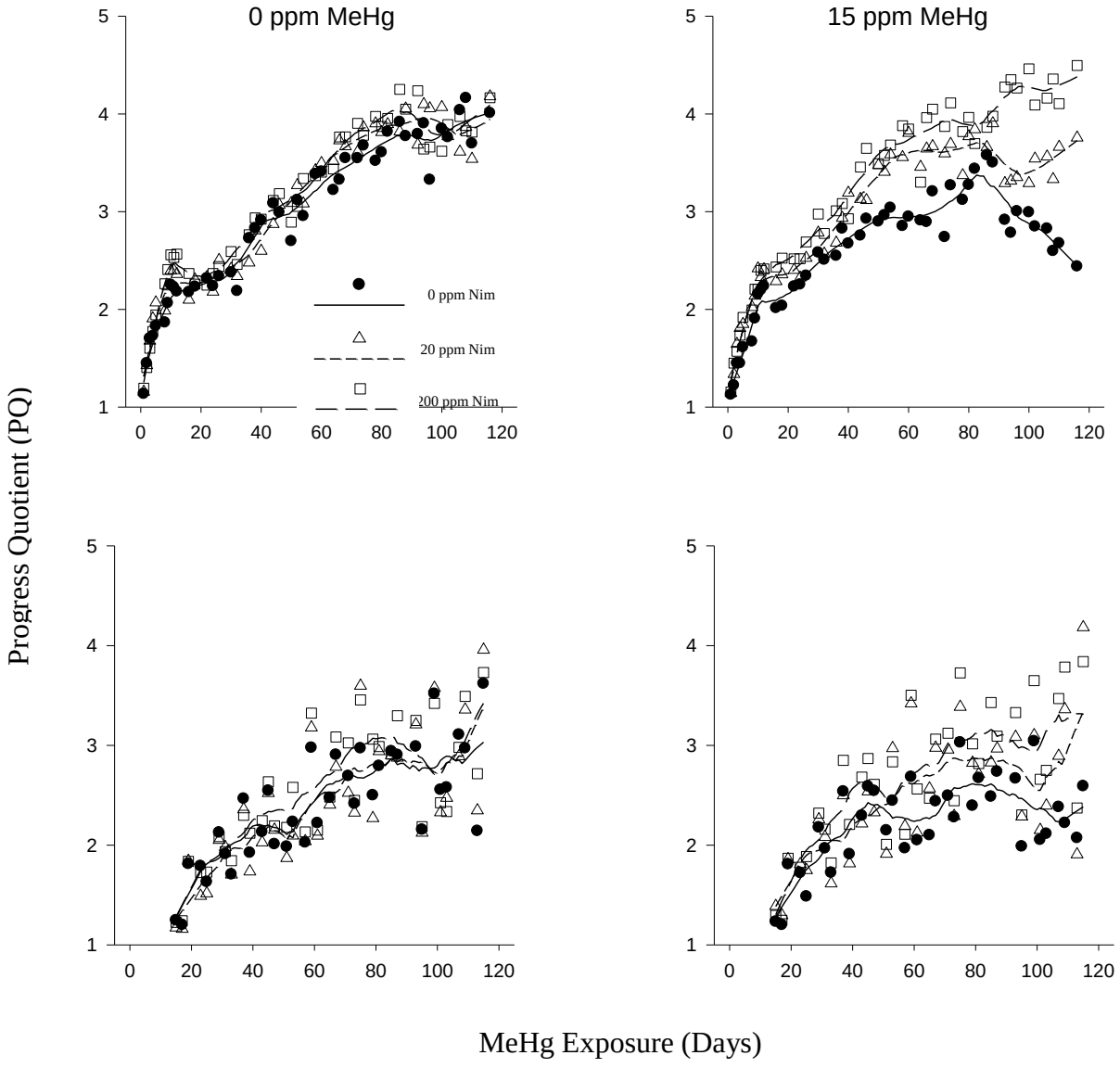

Figure 1. Top row is PQ score from the performance component and the bottom row is from the learning component of the IRA procedure. The left column contains data from the 0- ppm MeHg exposure group, for each of the three nimodipine condition (0, 20 and 200 ppm Nim). The right column contains data from the 15 ppm MeHg exposure group for each nimodipine condition. Data points represent the mean for each group as a function of MeHg exposure day. The curves represent a LOESS smoothing algorithm.

