

CELLULAR MECHANISMS FOR NIMODIPINE'S REDUCTION OF AGING-RELATED LEARNING DEFICITS

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Systemic administration of nimodipine, a 1,4-dihydropyridine Ca^{2+} channel antagonist, reverses aging-related behavioral deficits in rabbits, including impaired open-field behavior and slowed acquisition of associative eyeblink conditioning. The cellular mechanisms for these effects are the focus of current neurophysiological research in our laboratory, summarized here. Our working hypothesis is that regulation of intracellular Ca^{2+} in the hippocampal system is impaired by aging, and nimodipine reduces this impairment by blocking calcium entry through voltage-dependent calcium channels. In effect, nimodipine restores important biophysical properties in aging hippocampal neurons so as to mimic those observed in neurons from young animals.

This hypothesis is not new, nor ours alone. For example, Khachaturian (1984, 1989) has proposed that the aging brain loses the ability to regulate homeostatically intracellular calcium, leading to a cascade of problems, dependent upon the neuronal cell type and the degree of dysregulation involved. Cellular impairments consequent to this loss of intracellular calcium regulation include changes in phospholipid metabolism, alterations in many other cytosolic second and third messenger systems, chronic changes in synaptic transmitter release, and in a number of different model systems of necrotic events and cell death (Feig & Lipton, 1990). We have concentrated our efforts on the hippocampus, since neurons in this region exhibit profound functional changes following relatively small perturbations in intracellular calcium levels (see discussion below). They are among the first neurons to undergo degenerative changes after brief ischemic episodes (Johansen *et al.*, 1990; Onodera *et al.*, 1990), and are severely impacted in aging associated brain

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disorders including but not limited to Alzheimer's disease (Mani *et al.*, 1986; see also other papers in this volume). Although much attention has been given to aging-related deficits in cholinergic systems that provide major afferents to the hippocampus and neocortex, our data and that of others suggests that other biophysical alterations result in impaired intracellular calcium regulation and contribute to the learning impairments observed in aging subjects. The hippocampal region has long been studied as a region critically involved in learning and memory (Olton, 1988; Squire *et al.*, 1989). Since learning is impaired in many aging subjects (see Disterhoft *et al.*, this volume), and since the neuroanatomy (Geinisman *et al.*, 1986; Landfield *et al.*, 1977), the neurochemistry (Eldridge *et al.*, 1989) and the neurophysiology (Barnes, 1988; Lamour *et al.*, 1989) of the hippocampal region are profoundly affected by aging, it follows that amelioration of dysfunctional physiological changes in the region may lead to reductions in some of the behavioral and psychological dysfunctions commonly associated with aging.

The hippocampus plays a key role in associative learning

The hippocampal region exhibits the highest binding affinities and greatest density of dihydropyridine binding sites in the brain (Skattebøl & Triggle, 1987; van den Kerckhoff & Drewes, 1989), making it a prime target for calcium channel antagonist actions. Three lines of evidence derived from work in our laboratory (as well as that of others) relating behavior, *in vivo* physiology, and *in vitro* biophysics, suggest that the hippocampus is a key mediator of nimodipine's beneficial effects on age-related learning deficits. This is not to suggest that other brain areas are not impacted both by aging or by nimodipine, nor that other areas are not involved in learning. Instead, it has allowed us to investigate rigorously the cellular and subcellular mechanisms underlying both learning and aging-related impairments in learning, within the context of a well defined biological system, using classical neurophysiological approaches, coupled to the refined behavioral tools discussed in the preceding paper (Disterhoft *et al.*, this volume).

First, behavioral studies indicate that functional integrity of the hippocampus is required for many forms of learning in young animals, and that functional declines associated with aging also impair learning (see full discussion in Disterhoft *et al.*, this volume). Complete ablative lesions of the hippocampus result in learning deficits in trace eyeblink conditioning tasks similar to, although more severe than, those seen in aging rabbits (Moyer *et al.*, 1990b; Solomon *et al.*, 1986; Solomon & Graves, 1985). Pharmacological blockade of cholinergic or glutaminergic synapses, together the major afferent neurotransmitter systems to the hippocampus, produce profound deficits in a wide range of learning tasks, mimicking the effects of hippocampal lesions (Paylor & Rudy,

1990; Robinson *et al.*, 1989; Thompson & Disterhoft, 1991). Senescent animals exhibit deficits in many learning tasks similar to those observed in young animals after lesions or pharmacological blockade of hippocampal activity (Caprioli *et al.*, 1991; see also other papers, this volume).

