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Functional Aspects of Calcium-Channel Modulation

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Summary: Associative learning is accompanied by a number of changes in the brain, many mediated by calcium. We have used eyeblink conditioning, a well-controlled learning task in animals and humans, to elucidate these changes. Our studies have focused on the hippocampus, a temporal lobe structure known to be important for storage of new information during learning in mammalian brain. Hippocampal neurons show an enhanced firing rate during learning correlated with behavioral acquisition; they also show reduction in a calcium-mediated after-hyperpolarization (AHP), a likely mechanism for their enhanced activity. Aging animals and humans exhibit learning deficits; aging hippocampal neurons show increased AHPs and altered calcium buffering, which contribute to the behavioral learning deficits. Intravenous administration of the calcium antagonist nimodipine causes aging rabbits to learn the eyeblink conditioning task as quickly as young controls. Oral nimodipine enhances learning rates in aging rabbits, rats, and monkeys. In each case, the type of learning task analyzed is dependent on hippocampal processing for acquisition and is impaired with aging. Nimodipine also reverses aging-related alterations in open field behavior of both rats and rabbits. We have done a series of physiological studies focused on the possible role of nimodipine in enhancing neuronal activity in the hippocampus of aging rabbits. The purpose of these studies was to determine how nimodipine may be functioning at a cellular level to increase the learning rate. Four major conclusions may be drawn from our data: (a) Nimodipine strongly enhanced the firing rate of single hippocampal pyramidal neurons recorded in vivo in an aging- and concentration-dependent fashion. Other calcium-channel blockers, such as nifedipine and flunarizine, given to control for cerebral blood flow changes, had essentially no effect on the hippocampal firing rate. (b) The slow AHP, mediated by an outward calcium-activated potassium current, was markedly larger in pyramidal neurons in hippocampal slices prepared from aging rabbits. Nimodipine, at concentrations as low as 100 n*M*, reliably reduced the AHPs of aging pyramidal cells. Aging neurons also showed more spike frequency adaptation, or accomodation, than young neurons. Nimodipine partially blocked accomodation at concentrations as low as 10 n*M* in aging neurons. (c) The calcium action potential was larger

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in aging neurons. Nimodipine modulated the calcium action potential in an age- and concentration-dependent fashion; concentrations as low as 100 n*M* reduced the calcium action potential in aging CA1 neurons without effects on young cells. (d) Nimodipine blocked the high threshold, noninactivating calcium current (L-type calcium current) in acutely dissociated hippocampal pyramidal neurons. This effect quickly washed out and was reversed with application of Bay K 8644, a dihydropyridine calcium-channel agonist. These data, gathered both in vivo and in vitro, suggest that nimodipine acts directly on neuronal elements known to be importantly involved in eyeblink conditioning. Such direct neuronal action should help to improve learning in aging brain. The clinical implications of our work lie in the attempt to use nimodipine to treat Alzheimer disease or learning deficits in the aging. Many of the learning deficits in aging human brain may be importantly mediated by excess neuronal calcium and should be amenable to intervention with a calcium-channel antagonist. **Key Words:** Eyeblink conditioning—Hippocampus—CA1—Afterhyperpolarization—Calcium action potential—Calcium-channel antagonist—Nimodipine—Aging—Alzheimer disease.

A prominent theory regarding the neural substrates that may underlie the difficulties that aging mammals, including humans, have in learning many new associations is the "calcium hypothesis of aging" (1,2). This hypothesis suggests that cells in the aging brain have an excess of intracellular calcium. Normally, calcium levels are tightly regulated in neurons (3). The excess in aging neurons is attributable to alterations in their ability to buffer calcium with calcium-binding proteins or by storing it in the endoplasmic reticulum or other internal stores, to reduced ability to extrude calcium via the sodium–calcium exchange pump or an energy-dependent pump, or to altered calcium influx at the neuronal membrane via voltage-dependent calcium channels. This alteration in the ability to process calcium leads to levels of calcium in aging neurons approximately one order of magnitude higher than in young neurons. An increased intraneuronal calcium level over a relatively long period of time, even if the increase is relatively small, can have deleterious effects on neurons that can lead ultimately to membrane deterioration and cell death in the aging or Alzheimer diseased neuron (1). A variety of pathways including DNA breakdown, protein breakdown, or arachidonic acid and free radicals may be involved in the processes leading to membrane deterioration and cell death. These processes are conceptually similar to those that are postulated to cause cell death in an ischemic focus during stroke (4). Such processes, occurring slowly and over a long time course, may be involved in causing or contributing to the cellular alterations that occur during the course of Alzheimer disease. The studies we summarize here on learning in aging brain have relevance to the calcium hypothesis of aging. As we have reviewed our recent experiments in several recent publications (5–9), this article will concentrate on their main findings.