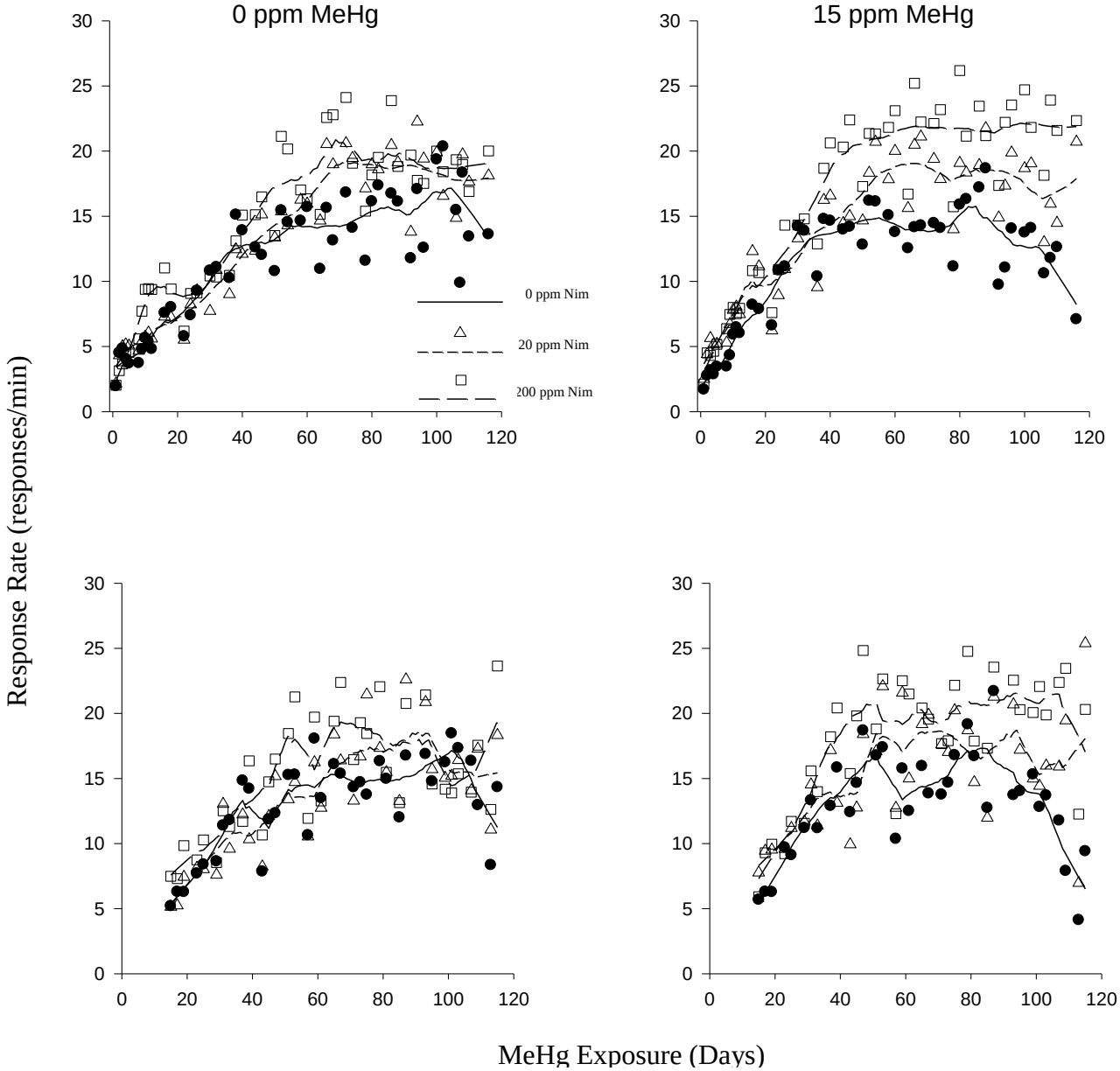

Figure 2. Top row is response rate from the performance component and the bottom row is from the learning component of the IRA procedure. The left column contains data from the 0- ppm MeHg exposure group, for each of the three nimodipine condition (0, 20 and 200 ppm Nim). The right column contains data from the 15 ppm MeHg exposure group for each nimodipine condition. Data points represent the mean for each group as a function of MeHg exposure day. The curves represent a LOESS smoothing algorithm.