Second, firing rates of hippocampal pyramidal cells model the conditioned eyeblink response in young rabbits. Investigations using extracellular recording techniques in intact behaving animals have shown that increases in single-neuron activity in the intact hippocampus are highly correlated with learning. Increased frequency and reliability of firing are observed as learning progresses, reaching asymptote shortly prior to behavioral asymptote (Berger & Thompson, 1977). The activity of hippocampal pyramidal cells increases above spontaneous baseline firing rates slightly in advance of the onset of a conditioned response, and continues above baseline until after the paired unconditioned stimulus is presented. Berger and Thompson described this dramatic correlation between the firing of hippocampal pyramidal neurons and the conditioned behavioral response as "neural modelling." Work in our own laboratory has shown that almost all identified pyramidal neurons isolated in the CA1 and CA3 subfields of the hippocampus in trace conditioned rabbits are functionally modulated during eyeblink conditioning (Akase *et al.*, 1988). Cells followed during 300 msec trace conditioning trials show clear modelling of the behavioral response, similar to that described by Berger and colleagues, but with a novel additional burst of firing shortly after onset of the CS, an apparent priming response to the tone CS that may be important for setting up a memory trace across the relatively long interstimulus trace interval. This non-habituating response to the CS (i.e. the response persists in overtrained animals) is almost certainly unique to trace conditioning, as it has not previously been reported. It may be a further indication of the critical involvement of the hippocampal circuitry in this relatively complex (compared to delay conditioning) associative learning task.

Third, the calcium-dependent afterhyperpolarization (AHP) responses of hippocampal CA1 pyramidal cells are reduced in animals that have been eyeblink conditioned (Disterhoft *et al.*, 1986; Coulter *et al.*, 1989; de Jonge *et al.*, 1990). Interestingly, this reduction in the AHP is not dependent solely on training, since animals that have begun training but not yet successfully learned the task exhibit no AHP reduction; only animals that successfully learn the task show the biophysical effects (Disterhoft *et al.*, 1988). The converse experiment, demonstrating that AHP reductions are necessary and sufficient for learning to occur, and that specific manipulations of the AHP result in highly correlated changes in learning, is not currently technically feasible in our rabbit eyeblink preparation, since *in vivo* AHP measurements (as well as manipulations via current

injection) during the course of learning are difficult to achieve and open to multiple interpretations (see Woody *et al.*, 1991, as an example).

The firing rates of CA1 (and other) neurons are regulated by slow AHPs, that act to "clamp" the cell membrane potential at a hyperpolarized level incompatible with recurrent firing (Madison & Nicoll, 1984). Reductions in the AHP make cells more excitable; the *in vitro* data on AHP reductions is thus congruent with the *in vivo* data indicating increased conditioning-specific firing activity. The AHP has been shown to be Ca^{2+} -sensitive, as intracellular buffering with EGTA or BAPTA or removal of Ca^{2+} from the extracellular media in *in vitro* hippocampal slice preparations abolishes or severely reduces the amplitude and duration of the AHP (Lancaster & Adams, 1986). These findings suggest that agents which alter calcium influx should alter the afterhyperpolarizing responses of hippocampal neurons. Further, direct or indirect pharmacological manipulation of the AHP may have a significant impact on learning.

Since hippocampal neurons from aged animals exhibit larger AHPs than those from young animals (see discussion below), learning deficits in aging animals and in aged humans may be partly attributable to greater difficulty in regulating calcium-dependent AHPs. The calcium-sensitive AHP in hippocampal neurons is functionally quite similar to the cholinergic-sensitive M-current (another macroscopic potassium conductance blocked by muscarinic agonists), in that it serves to inhibit excitation of neurons, holding them to more hyperpolarized resting potentials at which they are less likely to fire. It is interesting to note that both cholinergic and Ca^{2+} dependent systems in the same neurons show similar functional declines with aging. Whether a common subcellular mechanism links the two sets of changes is unknown, but is certainly open to further investigation.

