

The Calcium Rationale in Aging and Alzheimer's Disease

Evidence from an Animal Model of Normal Aging^a

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Calcium is required for the function of all cells in the body, including neurons. Considerable research has described the function of calcium in the regulation of numerous processes including neurotransmitter release, cytoarchitecture and growth, and activation of enzyme systems including kinases and phosphatases. Calcium is intimately involved in a variety of "plastic" changes in the brain. For example, during adaptive processes such as learning and development, changes in transmembrane calcium fluxes correlate with changes in neuronal excitability and structural connectivity. Calcium thus is likely to have key roles in the cellular processes underlying aging-related changes in the brain, including normal age-associated memory impairments as well as more severe dementias, including Alzheimer's disease.

The pivotal role of calcium in so many neuronal processes dictates the need for precise regulation of its intracellular levels. Any dysregulation, however subtle, could lead to dramatic changes in normal neuronal function. Recent studies from our laboratory and those of others have implicated altered calcium influx with aging-related changes at both the behavioral and the neurophysiological levels. These findings led to and continue to support the calcium hypothesis^{1,2} which posits that in the aging brain, transient or sustained increases in the average concentration of intracellular free calcium contribute to impaired function, eventually leading to cell death. The hypothesis suggests that the final common pathway that may contribute to cognitive deterioration of aging vertebrates, including persons with Alzheimer's disease or other aging-related dementias, is increased free calcium within neurons. The functional impairment that characterizes a patient at a particular time in the aging-related disease process may be relieved by reducing excessive calcium influx. Additionally, because calcium dysregulation terminating in cell death is likely to be

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a continuous process, with many cells exhibiting varying degrees of impairment, the rate of additional loss of neurons resulting from altered calcium influx may be reduced pharmacologically by calcium antagonists.

This chapter focuses on data from our laboratory illustrating the involvement of calcium in the cellular mechanisms of learning and memory. In addition, we provide evidence linking altered calcium influx with learning deficits and changes in the electrophysiological properties of hippocampal neurons using an animal model of normal aging. We shall consider the potential for calcium channel antagonists to ameliorate these aging-related changes and the relevance of these issues both to normal aging and to Alzheimer's disease.

REGULATION OF INTRACELLULAR CALCIUM AND AGING

Numerous cellular processes depend upon the maintenance of appropriate calcium concentrations for their normal function. A variety of intracellular mechanisms exist for maintaining calcium homeostasis in neurons, some of which are summarized in FIGURE 1. There are at least three major transmembrane sources of calcium influx: (1) voltage-gated calcium channels, of which there are at least four classes; (2) the NMDA receptor channel complex; and (3) activation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Additional sources of free intracellular calcium come from release of intracellular stores (including organelles such as calciosomes, the endoplasmic reticulum, and mitochondria) and release from Ca^{2+} -binding proteins such as calmodulin, calbindin, and parvalbumin. Efflux of intracellular calcium occurs via ATP-driven pumps and the $\text{Na}^+/\text{Ca}^{2+}$ exchangers. The calcium hypothesis for Alzheimer's disease posits that dysregulation of free intracellular calcium is causally linked to some of the processes that underlie the neuronal deterioration occurring in Alzheimer's disease as well as in normal aging.^{1,2} The root cause for Alzheimer's disease, whether genetic, environmental, or some interaction between the two, has not been definitively established. It has been postulated that excessive influx, raised levels, or poor buffering of intracellular calcium are results, not causes, of the molecular and cellular mechanisms leading to the development of Alzheimer's disease. If the calcium hypothesis is correct, the reason that some individuals have accelerated age-related deterioration should also be amenable to empirical determination and treatment. It is critically important to empirically test the hypothesis, both in animal models and in clinical settings, and to modify the hypothesis if necessary to fit the empirical data.

Considerable evidence shows that calcium is intimately involved in cell deterioration and death in a variety of disease processes (FIG. 2). One intensively studied example is glutamate neurotoxicity that accompanies ischemic episodes.^{3,4} Calcium influx is enhanced through NMDA-receptor channels and through voltage-gated calcium channels, both of which require sustained depolarization for opening. Calcium is also released from intracellular stores as a result of activation of metabotropic glutamate receptors. Free cytosolic calcium levels would be raised as a result of these processes, resulting in cellular damage from various causes including reduced cellular energy metabolism⁵ and activation of catabolic enzymes including a calcium-activated neutral protease (calpain) which can degrade neuronal structural proteins.^{6,7} Sustained elevation of intracellular calcium would also lead to a number of destructive cascades:

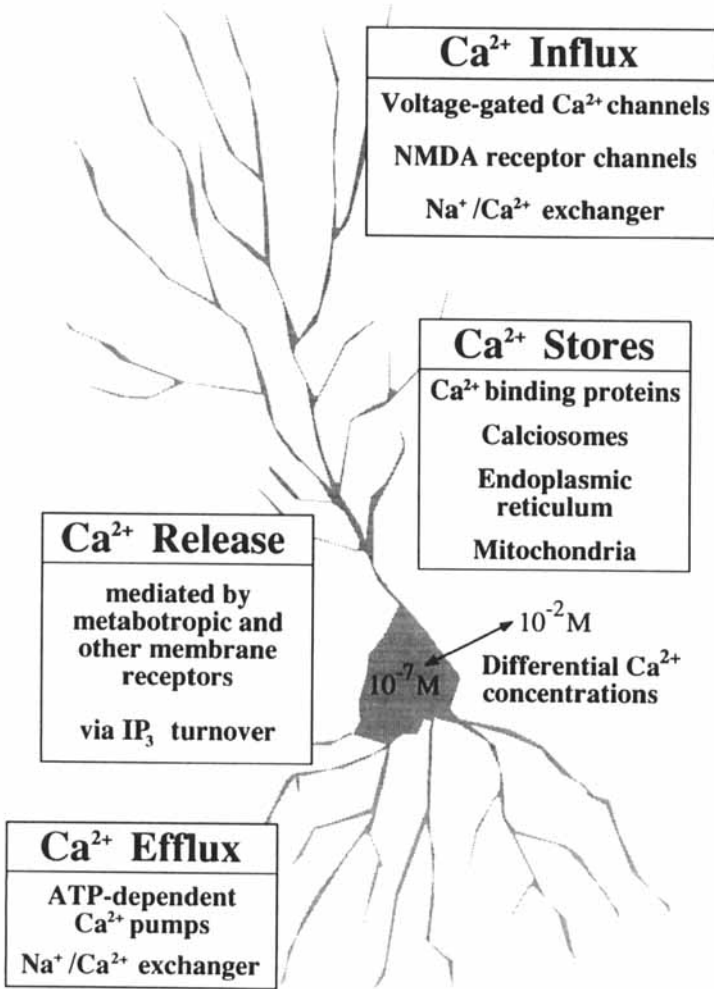


FIGURE 1. Calcium homeostasis in young neurons involves buffering of calcium from a number of sources (both intra- and extracellular) to maintain an intracellular free Ca²⁺ concentration in the nanomolar range. Calcium influx occurs via voltage-operated calcium channels (VOCCs, including the L-type channels blocked by nimodipine), via one type of excitatory amino acid receptor (*N*-methyl-D-aspartate receptors, NMDA-Rs), and via Na⁺/Ca²⁺ exchange proteins. Calcium is stored intracellularly in a variety of ways, including binding with Ca²⁺-binding proteins (CaBPs including calmodulin, calbindin, and parvalbumin), and sequestration in cellular organelles including calciosomes, the endoplasmic reticulum, and mitochondria. A number of membrane receptors activate second messenger cascades that liberate Ca²⁺ from intracellular stores, and Ca²⁺-dependent intracellular Ca²⁺ release has been described. Calcium efflux both via energy-dependent Ca²⁺ pumps and via Na⁺/Ca²⁺ exchange proteins also serve to regulate intracellular free Ca²⁺ concentrations. Disruptions of any of these processes can have profound consequences to both the neuron and the organism to which it belongs.