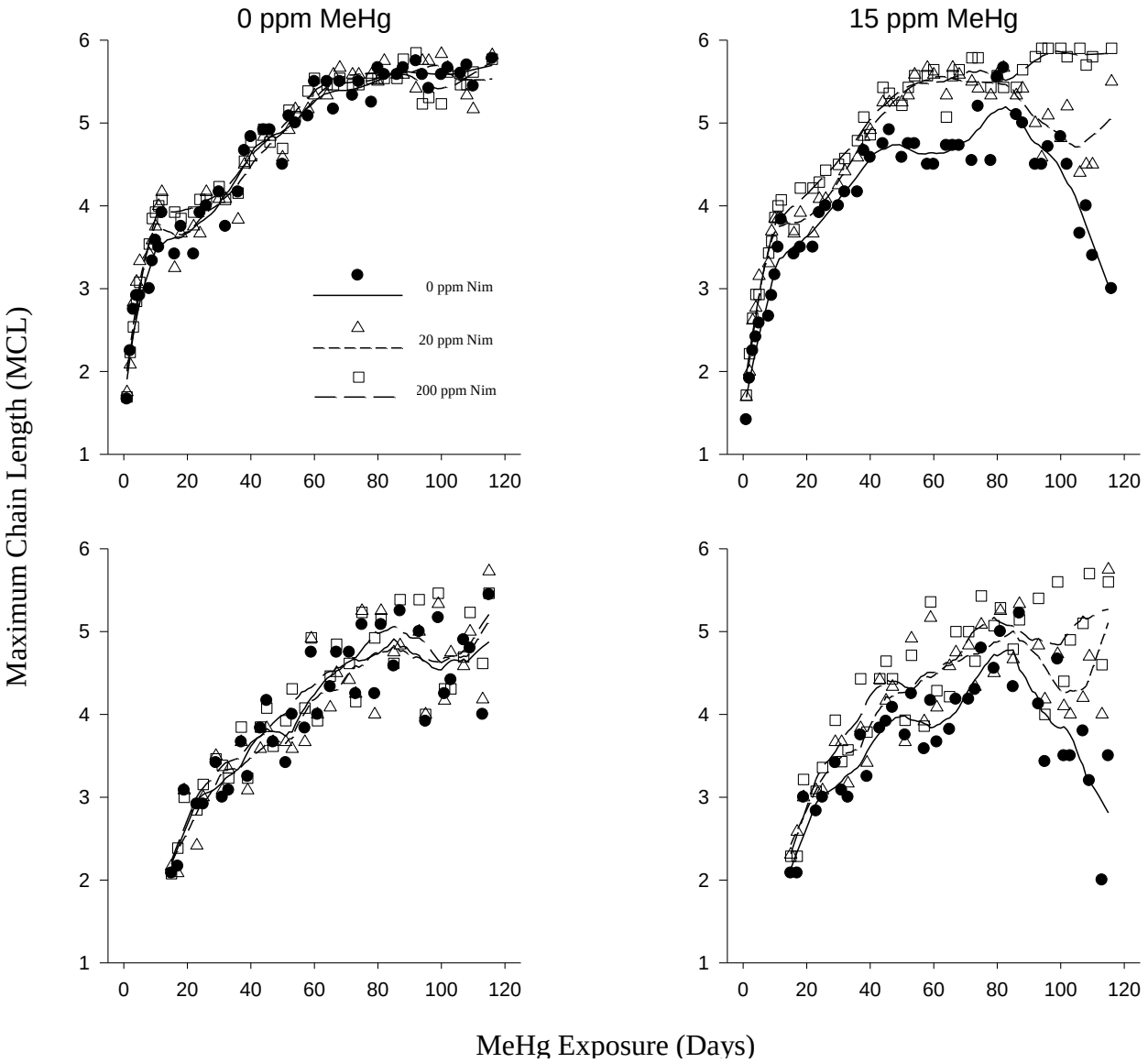

Figure 3. Top row is MCL score from the performance component and the bottom row is from the learning component of the IRA procedure. The left column contains data from the 0- ppm MeHg exposure group, for each of the three nimodipine condition (0, 20 and 200 ppm Nim). The right column contains data from the 15 ppm MeHg exposure group for each nimodipine condition. Data points represent the mean for each group as a function of MeHg exposure day. The curves represent a LOESS smoothing algorithm.

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