Changes in hippocampal calcium conductances with aging

The calcium-dependent AHP is increased in both peak amplitude and in duration in pyramidal cells recorded intracellularly in hippocampal slices taken from aging rats (Landfield & Pitler, 1984) and aging rabbits (Moyer *et al.*, 1991; see Figure 1). The AHPs in aging neurons are of extremely large amplitude and long duration (i.e. more than 5 mV in amplitude and 800 msec in duration after a 4-spike burst), which effectively eliminates spontaneous or induced activity for the duration of the AHP. The larger AHP thus increases the interspike interval and decreases the observed firing rate in the whole animal. In fact, the AHPs observed in aging neurons are remarkably similar to those seen in young neurons in the presence of high levels of extracellular calcium, which presumably increases calcium influx during depolarizing action potential generation (Landfield & Pitler, 1984). Similarly, calcium potentials (slow onset potentials seen during depolarization with TTX

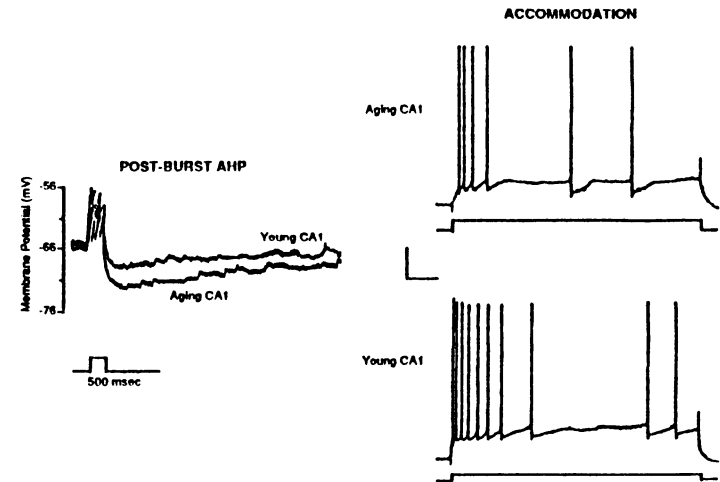


Figure 1. CA1 pyramidal neurons in hippocampal slices taken from aging rabbits are less excitable than neurons in slices from young rabbits. Afterhyperpolarization (AHP) responses following a burst of 4 spikes evoked by depolarizing intracellular current injections are significantly larger in neurons recorded intracellularly in slices from aging rather than young rabbits. Both AHP peak amplitude and AHP integrated area (a measure combining both amplitude and duration) are increased significantly in neurons from aging rabbits. Spike accommodation is also greater in aging neurons than in young ones, as young neurons fire more spikes to a prolonged (800 msec) depolarizing current pulse before accommodating than do aging ones. All of these biophysical alterations may make major contributions to the aging-related learning deficits discussed. Calibration: 20 mV, 100 msec.

blockade of sodium potentials; Wong & Prince, 1978) are increased in hippocampal neurons from aging rats (Pitler & Landfield, 1990) and aging rabbits (Moyer *et al.*, 1990a). Potentiation of the Schaffer collateral synapse from CA3 to CA1 pyramidal neurons is also impaired in aging rats (Landfield *et al.*, 1978). Interestingly, high levels of magnesium, a non-specific competitive antagonist of calcium entry, reverses some learning deficits observed in aging rats and also reverses these and other aging-related biophysical changes (Landfield & Morgan, 1984). Although alterations in intracellular calcium buffering, changes in sequestering within organelles, or reduced ATP-dependent extrusion from the neuronal membrane cannot be ruled out, the data presented are consistent with the hypothesis that increased calcium entry via voltage-dependent calcium channels is a hallmark of neurons in aging, learning deficient animals (Landfield *et al.*, 1989).