(1) the generation of phospholipase A₂ and then superoxide radicals, leading to cellular damage; (2) activation of phospholipase C which activates protein kinase C, leading to increased calcium channel activity, and to IP₃ generation, releasing additional calcium from internal stores; (3) generation of calcium-calmodulin-dependent protein kinase II, which phosphorylates presynaptic synapsin I, leading to further glutamate release; (4) activation of endonucleases that cause DNA fragmentation; and (5) production of nitric oxide synthase, which inhibits mitochondrial respiration, the citric acid cycle enzyme aconitase, and DNA synthesis.^{3,4} Thus, it is likely that even a small disruption of normal calcium homeostasis could have devastating consequences for neurons. These kinds of cascades and the evidence for them are discussed at length in other chapters within this volume.

At the same time, numerous studies suggest a role for calcium channel blockers as treatments for various neuropathologies, including the functional sequelae of ischemic episodes.⁵ For example, preclinical studies demonstrate that discrimination learning and memory deficits associated with prenatal hypoxia in developing rats is markedly reduced in pups whose mothers received oral nimodipine (a 1,4-dihydropyridine, L-type calcium channel antagonist) during induction of prenatal hypoxia.⁸ In addition, animals whose mothers received nimodipine showed normal densities of serotonin and of acetylcholine staining fibers in their hippocampi, whereas hypoxic controls did not. In two different clinical trials, it was shown that nimodipine has a beneficial effect, especially on functions involving learning, in patients with chronic cerebrovascular disorder or vascular dementia.^{9,10} These preclinical and clinical studies suggest that calcium channel blockers may be useful in countering the effects of the neurotoxic events that accompany ischemic episodes. As will be discussed, these findings also suggest that Ca²⁺ antagonists may be useful adjuncts to treatments for age-associated impairments including Alzheimer's disease.

CALCIUM-MEDIATED PROCESSES IN NEURONS

A variety of processes are governed by the concentration of available intracellular free Ca²⁺. For example, during development, neurons extend growth cones for generating and extending new processes, and recent research has shown that calcium is vital to this process.¹¹ Studies in cultured neurons further suggest that calcium mobilization from caffeine-sensitive intracellular stores may be important in growth cone activity.¹² It is thought that similar processes may be involved in the formation of new connections and/or the strengthening of preexisting connections that occur during learning and memory in the adult brain.¹³ Other examples in which neurons require intracellular calcium include the regulation of membrane currents and second messenger cascades as well as the activation of various kinases and phosphatases. Disturbances in calcium regulation have been implicated in a number of neuropathological syndromes.

When Ca²⁺ concentrations reach high levels, calcium-activated regulatory processes are stimulated to restore calcium to resting levels. A good example of such a calcium-activated process is the afterhyperpolarization (AHP), a hyperpolarizing voltage shift from the resting membrane potential that occurs after a burst of action potentials.^{14,15} The burst depolarization activates voltage-dependent calcium channels. The calcium influx through these channels in turn activates outward calcium-depen-

dent potassium currents. When these outward currents hyperpolarize the neuronal membrane, voltage-sensitive calcium currents are inhibited. This feedback sequence prevents uncontrolled firing activity in healthy neurons.

It should be recognized that calcium-dependent systems can show saturation. Under pathological conditions, intracellular calcium concentrations can in theory swamp homeostatic control mechanisms, rapidly leading to edema and cell death. As currently formulated, the calcium hypothesis does not presume that massive acute calcium overloads are the primary source of age-associated pathologies. Both aging and Alzheimer's disease may represent the cumulative effects of small disturbances in calcium homeostasis, with some disturbances resulting in further small but significant breakdowns of the regulatory process.

AGING-ASSOCIATED LEARNING DEFICITS

Aging mammals are clearly impaired in learning a variety of tasks. Learning in our behavioral model task, eyeblink conditioning, is impaired for both aging rabbits and aging humans.¹⁶⁻¹⁹ Eyeblink conditioning is further impaired in Alzheimer's patients as compared to age-matched controls.^{20,21} We used trace eyeblink conditioning to study age-associated learning deficits of aging rabbits and aging humans. In this paradigm, the subject must form a short-term memory trace of a brief tone (the conditioned stimulus) in order to appropriately time the occurrence of a conditioned eyeblink response. We chose this paradigm because trace eyeblink conditioning depends on an intact hippocampus for its successful acquisition^{22,23} (FIG. 3A). Early work from our own laboratory, to be discussed in more detail, demonstrated that age-associated deficits in learning this task could be reversed by treatment with a centrally active Ca²⁺ channel antagonist (FIG. 3B). Consequently, we pursued a convergent series of studies examining both behavioral and physiological effects of Ca²⁺ channel blockade in aging rabbits.

It has been hypothesized that hippocampally dependent learning tasks are impaired in aging animals and humans owing to the early involvement of the hippocampus in aging-associated changes that affect learning and plasticity.^{17,24} In our animal experiments, aging rabbits are impaired in acquisition of trace eyeblink conditioning. A recent detailed analysis of aging-related changes revealed that rabbits show a systematic age-related decline in their learning ability, beginning as early as 24 months of age with a plateau at around 30-36 months of age.¹⁷ Additionally, with increasing age an increasingly larger percentage of the rabbit population was severely impaired in learning the trace conditioning task (ie, exhibiting much more severe learning deficits than did their age-matched cohorts). This inherent heterogeneity among the aging rabbit population is comparable to that seen in aging humans, where most individuals acquire the task, but where learning deficits become more obvious in older individuals.^{18,21,25}

We have not restricted our model system studies solely to eyeblink conditioning, but have also evaluated the performance of aging rabbits in an "open field" environment.²⁶ Recall that rabbits are prey animals. Thus, it is not surprising that young rabbits, when placed in an open field, tended to stay on the border next to the walls, not explore much, and spent considerably more time sitting and observing their