EYEBLINK CONDITIONING

The behavioral paradigm we have used in our work is an associative learning task called eyeblink conditioning. This relatively simple learning task serves as a "model system" that is amenable to a neurobiological analysis of the systems and cellular

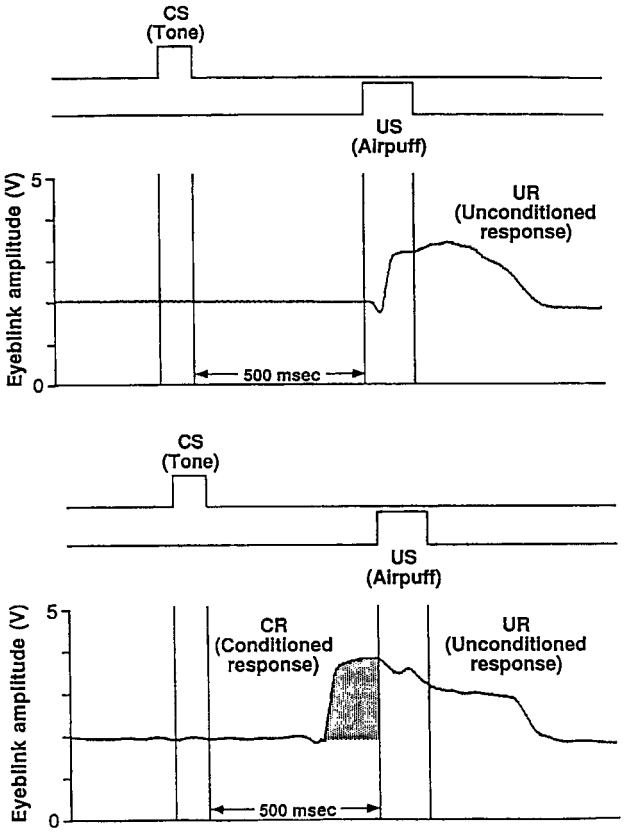

FIG. 1. The trace eyeblink conditioning paradigm used with both rabbits and humans. A 100-ms pure tone conditioned stimulus (CS) is presented with binaural headphones. After a 500-ms interstimulus trace interval, the 150-ms air puff unconditioned stimulus (US) is presented to the cornea of the eye. The term "trace interval" was introduced by Pavlov and describes the fact that the subject must form a short latency memory or "stimulus trace" of the CS in order to successfully time the conditioned eyeblink response, which serves to minimize the US impact on the eye. In rabbits, extension of the third eyelid (nictitating membrane response) is measured in our laboratory with a noninvasive phototransistor that detects the increased reflection of light from a light-emitting diode during the blink. In humans, eyeblinks are measured with the same noninvasive system. Eyeblink responses in two individual trials in a human subject are indicated. Early in training (**upper panel**), unconditioned responses occur to air puff onset but no eyeblink occurs to tone CS presentation. Later in training (**lower panel**), conditioned eyeblinks occur to the tone presentation prior to air puff US presentation. These conditioned responses occur with greater frequency, shorter latency, and larger amplitude as conditioning proceeds (5).

substrates of learning (10). In our version of the task (see Fig. 1), a short tone conditioned stimulus (CS) is paired after a 500-ms trace interval with an air puff to the eye (unconditioned stimulus, or US) sufficient to elicit a reliable eyeblink. Early in training, presentation of the tone CS causes no eyeblink; as training proceeds, tone CS presentation causes a relatively high percentage of eyeblinks that occur prior to the onset of the US and that tend to be timed so that their maximum amplitude occurs at about the onset of the air puff US. These conditioned responses (CRs) represent an indication on a trial-by-trial basis that an association has occurred between the tone

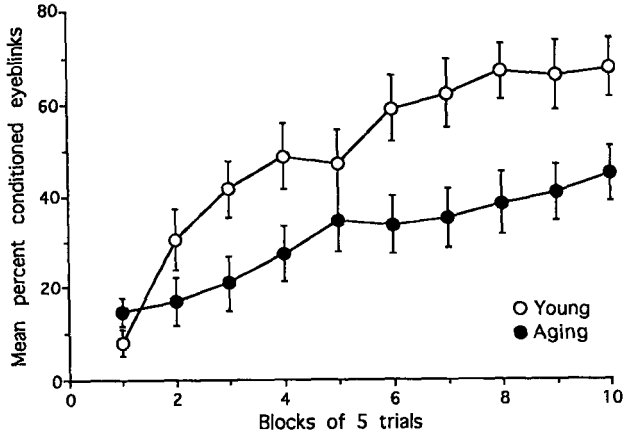

FIG. 2. The percentage of conditioned eyeblink responses in young (n = 25, mean age = 24.9 years) compared to aging (n = 25, mean age = 67.8 years) humans during trace eyeblink conditioning with parameters as in Fig. 1. Average percent conditioned responses are given in successive five-trial blocks of training. A clear aging effect ($p < 0.02$) was evident within the second block of five trials. By the fourth block of trials, the young subjects reached a higher performance level than the aging subjects achieved by the end of training. (Adapted from ref. 15.)