Nimodipine blocks L-type calcium currents in hippocampal neurons

Nimodipine (see Figure 2) is a dihydropyridine calcium channel antagonist (Scriabine & van den Kerckhoff, 1988). It was originally tested in humans to reduce the consequences of ischemic stroke. Interestingly, a preliminary report suggested that nimodipine enhanced learning in aging humans after ischemic episodes, which led to further testing in normal aging populations (see discussion in Disterhoft *et al.*, this volume). Nimodipine blocks L-type calcium channels in vascular smooth muscle, resulting in dose-dependent vasodilation. Nimodipine has high specificity for cerebrovascular smooth muscle, increasing cerebral blood flow in a dose-dependent fashion (Haws & Heistad, 1984). It appears to have utility in animal models of ischemia, reducing infarct size and edemic sequelae secondary to neuronal and glial necrosis (Mossakowski & Gadamski, 1990; Nuglich *et al.*, 1990), and is currently available for use in human patients for treatment of subarachnoid hemorrhage. Our interests in nimodipine's effectiveness, however, go beyond its abilities to improve cerebrovascular perfusion.

Instead, we hypothesize that it directly blocks neuronal calcium channels *in vivo*, so as to reverse specifically the aging-related changes in neuronal excitability discussed above. Several studies (Hoffmeister *et al.*, 1985; van den Kerckhoff & Drewes, 1989) have shown that nimodipine, which is extremely lipophilic, crosses the blood brain barrier to a greater extent than other dihydropyridines, making central dihydropyridine binding sites accessible to peripherally delivered nimodipine. The fact that the drug has access to central nervous system binding sites, however, is not sufficient to demonstrate that its effects are a result of ligand-receptor interactions at these sites (nor a result of a chain of events initiated by these binding events). Thus, the following neurophysiological experiments were carried out to demonstrate nimodipine's actions on neuronal tissue, in several *in vitro* preparations, as well as in the awake intact rabbit preparation identical to that used for our behavioral studies.

One study in our laboratory (Black *et al.*, 1990) demonstrated that the 1,4-dihydropyridine calcium channel antagonist nimodipine partially blocked high-threshold non-inactivating (L-type) calcium currents in acutely dissociated hippocampal pyramidal cells. The kinetic and pharmacologic properties of these currents were studied using patch electrodes to provide whole-cell voltage-clamp recordings, with records obtained from cells for periods up to 4 hr after dissociation, allowing complex voltage command protocols to be carried out at several holding potentials in the presence of a series of solutions. Nimodipine and BAY-K-8644, a dihydropyridine calcium channel agonist, were pressure-ejected from pipettes placed under microscopic control near the cell. As in other studies of the effects of dihydropyridines on Ca^{2+} currents in hippocampal CA1 and CA3 pyramidal cells in a number of preparations (Docherty & Brown 1986; Gähwiler & Brown 1987;

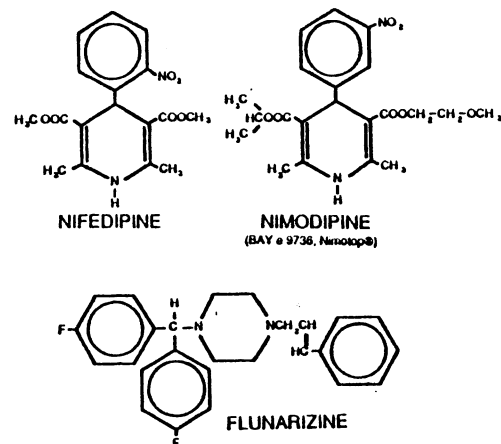


Figure 2. The 1,4-dihydropyridine high-voltage activated non-inactivating (L-type) calcium channel antagonists *nifedipine*, widely used as an antihypertensive vasodilator, and *nimodipine*, which exhibits greater specificity for cerebrovascular smooth muscle and for neuronal tissue, and the diphenylalkylamine calcium channel blocker *flunarizine*, which blocks transient low-voltage activated transient (T-type) calcium currents. These three calcium channel antagonists were tested in the awake rabbit preparation described earlier (Disterhoft *et al.*, this volume), to see if nimodipine's effects on neuronal activity differed from those of other drugs that had similar peripheral effects.