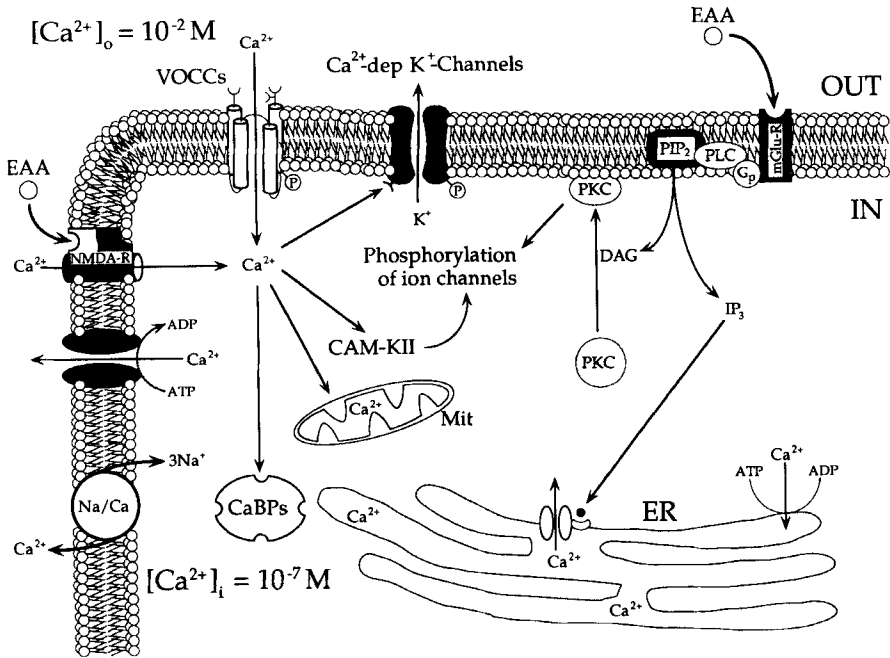


FIGURE 2. Altered intracellular free Ca^{2+} concentrations need not be directly excitotoxic in order to severely compromise neuronal functioning. As seen, excess Ca^{2+} influx (from sources such as voltage-operated calcium channels [VOCCs] and *N*-methyl-D-aspartate receptors [NMDA-Rs]) can rapidly and reversibly alter responses such as the postburst AHP generated by a Ca^{2+} -dependent K^+ conductance. Increased intracellular free Ca^{2+} would also alter numerous second-messenger cascades. For example, increased activity of the calcium-dependent enzyme protein kinase C (PKC) would increase protein phosphorylation. Excess phosphorylation of the microtubule-associated tau protein is one consequence observed in Alzheimer's brains. Excess phosphorylation of receptor and ion channel proteins could further amplify initial increases in free Ca^{2+} concentrations. Phosphorylation of calcium-binding proteins (CaBPs) including calbindin, calmodulin, and parvalbumin may reduce their capacity for Ca^{2+} buffering. Increased intracellular free Ca^{2+} would also increase the activity of calcium-dependent enzymes, including calcineurin, calpains I and II, phospholipase A2 (PLA2), Ca^{2+} -calmodulin-dependent protein kinase (CAM-KII), and phospholipase-C (PLC), altering metabolism of both proteins and phospholipids. Multiple ligand-gated membrane receptors, including metabotropic receptors (mGlu-R) could activate PLC and liberate inositol triphosphate (IP_3) and diacylglycerol (DAG) via G-protein (G_p)-mediated breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2). Increased IP_3 production would increase release of Ca^{2+} from intracellular stores such as endoplasmic reticulum (ER). Amplified depolarizing responses to excitatory amino acids (EAAs) would increase Na^+ influx, altering activity of the Na^+/Ca^{2+} -exchange antiporter. Altered metabolic demands and an overload of intracellular free Ca^{2+} could overwhelm the calcium pump (Ca^{2+}/Mg^{2+} -ATPase). As noted, the effects of both aging and Alzheimer's disease are cumulative, and the severity of the functional deficits observed is the product of multiple dysfunctions of varying severities.

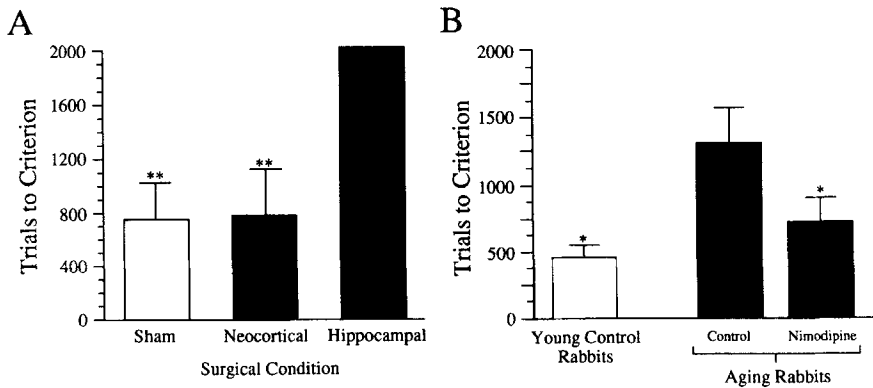


FIGURE 3. The functional integrity of the hippocampal region is notably impaired by aging and is severely altered in Alzheimer's disease. Hippocampal lesions severely impair or block acquisition of 500 msec trace eyeblink conditioning in young rabbits (A; Moyer *et al.*²²). Aging rabbits are impaired in trace conditioning as compared to young controls, but daily ingestion of nimodipine-treated food pellets (860 ppm) largely reversed this deficit (B; Straube *et al.*⁶⁹). Since Ca^{2+} channels are particularly abundant within the hippocampus and since Ca^{2+} serves important roles in a number of cellular functions, we have utilized a broad range of techniques to assess the functional consequences of altered Ca^{2+} influx in a primary cell type of the hippocampus, the CA1 pyramidal neuron, and to delineate links between Ca^{2+} and aging-associated learning or memory impairments.

environment. On the other hand, aging control rabbits wandered aimlessly around the open field. Their tendency to expose themselves in the center of the open space, and to move around a lot, would make them easy prey in the wild. The open field test has components of general cognitive functioning; it evaluates awareness of the rabbit to its surroundings and its level of alertness. As will be discussed later, calcium appears to be an important contributor to these and other age-related learning deficits.

CALCIUM AND CELLULAR MECHANISMS OF LEARNING

The calcium hypothesis argues that intracellular free Ca^{2+} plays a significant role in many forms of neural plasticity, including the models of learning studied in our laboratory. For example, single neuron recording *in vivo* demonstrated that the activity of hippocampal pyramidal neurons is increased during and after acquisition of the conditioned eyeblink response.²⁷ We used intracellular recording techniques and the *in vitro* rabbit hippocampal slice preparation to delineate the cellular mechanisms involved in this observed increase in excitability *in vivo* (FIG. 4). By using hippocampally dependent trace eyeblink conditioning,²² we demonstrated that both the afterhyperpolarization (AHP; mediated by a Ca^{2+} -dependent K^+ current) which follows a burst of action potentials and spike frequency accommodation are reduced in CA1 and CA3 pyramidal neurons after acquisition.^{28,29} These reductions increase neuronal excitability and are well correlated with behavioral acquisition as they are not observed

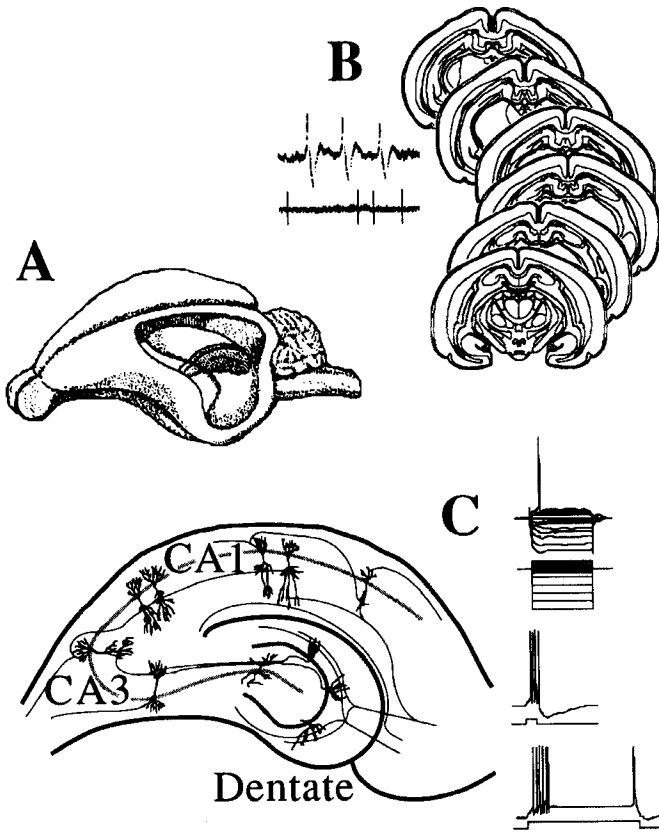


FIGURE 4. The structure and circuitry of the hippocampus lend themselves well to neuroscientific study. A large bilateral telencephalic structure (A), the hippocampus has a long and well-documented history investigating its functional role in behavioral plasticity (learning). Pyramidal neurons are arranged in curved sheets, with aligned basal and apical dendritic fields receiving afferents from defined sources. This arrangement facilitates replicable recordings of field potentials evoked by mass activation of afferent fiber bundles, a method that spawned the study of long-term potentiation of excitatory synaptic transmission within the hippocampus. It is also possible to place extracellular microelectrodes into specified regions and replicably isolate and record pyramidal neuron activity from a number of individual neurons across individual subjects (B). Pyramidal cell activity is distinguished extracellularly by occasional complex-spike (decremental bursting) activity, by waveform analysis, and by typical slow spontaneous rates of activity. The anatomical arrangement of hippocampal neurons yields a relatively uniform *in vitro* preparation, the transverse hippocampal slice (C). Using current-clamp protocols, a number of different biophysical measurements can be obtained from CA1 pyramidal cells, including current-voltage relationships, the afterhyperpolarization (AHP) following burst firing (typically, the AHP is measured following a burst of four action potentials elicited by injection of a short pulse of depolarizing current), and accommodation (or spike frequency adaptation, the decremental bursting observed with a longer depolarizing current injection).

in hippocampal slices taken from pseudoconditioned or poorly conditioned rabbits (FIG. 5). Furthermore, these alterations are localized to the hippocampus, as they are observed using *in vitro* slices separated from their normal afferent and efferent connections.^{30,31} They are also postsynaptic, as they are evoked by intracellular current injection and persist even after block of sodium spike-dependent synaptic transmission.³² Our evidence strongly supports involvement of Ca^{2+} in the learning-related changes observed in the hippocampus after trace eyeblink conditioning.

Calcium also appears to be critically involved in several other measures of hippocampal plasticity. For example, long-term potentiation (LTP)³³ has been extensively studied as an experimental model for synaptic changes that occur during learning and memory. The most commonly studied (and best understood) form of LTP depends upon the activation of NMDA (*N*-methyl-D-aspartate) receptors for its induction. Evidence has shown that NMDA channels are permeable to Ca^{2+} , whose influx is necessary for induction of LTP. Several other forms of plasticity, including those that depend on the hippocampus and temporal lobe for their acquisition, also require NMDA-dependent transmission.³⁴ When neurons are sufficiently depolarized by summated inputs, NMDA channels open, resulting in additional calcium influx. This calcium flows into very localized zones in dendritic spines, and calcium-dependent postsynaptic processes, such as stimulation of kinases and phosphatases, or mobilization of calcium from intracellular stores are activated. These result in synaptic changes hypothesized by many researchers to contribute to memory storage.³⁴ For example, in CA1 neurons, PKC activity associated with the membrane fraction was increased (while PKC activity associated with the cytosolic fraction was decreased) after eyeblink conditioning.³⁵ Immunocytochemical data from our laboratory further demonstrated that staining for the PKC-gamma subunit is significantly increased after hippocampally-dependent trace eyeblink conditioning.³⁶ Subsequent western blot or biochemical analyses should allow us to determine whether the PKC-gamma subunit staining observed after learning results from altered *de novo* synthesis of the protein, from translocation of the protein, or from changes in the conformation of the protein. Since activation of PKC by phorbol esters reduces the AHP,³⁷ these data suggest that the AHP reductions we observed after learning result from phosphorylation of calcium-dependent potassium channels, possibly by PKC or other kinases.

CALCIUM AND CELLULAR MECHANISMS OF NORMAL AGING

The calcium hypothesis posits that during normal aging, neurons become more vulnerable to Ca^{2+} dysregulation, with possible elevation of intracellular free calcium.³⁸ Our current understanding of neurotoxicity suggests that this Ca^{2+} dysregulation would make neurons vulnerable to other insults and disease processes, including the multiple factors contributing to Alzheimer's disease. In its most controversial form, the calcium hypothesis asserts that a breakdown in calcium homeostasis is the primary cause of aging-associated pathologies, including Alzheimer's disease. It should be noted that this extreme calcium hypothesis is probably incorrect and that elevated intracellular free Ca^{2+} is only one causative factor in the development of Alzheimer's disease. Even so, if neurons can be protected by appropriate calcium channel blockers against

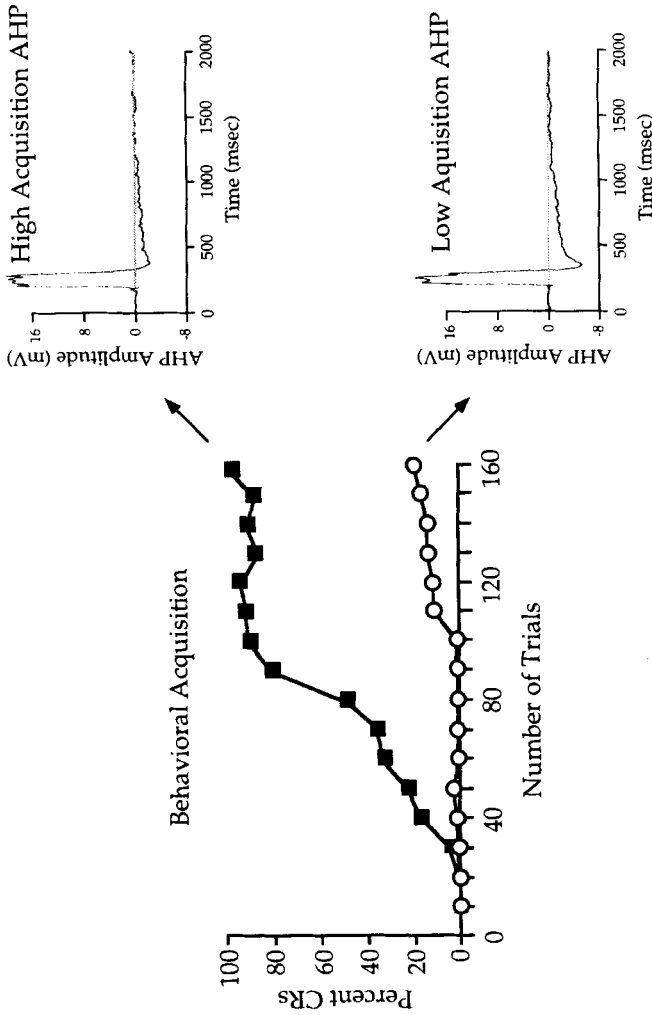


FIGURE 5. The postburst AHP reductions are learning-specific. (*Left panel*) Learning curves for rabbits receiving delay conditioning for two consecutive sessions. Note that some rabbits (high acquisition, ■) reached the criterion of 80% CRs during the second session while others (low acquisition, ○) showed relatively few CRs. CA1 pyramidal cells in slices taken from the high acquisition population (*top right panel*) exhibited a significantly reduced postburst AHP as compared to cells from the low acquisition population (*lower right panel*). Both the peak amplitude and the integrated area of the AHP were reduced after acquisition. (Adapted from Disterhoft *et al.*³¹).

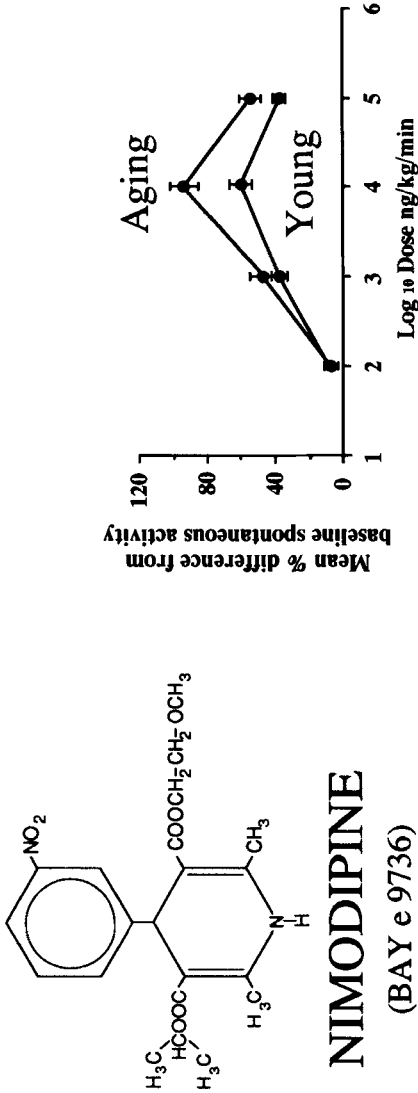


FIGURE 6. Calcium channel antagonists vary in their specificity for blocking different voltage-operated calcium channels (VOCCs) and in their tissue specificity (eg, cardiac, smooth muscle, or neuronal). Both factors are thought to be related to their physical structure and influenced by a number of factors, including their lipophilicity. Nimodipine (A), a 1,4-dihydropyridine with high specific activity in cerebrovascular smooth muscle, also crosses the blood brain barrier more readily than do many other calcium antagonists, giving it ready access to the central nervous system after peripheral administration. Intravenous administration of varying doses of nimodipine (B) significantly enhanced the spontaneous firing activity of hippocampal neurons in awake rabbits. Other calcium antagonists that increase cerebral blood flow (as does nimodipine) but do not readily cross the blood brain barrier or that cross the blood brain barrier but block non-L-type Ca^{2+} channels had no effect on CA1 pyramidal cell firing activity. The effects of nimodipine were much greater in aging (mean 47.3-month-old) rabbits than in young (mean 4.1-month-old) rabbits. These results were suggestive of differential Ca^{2+} regulation in the brains of aging rabbits. (Adapted from Thompson *et al.*⁴¹)