Mogul & Fox, 1991), 10 μ M nimodipine reduced the peak current by about 50% in dissociated neurons, while 10 μ M BAY-K-8644 potentiated the current by an approximately equal amount. These effects were reversible after washing (see Figure 3). Thus, when applied directly to the soma of dissociated CA1 neurons under good voltage control, nimodipine blocked L-type Ca^{2+} currents. The next question addressed, therefore, was whether it has similar effects in more intact preparations.

Nimodipine's effects on hippocampal neuronal activity *in vivo*

A chronic extracellular multiple-electrode assembly was used for simultaneous isolation of a large number of single-units in the awake, behaving rabbit before, during, and after infusion of different doses of nimodipine, the drug vehicle alone, or of other calcium channel antagonists. Nimodipine enhanced extracellular firing activity in an aging- and dose-dependent fashion in awake animals, with the greatest enhancement at the dose of nimodipine previously shown to reverse aging-related learning deficits (Thompson *et al.*, 1990). Reliable rate increases were noted within 8 min after drug infusion began, and were stable within 20 min. Spontaneous firing activity returned to baseline rates within 20 min after nimodipine infusion ended. Significantly greater enhancements in firing rates were seen in cells recorded in aging subjects than in young ones (see Figure 4).

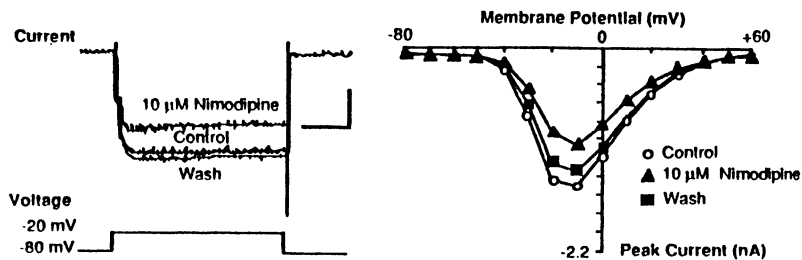


Figure 3. Nimodipine blocks non-inactivating high-voltage activated (L-type) calcium conductances in voltage-clamped acutely dissociated hippocampal pyramidal neurons from guinea-pigs. 10 μ M nimodipine reduced the calcium conductance activated by a voltage step from -80 mV to -20 mV by approximately 35%. The effects of the dihydropyridine was almost fully reversible after a brief wash.

dihydropyridine that has similar effects on cerebral blood flow but enters the brain to a much lesser extent; van den Kerckhoff, & Drewes, 1989) on hippocampal single-unit activity, specifically to address the issue of cerebral blood flow effects (Thompson *et al.*, 1990). The lack of effect of either of the other two calcium channel blockers (Figure 4C and 4D) and the dramatic effects of nimodipine on hippocampal neuronal activity in the awake rabbit strongly argues against cerebrovascular perfusion changes as a primary mechanism of its behavioral actions.

The next step in our analyses of the effects of nimodipine on hippocampal physiology has been to define the specific cellular excitability changes nimodipine induces in neurons in hippocampal slices. Since aging neurons exhibit increased AHPs, and since learning reduces the AHP in young rabbits, AHP amplitude and duration were studied before and after nimodipine treatment. Intracellular current-clamp recordings were used to

An important qualification that should be considered is that we have concentrated our hypotheses on nimodipine's blockade of neuronal calcium channels. Nimodipine and other dihydropyridines also block calcium channels in smooth muscle, thereby increasing cerebral blood flow in rabbits at the doses tested (Haws *et al.*, 1983). It is possible that the behavioral effects we have noted in aging rabbits are primarily mediated by enhanced cerebral blood flow, rather than direct blockade of neuronal calcium channels. In reply, it should be noted that nimodipine readily crosses the blood-brain barrier (Hoffmeister *et al.*, 1985) and binds with high affinity to dihydropyridine receptor sites, which are most concentrated in the hippocampal region (Skattebøl & Triggle, 1987). Further, we directly compared the effects of *nimodipine* (which readily crosses the blood-brain barrier) with that of *flunarizine* (which blocks T-type rather than L-type calcium channels; Takahashi & Akaike, 1991; and thereby also alters cerebral blood flow) or *nifedipine* (another