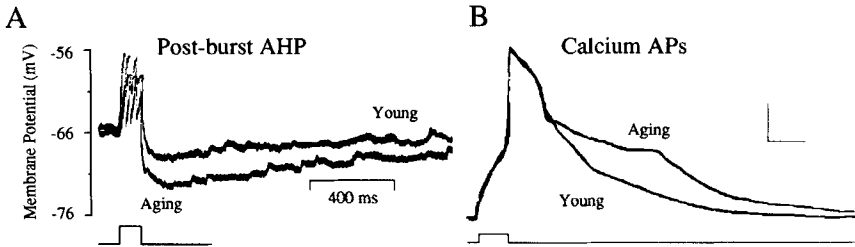


FIGURE 7. The slow postburst AHPs of aging CA1 pyramidal neurons were significantly enhanced in comparison with those of young neurons at the same resting potentials, being both longer in duration and greater in amplitude (A). Interestingly, the slow plateau phase of Ca^{2+} action potentials of hippocampal neurons was also greatly enhanced in aging (B), indicating a significant increase in calcium influx during depolarizing events. Sustained increases in Ca^{2+} influx are a likely basis for the enhanced calcium-dependent AHP observed in aging hippocampal neurons. (Adapted from Moyer *et al.*⁴⁴ and Moyer and Disterhoft.⁴⁵)

factors in aging that make them more susceptible to insult and disease, their function should be enhanced and they should be less vulnerable to other processes contributing to cell death.

Magnesium, a divalent calcium channel antagonist, has been shown to facilitate maze reversal learning and to improve hippocampal frequency potentiation in aging rats.³⁹ In addition, blocking calcium influx by altering Mg^{2+} to Ca^{2+} ratios improved intracellular and extracellular measures of frequency potentiation in hippocampal slices prepared from aging rats.⁴⁰ These experiments strongly suggest that reducing calcium influx in aging hippocampal neurons can reverse some of the functional and learning deficits observed and that behavioral and physiological effects of calcium channel blockade go hand in hand. As described, we studied the effects of Ca^{2+} channel blockers on eyeblink conditioning and on performance of other behaviors. Parallel work has examined the effects of calcium antagonists on a number of physiological measures, ranging from single-unit recording in the same intact awake rabbit preparation used for our eyeblink conditioning studies, to biophysical studies of neuronal excitability and of calcium influx in the isolated hippocampal slice or in acutely dissociated neurons. Convergent data, consistent with the calcium hypothesis of aging, was obtained and is summarized below.

We reasoned that the resting activity of hippocampal neurons in conscious aging rabbits should be influenced by calcium influx, if our hypothesis was correct. Reducing Ca^{2+} influx should increase the excitability of hippocampal neurons, especially in aging animals, by reducing feedback activation of the Ca^{2+} -dependent AHP. We tested this corollary, with the result that nimodipine dose- and age-dependently increased the baseline activity of single CA1 hippocampal neurons (FIG. 6).⁴¹ Nimodipine increased neuronal baseline firing rates at the same doses that facilitated acquisition of trace eyeblink conditioning in aging rabbits.⁴² The increase was much larger in aging rabbits than in young animals. So, a physiological effect of nimodipine was to enhance pyramidal neuron firing rates, an effect that might be expected to contribute to enhanced eyeblink conditioning in aging animals.

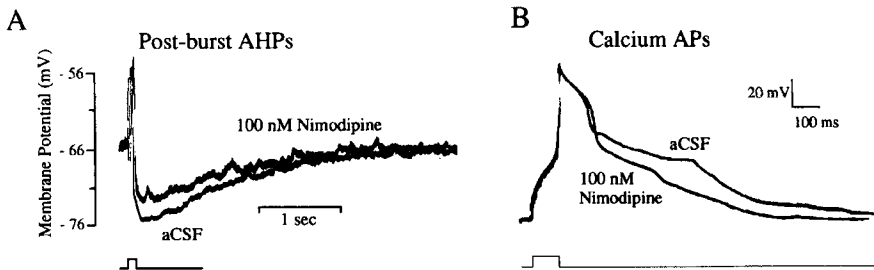


FIGURE 8. Although greatly enhanced in aging CA1 neurons, both postburst AHPs (A) and Ca^{2+} action potentials (B) were significantly reduced by low concentrations (100 nM) of nimodipine. Again, concentrations in the micromolar range were required for effect in young CA1 neurons. Treatment with nimodipine restored Ca^{2+} influx to levels typical of young neurons. The Ca^{2+} -dependent AHP was also restored to levels typical of young pyramidal cells. (Adapted from Moyer *et al.*⁴⁴ and Moyer and Disterhoft.⁴⁵)

The Ca^{2+} -dependent afterhyperpolarization is prolonged in the hippocampus of aged mammals due to excess calcium influx through voltage-activated, L-type calcium channels and/or to tonically elevated levels of free intracellular calcium in neurons^{43,44} (FIG. 7A). The postburst afterhyperpolarization is mediated by an outward calcium-dependent potassium current, activated by the Ca^{2+} influx during the burst of action potentials. It seemed likely that the enhanced AHP in aging CA1 neurons results from an increase in the amount of calcium entering the neuron during the burst. Thus, we next determined that the calcium action potential was indeed enhanced in CA1 neurons from aging rabbits⁴⁵ (FIG. 7B). Note that the enhanced depolarizing plateau of the calcium action potential is ideally timed to underlie the enhanced slow AHP seen in aging neurons.

The prolonged AHP of hippocampal principal neurons of aged mammals causes this structure to be less excitable and would act to impede information transfer through the hippocampus. This impaired function could contribute to age-associated learning impairments.^{16,17} The hippocampus, critical for learning and memory processes, is often the first neuronal structure to be affected by the pathologies associated with Alzheimer's dementia.⁴⁶ Thus, learning and memory deficits are often a cardinal feature of Alzheimer's disease even in its early stages. Prolonged elevation of free Ca^{2+} during the period following bursts of action potentials in aging neurons is linked to larger AHPs and to impaired learning. Tonically enhanced intracellular Ca^{2+} within hippocampal neurons over time, even if slight, could contribute to cell death in this pivotal region.^{1,2}

We next explored whether nimodipine could reduce the postburst AHP, carried by a Ca^{2+} -activated outward potassium current, an effect that would increase aging neuronal excitability. Again, we were particularly interested in this type of functional change, as we have shown that the postburst AHP is reduced in pyramidal neurons after eyeblink conditioning in both young²⁸⁻³² and aging rabbits.⁴⁷ Our recent studies indicate that this AHP reduction in both CA1 and CA3 neurons is related to the acquisition and consolidation of the trace eyeblink conditioned response rather than

its longer term storage during the learning process.^{28,29} We also know that the slow AHP is markedly enhanced in CA1 pyramidal neurons from aging rats and rabbits, an enhancement that has been suggested would reduce hippocampal neuron functional excitability and thus slow learning rate.^{43,44}

Nimodipine, in concentrations as low as 100 nM, reduced the slow afterhyperpolarization of aging CA1 neurons in rabbit hippocampal slices (FIG. 8A).⁴⁴ Concentrations this low had no effect on the AHPs of CA1 neurons from young rabbits. We similarly showed that nimodipine, in concentrations as low as 100 nM, significantly reduced the Ca²⁺ action potential in aging but not young neurons (FIG. 8B).⁴⁵ It is important to note that nimodipine affected the late depolarizing plateau of the calcium action potential, that same portion that was noticeably enhanced in aging neurons. This slow component, apparently mediated by L-type channels, is ideally suited temporally to underlie the enhanced slow calcium-activated postburst AHPs observed in aging neurons.