examine the effects of bath applied nimodipine in the hippocampal slice (Moyer *et al.*, 1991). In cells held at similar resting membrane potentials, AHPs in pyramidal cells from aging animals were significantly larger than those from young animals. Nimodipine caused a dose-dependent reduction in the size and integrated area of the slow AHP of CA1 pyramidal neurons, with significantly greater AHP reductions in aging cells (see Figure 5). AHP reductions were seen in aging cells at concentrations of nimodipine as low as 100 nM. Additionally, nimodipine produced a marked increase in the number of action potentials elicited by a long depolarizing pulse (i.e. decreased spike accommodation) in aging animals, at doses as low as 10 nM. These changes were not accompanied by alterations in input resistance. The current required for orthodromic synaptic activation was typically decreased by nimodipine in aging neurons (see also O'Regan *et al.*, 1991). Reduced spike accommodation, coupled with the reductions in both the amplitude and the duration of calcium-sensitive AHPs, provides further evidence for nimodipine's enhancement of hippocampal neuronal excitability. An increased number of spikes are fired by aging neurons treated with nimodipine, for any given depolarizing stimulus strength, as compared to age-matched controls. This is precisely the effect the earlier findings (discussed above) relating hippocampal neuronal activity to learning would predict as a necessary condition for successful associative learning to occur.

CONCLUSIONS

Nimodipine acts to reverse aging-induced reductions in hippocampal neuronal excitability, presumably via its effects on hippocampal voltage-dependent calcium channels. We have demonstrated that the dihydropyridine calcium antagonist nimodipine markedly facilitates associative learning in aging rabbits. Further, we have demonstrated in young adult rabbits that one change induced by classical conditioning is a reduction in the afterhyperpolarization (AHP) that follows a burst of action potentials in hippocampal CA1 neurons. This AHP is generated via one or more calcium-sensitive potassium conductances. Nimodipine blocks L-type calcium conductances and reduces AHPs in hippocampal neurons of aging rabbits, thereby giving them biophysical characteristics similar to those found in young adult hippocampal neurons. Pharmacologically-induced reductions of the AHP coupled with reductions in spike accommodation may in turn facilitate learning, by enhancing the ability of hippocampal neurons to increase their firing activity adaptively when associative stimuli are presented. Nimodipine's actions on hippocampal neurons *in vivo* are consistent with observations that these cells have increased spike activity during and after associative eyeblink conditioning, that learning is impaired in aging animals, and that nimodipine treatment reverses this impairment, perhaps via changes in hippocampal neuronal activity.

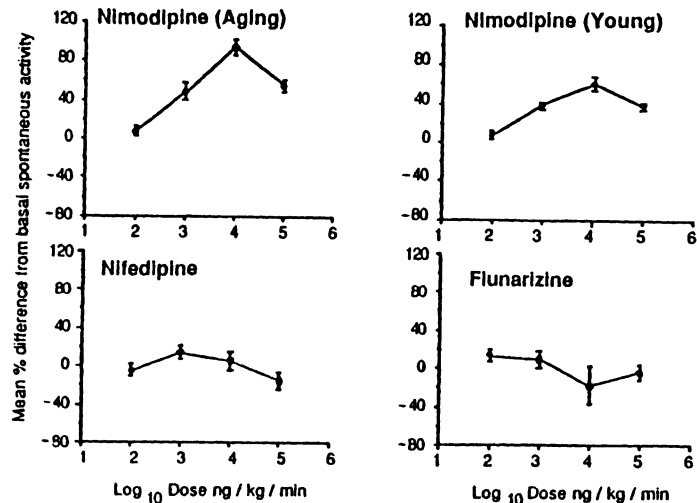


Figure 4. In intact rabbits, the L-type calcium channel blocker nimodipine has a greater effect on neuronal excitability than either nifedipine or flunarizine, with greater effects seen on aging than on young neurons. Nimodipine significantly enhanced the spontaneous firing rates of hippocampal CA1 pyramidal neurons in aging rabbits across a range of doses, with the greatest effect at the behaviorally most effective dose. Similar but quite reduced effects were seen in the younger age group. Neither nifedipine nor flunarizine had significant effects on pyramidal cell firing rates across the range of doses tested, even in aging animals.

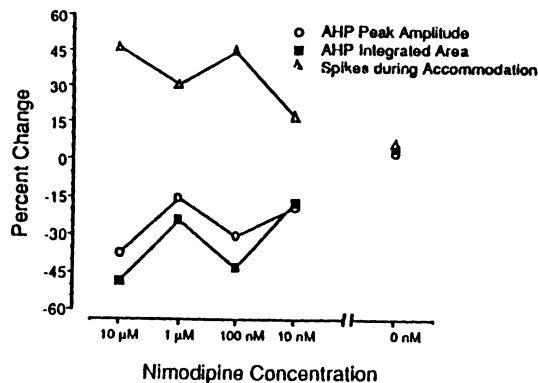


Figure 5. Dose-response curves showing that nimodipine reduces both the post-burst afterhyperpolarization (AHP) and spike accommodation in aging hippocampal CA1 pyramidal cells. All values are shown as a percent change after bath application of nimodipine in aCSF. Both the peak amplitude and integrated area of the AHP were reduced, with significant effects seen at concentrations as low as 100 nM. Nimodipine similarly caused aging neurons to fire more action potentials during an 800 msec depolarizing current pulse, with significant effects at doses as low as 10 nM. When 0.01% ethanol vehicle (0 nM) was substituted for nimodipine, no changes were observed in any of the measures.

We are well aware of some qualifications that must be considered when evaluating and interpreting the data discussed above. We have concentrated our discussion on the hippocampus, a brain region profoundly affected by aging. And, we have used trace eyeblink conditioning, a hippocampally-dependent associative learning task, in our behavioral studies of aging rabbits and aging humans. But it is clear that the aging process affects all brain regions, not the hippocampus alone. Given the methods of nimodipine administration in our behavioral and *in vivo* single neuron recording studies, the drug reaches the entire brain, and could have positive behavioral effects mediated by brain regions other than the hippocampus. This is undoubtedly true, although further accumulation of evidence linking hippocampal changes in calcium regulation with learning will continue to strengthen our hypothesis that the hippocampus is critically involved in the learning deficits associated with aging. As noted above, nimodipine has cerebrovascular effects, which may play a significant role in other brain areas than in the hippocampus. Although such effects could contribute to global measures of cognitive and attentional enhancement by nimodipine, by concentrating on hippocampally-dependent learning tasks such as trace eyeblink conditioning (Moyer *et al.*, 1990b), we have reduced their contributions to a minimum in our measurements. We are beginning to more directly address this issue, utilizing a combined approach to examine nimodipine's effects after hippocampal lesions.

At this time, it appears that pharmacological blockade of neuronal calcium channels can have positive effects, reversing some forms of aging-related learning deficits. We have demonstrated specific examples, using the rabbit eyeblink conditioning paradigm, in which both oral and intravenous administration of a dihydropyridine calcium channel antagonist (nimodipine) facilitates learning in aging animals (see Diserhoft *et al.*, this volume). We have presented evidence that indicates that the hippocampus is both profoundly affected by aging, and is critically involved in specific forms of learning, including trace eyeblink conditioning. We have seen that some physiological properties of hippocampal neurons are regulated by calcium-sensitive mechanisms, that hippocampal pyramidal neurons model associative eyeblink responses, and that nimodipine changes the activity of these neurons *in vivo*. Further, we have shown that learning and aging induce opposing changes in a calcium-dependent AHP response localized to hippocampal neurons. Nimodipine reverses the aging-related increase in the AHP, allowing learning-dependent decreases in the AHP to occur more readily. Each line of evidence presented is convergent with the hypothesis that disturbances in neuronal calcium regulation, perhaps centered in the hippocampal region, underlie many of the deficits in learning and memory which frequently accompany the process of aging. Thus, our rabbit model of associative learning should be relevant to scientists and clinicians concerned with human age-related learning deficits and/or the

behavioral consequences of Alzheimer's disease. Our studies indicate that that nimodipine or other similar calcium channel blockers may have important applications in the treatments of such disorders.

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