Concentrations of nimodipine as low as 10 nM were also effective in reducing spike frequency accommodation to a long depolarizing current injection in aging but not young CA1 neurons (FIG. 9).⁴⁴ This is another measure of excitability change in hippocampal neurons, one more sensitive to neuronal depolarizations due to the repeated action potentials that are evoked by sustained depolarization. Spike frequency accommodation is also reduced after trace eyeblink conditioning in CA1 and CA3 pyramidal neurons.^{28,29} The final portion of our physiological analyses which has been completed indicates that nimodipine markedly reduces the high threshold, non-inactivating component of the Ca²⁺ current which is presumably the L-type current, as evaluated with voltage-clamp recordings of neurons acutely dissociated from young hippocampus.⁴⁸

Thus, a lipophilic dihydropyridine, nimodipine, which has ready access to the hippocampal neurons presumably involved in the learning process, may facilitate learning in aging rabbits by reducing age-associated increases in Ca²⁺ influx. Nimodipine restored these hippocampal neurons to a level of excitability comparable to that observed in young hippocampal neurons. An extension of these findings, to be discussed, is whether long-term treatment with a centrally active calcium antagonist would have long-term beneficial effects in aging.

The functional characteristics of L-type calcium channels (those blocked by dihydropyridines) change with age. As discussed, there is evidence that the conductance through these channels increases in aging hippocampal CA1 neurons and is selectively blocked by nimodipine.^{45,49} L-type Ca²⁺ channels may be especially important in age-associated changes, because they are open a long time and pass a relatively large amount of current.⁵⁰ Thus, their alteration can contribute in a major fashion to tonic increases in free intracellular Ca²⁺ that may lead to neuronal degeneration. Note that L-channels are concentrated on the neuron soma and proximal dendrites and are not directly involved in synaptic transmission.⁵¹ This is important to stress because it implies that an L-type calcium channel blocker will not slow synaptic transmission. Instead, L-type calcium channel blockers will influence cellular function through their effects on postsynaptic excitability and neuronal calcium concentrations at low doses that will not interfere with synaptic transmitter release. More specifically, if cellular excitability is reduced as a result of excess Ca²⁺ or larger AHPs, an L-type

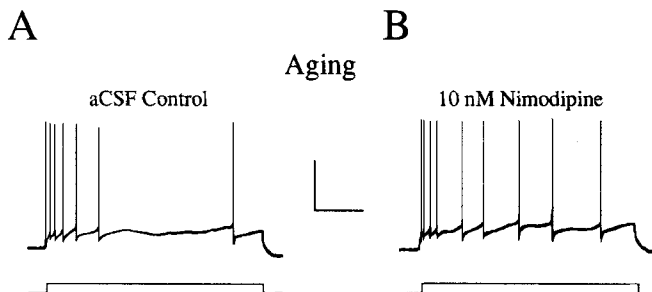


FIGURE 9. Bath application of nimodipine to CA1 pyramidal neurons in slices from aging (mean 39.3-month-old) rabbits reduced accommodation to prolonged depolarization at concentrations as low as 10 nM. Concentrations this low were ineffective in slices from young rabbits (effects were seen on young neurons only at concentrations in the micromolar range). This increase in cellular excitability *in vitro* models that observed *in vivo*, with differential sensitivity exhibited by aging neurons. Because hippocampal pyramidal cell excitability increases during conditioning, the findings summarized in this and the previous figure provide a hypothetical mechanism for the ability of centrally active calcium channel antagonists to restore learning capacity in aging animals.

calcium channel blocker could enhance neuronal function in an age-dependent fashion at levels that will have no deleterious side effects on neuronal function.^{41,44,45}

Potential interactions of Ca^{2+} influx through NMDA-receptor channels with Ca^{2+} influx through L-type calcium channels or with release of intracellular calcium by other excitatory amino acids are important when considering the calcium hypothesis of aging and Alzheimer's disease. The calcium hypothesis posits that Ca^{2+} channel blockers can enhance postsynaptic excitability by reducing depolarization-induced Ca^{2+} influx and thus restore neuronal function to a level more like it was in young neurons. As mentioned above, NMDA-receptor channels are known to be critically important for some forms of learning and act to enhance Ca^{2+} entry into the cell. This may seem counterintuitive, unless it is considered that L-type calcium channels and NMDA glutamate receptors are located at two very different spatial sites on neurons, the cell body and proximal dendrites for L-type channels and out on the spines of distal dendrites for NMDA channels, respectively.⁵¹ Thus, L-type Ca^{2+} antagonists can enhance cellular excitability by reducing calcium influx in the relatively large soma and not affect Ca^{2+} influx in synaptic regions where the NMDA receptor complex is located. Interactions between these two significant sources of cellular calcium influx obviously require further study.

LINK BETWEEN NORMAL AGING AND ALZHEIMER'S DISEASE

As just noted, aging animals show dramatic deficits in learning many hippocampally dependent tasks,¹⁷ and calcium channel blockade effectively reverses these learning deficits. Recent findings from our laboratory confirm that aging humans are also impaired in acquisition of the hippocampally dependent eyeblink conditioning

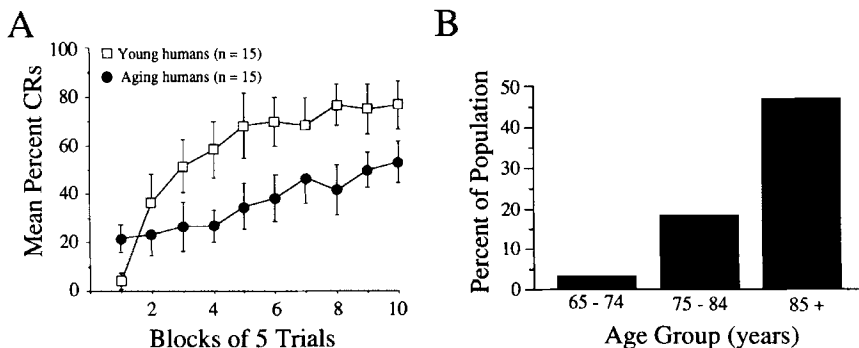


FIGURE 10. Treatment with Ca²⁺ channel antagonists may be useful in reversing the behavioral deficits associated with both normal aging and Alzheimer’s disease. Even relatively simple measures of human learning ability, such as eyeblink conditioning, indicate impairments in aging (mean 65 years) as compared to young controls (mean 25 years)²⁵ (A). Alzheimer’s incidence in the human population increases significantly with increasing age (B), with subsequent and eventually disabling impairment in learning and in cognitive functions required to perform even simple daily tasks.⁵² Because Ca²⁺ antagonists can restore neuronal function in aging to mimic that seen in young cells, there is considerable hope that they can also be valuable in impeding the progression or even reversing some of the symptoms of Alzheimer’s disease.

task used in our rabbit studies^{18,25} (Fig. 10A). Impaired learning was observed early in training and throughout the duration of the experiment. The preponderance of evidence suggests a progressive decline in learning ability with increasing age, with considerable individual variability. The data can be extrapolated to hypothesize that all members of the human population would eventually develop Alzheimer’s disease, if they survived long enough. Although this extreme view is untestable, several lines of evidence suggest that aging and Alzheimer’s disease may be causally linked:

1. *The incidence of Alzheimer’s disease increases exponentially in the 65+ age range.* For example, an important study⁵² evaluated the prevalence of Alzheimer’s disease in aging individuals living in a community setting. This study estimated that approximately 10% of individuals over 65 had cognitive deficits related to Alzheimer’s disease. The estimated incidence of probable Alzheimer’s disease increased from 3% in the 65-74-year age range to 47% for the group over 85 years old (Fig. 10B). Some feel that this study overestimated the incidence of Alzheimer’s disease; however, even more conservative estimates are unsettling. For example, a 1992 Framingham study⁵³ estimated that the incidence of Alzheimer’s disease increased from 0.5% in the 65-74-year age range to 13.1% in the 84+ age group. This is actually a larger proportional increase than the estimate of Evans *et al.*⁵² Estimates of dementia from all causes in the aging population are similarly disturbing. One study⁵⁴ diagnosed 14% of a sample of 3,777 community residents in France over 65 years old as demented. The Framingham study⁵³ estimated that the cause for dementia in 55% of the aging population was Alzheimer’s disease. If this estimate is generalizable to other parts of the world, somewhere between 7 and 8% of the aging population over 65 years of age have Alzheimer’s disease.

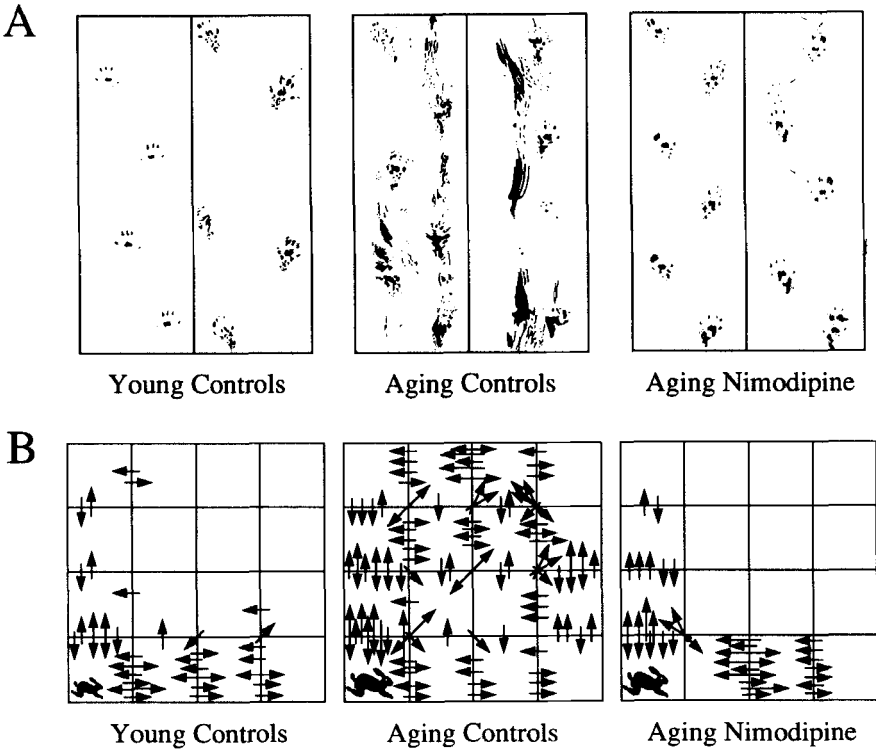


FIGURE 11. Calcium channel antagonists have proven effective in reversing a broad range of behavioral deficits associated with aging. For example, treatment with nimodipine, an L-type Ca^{2+} channel antagonist, restored a youthful gait to aging-impaired rats (A; Scriabine *et al.*⁷⁰). Work in our laboratory has shown that daily oral treatment with nimodipine reversed the impairments in open-field behavior exhibited by aging (more than 36-month-old) rabbits (B; Deyo *et al.*²⁶).

2. *Age-related changes in calcium influx may be an important risk factor for Alzheimer's disease.* As just discussed, calcium action potentials are enhanced in aging hippocampal CA1 pyramidal neurons.⁴⁵ This enhancement may be due to a reduction in the amount of calcium-mediated inactivation of calcium currents which occurs in aging neurons⁵⁵ or to an increase in the number of repolarization openings of L-type calcium channels.⁵⁶ The portion of the calcium action potential that is most increased in aging neurons is the late depolarizing plateau phase. In aging CA1 neurons, a likely consequence of this increase is the reported enhancement of the calcium-activated slow afterhyperpolarization following a burst of action potentials.^{43,44} An increased AHP can markedly reduce neuronal excitability and thus, presumably, should impair hippocampal function. Poor buffering of intracellular Ca^{2+} or impaired inactivation of Ca^{2+} channels in aging neurons may contribute to these observations. Indirect evidence for impaired calcium buffering stems from observed

decreases in immunoreactivity for the calcium binding protein calbindin-28K in aging rat and rabbit hippocampus.^{57,58} Calbindin-28K is also reduced in several cortical regions, including temporal cortex, in the brains of demented individuals diagnosed as having either Alzheimer's or non-Alzheimer's dementia.⁵⁹ Increased calcium influx has been demonstrated in soleus muscle motor nerve terminals in aging mice, resulting in increased quantal content at the terminals.⁶⁰ Finally, synaptosomal Ca^{2+} uptake has been demonstrated to decrease in aging rats.⁶¹ A subsequent study⁶² demonstrated that both the mitochondrial (digitonin-resistant) and non-mitochondrial (digitonin-labile) compartments of the synaptosomes showed reduced calcium uptake in aging mice, which could lead to increased intracellular free Ca^{2+} in axon terminals. Thus, multiple lines of evidence indicate that free intracellular calcium may not be well regulated in aging neurons. Further research is required to determine if this imbalance is qualitatively or quantitatively different in persons with Alzheimer's disease. Lack of a solid animal model for Alzheimer's disease has certainly not facilitated progress in this area.

3. *Pathological and functional characteristics of Alzheimer's disease have been observed in the normal aging brain.* It has been reported that beta amyloid-protein plaques and neurofibrillary tangles occur, although to a lesser degree, in normal aging and that the difference between the Alzheimer's brain and the normal aging brain may be quantitative, not qualitative.⁶³ Although this view of the development of Alzheimer's disease is speculative, in early stages of the disease process, neurofibrillary tangles and plaques are especially concentrated in the subiculum and the hippocampus.⁶⁴ These structures are known to be essential for normal learning and memory functions.^{22,23,65,66} Because the earliest neuropathological changes in Alzheimer's disease tend to be concentrated in these areas, learning deficits are an early and sustained cardinal feature of the disease. Further evidence that processes mediated by the hippocampus and the temporal lobe, such as learning, are critically affected by the aging process comes from animal studies that illustrate that physiological properties such as frequency potentiation and LTP are altered by aging^{24,35} and that such changes are especially pronounced in animals demonstrating the largest age-related learning deficits.⁶⁷

In fact, it is difficult to discriminate Alzheimer's disease from advanced aging or from other disease processes. Careful psychological testing, including a demonstrable memory loss and gradual onset of the disease, must be combined with postmortem neuropathological studies of brain tissue to determine the prevalence and distribution pattern of currently accepted markers such as β -amyloid plaques and neurofibrillary tangles for a definitive diagnosis of the disease. Even with these precautions, there are a variety of cases in which the diagnosis is not clear.⁴⁶ Although numerous animal models of Alzheimer's disease have been proposed, it should be emphasized that none has full attributes of the disease. Extensive research efforts must continue to develop and explore different animal models of aging and Alzheimer's disease. This is critical for researchers to understand how the disease develops, to identify persons at risk, and to provide hope for reasonable treatment.

CLINICAL APPLICATIONS OF CALCIUM ANTAGONISTS

There are several families of calcium channel blockers, including the dihydropyridines nifedipine and nitrendipine in current clinical use for the management of hypertension. Are these drugs likely to reduce excess calcium levels in neurons and to counteract neuronal dysfunction and possibly cell death? For a majority of such compounds, the answer is probably no, because a necessary feature to subserve such function is the capacity to cross the blood brain barrier when given systemically. Peripherally active dihydropyridines such as nifedipine and nitrendipine cross the blood brain barrier with low efficiency. Centrally acting dihydropyridines like nimodipine, on the other hand, are more lipophilic and can readily cross the blood brain barrier.⁶⁸ Other compounds also exhibit this type of selectivity with reference to their compartments of distribution and may also exhibit clinical efficacy when tested.

Several studies have used Ca^{2+} channel blockers to examine the hypothesis that an inability to reduce the calcium-dependent AHP and thus modulate neuronal excitability contributes to learning deficits in aging animals. Elevation of plasma magnesium (a competitive inhibitor of calcium) improved reversal learning in both aged and young rats.³⁹ Because of its ability to cross the blood brain barrier,⁶⁸ the calcium antagonist nimodipine has been tested in a variety of learning and behavioral tasks in aging mammals. A series of studies from our own laboratory (see FIG. 3, for example) indicate that nimodipine markedly facilitates acquisition of the trace eye-blink conditioned response in aging-impaired rabbits,^{16,41,69} with improvements in learning whether nimodipine was given orally or intravenously. Optimal behavioral effects were obtained at the same dose that maximally enhanced hippocampal neuronal activity *in vivo*,⁴¹ with calculated circulating concentrations equivalent to those that enhanced hippocampal excitability *in vitro*.⁴⁴ Nimodipine (FIG. 11) also improved sensorimotor behaviors in aging rats⁷⁰; reversed open field deficits in aging rabbits²⁶; improved delayed matching-to-sample performance in aging primates⁷¹; and improved spatial learning in aging rats.⁷²

Double-blind, placebo-controlled clinical trials indicate that nimodipine successfully reverses some of the cognitive deficits of dementia and Alzheimer's disease or slows the progression of these diseases in aging populations. With fairly low oral doses (30 mg tid) cognitive improvements are seen. A large double-blind placebo-controlled study of 228 patients separated into probable primary degenerative dementia (a large percentage of whom were likely to have Alzheimer's disease) and into multi-infarct dementia was summarized by Fischhof *et al.*⁷³ After 12 weeks of treatment, patients receiving nimodipine showed improved cognitive abilities. Schmage *et al.*⁷⁴ did a so-called "metanalysis," pooling data from 11 double-blind studies in which a total of 391 patients diagnosed with organic brain syndrome received nimodipine and 398 patients received placebo. In 7 of the studies, a low dose of 30 mg tid of nimodipine was used. The nimodipine-treated subjects improved in 9 of the 11 studies. Improvement was particularly marked on the cognitive subtests of the Sandoz clinical assessment geriatric scale.

In another double-blind multicenter trial of 178 aging patients with primary degenerative dementia, multi-infarct dementia, or a mixed syndrome, 30 mg tid of nimodipine was found to be superior to placebo during a 12-week trial.⁷⁵ Nimodipine-treated patients improved on the Wechsler memory scale, the Folstein mini-mental

status examination, the Sandoz clinical assessment geriatric scale, the Plutchick geriatric rating scale, the severity of illness and global improvement scales of the clinical global impression test, and the Hamilton psychiatric rating scale for depression. In another example in which low doses of nimodipine were used, 30 patients characterized as having organic brain syndrome were compared to an age-matched control group of 20 patients.⁷⁶ A high percentage of the nimodipine-treated patients improved on a series of psychometric tests for cognitive function, memory capacity, concentration, orientation, and degree of autonomy, whereas similar improvement was not observed in control patients.

Tollefson⁷⁷ summarized a multicenter trial of 227 patients who received nimodipine (30 mg tid) or placebo for 12 weeks following a 2-week placebo baseline. Nimodipine slowed the cognitive decline associated with Alzheimer's disease progression. Assessments that were improved relative to the other two groups included the Buschke long-term storage and retrieval test, first and last names recall test, activities of daily living subtests on personal care, housing and communications, and the relative's assessment of global symptomatology subtests on anxiousness and speech. It should be noted, however, that one of the site investigators involved with this study was concerned to stress its limitations in terms of the magnitude of the behavioral effects.⁷⁸ In other words, although improvement was noted, it should not be construed that nimodipine treatment "cured" the patients or restored them to a state approximating normal function. A higher dose of nimodipine (60 mg tid) produced no significant improvements in cognition.

We recently completed a double-blind placebo-controlled study evaluating the effects of nimodipine on eyeblink conditioning in both normal aging and young human subjects.⁷⁹ The subjects in this 3-month trial were assessed for normal learning ability. We found that aging, but not young, people who received nimodipine (60 mg tid for 3 months) acquired conditioned eyeblink responses faster than did their age-matched placebo controls. This effect was a trend after 1 month and was statistically significant after 3 months of treatment. This study demonstrates facilitation of learning by a pharmacological agent using exactly the same learning task in both humans²⁵ and an animal model.^{16,43,69} The results of these studies are consistent with the calcium hypothesis of aging, as discussed above and in other chapters throughout this volume.

It should be stressed that in all of the nimodipine clinical trials, the adverse side effects reported were relatively few and very mild. Similarly, essentially no drug-related changes were noted in patients' normal physiological functions. From a clinical perspective, nimodipine appears to be an extremely safe agent for use in humans and exhibits some degree of efficacy.

Because nimodipine is so safe and well tolerated, the possibility of using it in a prophylactic context is appealing. Unfortunately, it is possible that L-type calcium channel blockers would have only small therapeutic effects on patients with advanced stages of Alzheimer's disease owing to the likelihood that significant irreversible cell loss in the hippocampus and other brain regions may have already occurred. If therapy can begin early enough in the development of the disease, it is theoretically possible to slow progression of degenerative disease processes with a calcium channel blocker. This could either slow or reverse the progression of the disease and enhance the quality of life in these patients.

COMMENTS AND SUMMARY

We have attempted to summarize both hypothetical and experimental relationships between the calcium hypothesis, our current understanding of the neurobiological basis of aging, and proposed mechanisms leading to the etiology of Alzheimer's disease. Our own work has attempted to relate alterations in calcium-mediated events in neurons during the aging process to their potential impact on behavioral learning. As can be seen in the various chapters contained in this volume, a variety of approaches can be taken relating calcium, aging, and dementia. Our own approach uses an animal model of normal aging, with a behavioral assay designed to tap hippocampal/temporal lobe function. We know that the temporal lobe is importantly involved in learning and its impairments, including those that accompany aging, and therefore have concentrated our work on cellular changes in this region.

Our preclinical work has used trace eyeblink conditioning to study learning in aging rabbits. We have shown that acquisition of this task is slowed in aging animals. We have also found that calcium action potentials and calcium-mediated AHPs are augmented in hippocampal neurons in the same age ranges in which learning performance is impaired. These effects can be reduced using a lipophilic dihydropyridine calcium antagonist, nimodipine, at concentrations that we have also shown accelerate learning in aging rabbits. It is quite striking that this same Ca^{2+} channel blocker can reduce the behavioral effects of cerebral ischemia during development; can improve performance in a variety of learning tasks in aging animals; can improve learning in ischemic or demented adults; and, as we have shown, can enhance the ability of normal aged humans to learn our eyeblink conditioning task.

The fact that aging and Alzheimer's disease are so intimately related is probably not accidental. The calcium hypothesis asserts that increases in free intracellular Ca^{2+} during aging likely contribute to the cellular deterioration leading to age-associated dementias. The preclinical data that we and others have gathered *in vitro* and *in vivo* are tantalizing, because they suggest it may be possible to reverse or retard the impact of aging both behaviorally and physiologically by reducing calcium influx. More work is needed to explore this hypothesis. It is necessary to determine mechanisms that lead to excess Ca^{2+} influx and to explore potential dysfunctional changes in regulatory mechanisms that may contribute to a breakdown in calcium homeostasis in aging. Further work is needed to determine if calcium antagonists can be effective over very long-term periods (years) with prophylactic administration. Finally, more work is needed to meet the challenge of designing more effective pharmaceutical agents than are currently available for treatment of learning impairments in normal aging and in states of dementia such as Alzheimer's disease.

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