CS and the air puff US. We have shown that this trace eyeblink conditioned task, in which the rabbit or human must maintain a "stimulus trace" of the CS to blink successfully and minimize US presentation, requires an intact hippocampus in the rabbit for successful learning to occur (11). We know that the hippocampus is a structure very much affected by aging as well as by the neural degenerative processes of Alzheimer disease (12–14). Thus, we have utilized a paradigm that we know is affected by hippocampal damage, at least in our animal preparation. In preliminary work, we have observed that normal aging humans showed lower levels of eyeblink conditioning in the trace paradigm than did young controls (see Fig. 2) (15).

NIMODIPINE ENHANCES LEARNING RATE IN AGING RABBITS

The outward, calcium-activated potassium current that occurs after a burst of action potentials [the afterhyperpolarization (AHP)] is increased in neurons from aging brain (16,17). This current is thought to be an important controller of excitability in central neurons (18–21). The initial observation that this current is increased in duration in hippocampal CA1 neurons from aging rats was made by Landfield (16), who suggested that an increase in this current would reduce cellular excitability and possibly cause learning deficits. Our own work has replicated and extended Landfield's observations to show that the AHP is markedly increased in amplitude as well as duration in aging rabbit hippocampal CA1 neurons (17). Such an increase in the AHP, especially in a structure such as the hippocampus that is so importantly involved in a variety of associative learning processes in mammalian brain (22,23), should make learning more difficult in the aging brain.

That aging hippocampal neurons had increased AHPs was especially interesting to us, because we had shown that this same calcium-activated AHP was reduced as part

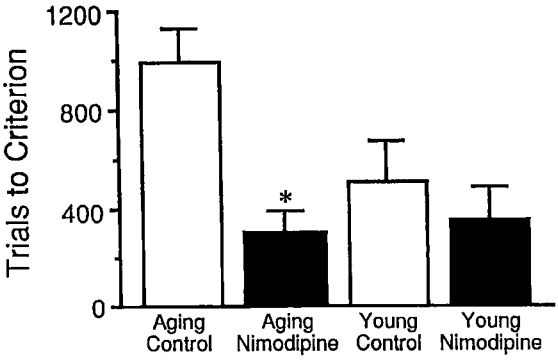

FIG. 3. Nimodipine facilitated acquisition of trace eyeblink conditioning in aging rabbits. Note that aging control rabbits required more trials to reach the criterion of 8 conditioned responses in any block of 10 trials than did young or aging nimodipine-treated rabbits (Adapted from ref. 32.)

of the cellular substrates of formation of new eyeblink associations in the hippocampus in young rabbits (24–28). An increase in the postburst AHP is one consequence of increased calcium in aging neurons, referred to above. We hypothesized that a pharmacological intervention that would reduce the AHP in aging hippocampal neurons could enhance the learning rate in aging animals. This would occur through an increase in aging neuron excitability so that the aging neurons more closely resembled the young neurons in level of excitability. This, of course, assumed that the slowed learning rate in aging animals could be attributed to enhanced calcium levels. The pharmacological manipulation we attempted was intravenous administration of the L-type calcium-channel antagonist nimodipine, a dihydropyridine that crosses the blood–brain barrier with relative ease and thus may be given peripherally (29–31). As can be seen in Fig. 3, we observed that aging rabbits given nimodipine learned the trace eyeblink conditioning task at the same rate as young vehicle controls (32). Their learning rate was clearly enhanced compared to aging vehicle control rabbits. Nimodipine did not enhance the learning rate in young animals. A follow-up study showed a behavioral enhancement of learning rate when nimodipine was administered orally via food pellets for 1 month prior to training (33). The effect was not as large in the oral study, possibly reflecting a nonoptimal dose of the drug. An ongoing dose–response study suggests that intravenous nimodipine in concentrations from 0.5 to 5.0 μg/kg/min is sufficient to enhance the learning rate above vehicle controls (34). Measurements of serum levels of nimodipine are not yet complete in that study.

NIMODIPINE INCREASES EXCITABILITY OF AGING HIPPOCAMPAL NEURONS

The dramatic behavioral effects we have observed with nimodipine administration suggest that the hypothesis that prompted our attempt to investigate nimodipine's effects on learning in aging brain may have been correct. We have conducted a series of neurophysiological and biophysical experiments designed to address this question. Our experiments were directed at the hippocampus in the rabbit. They suggest that this structure may be an important mediator of the enhancement of learning in aging rabbits by nimodipine. They have shown that nimodipine enhances CA1 hippocampal pyramidal neuron excitability in an age- and concentration-dependent fashion

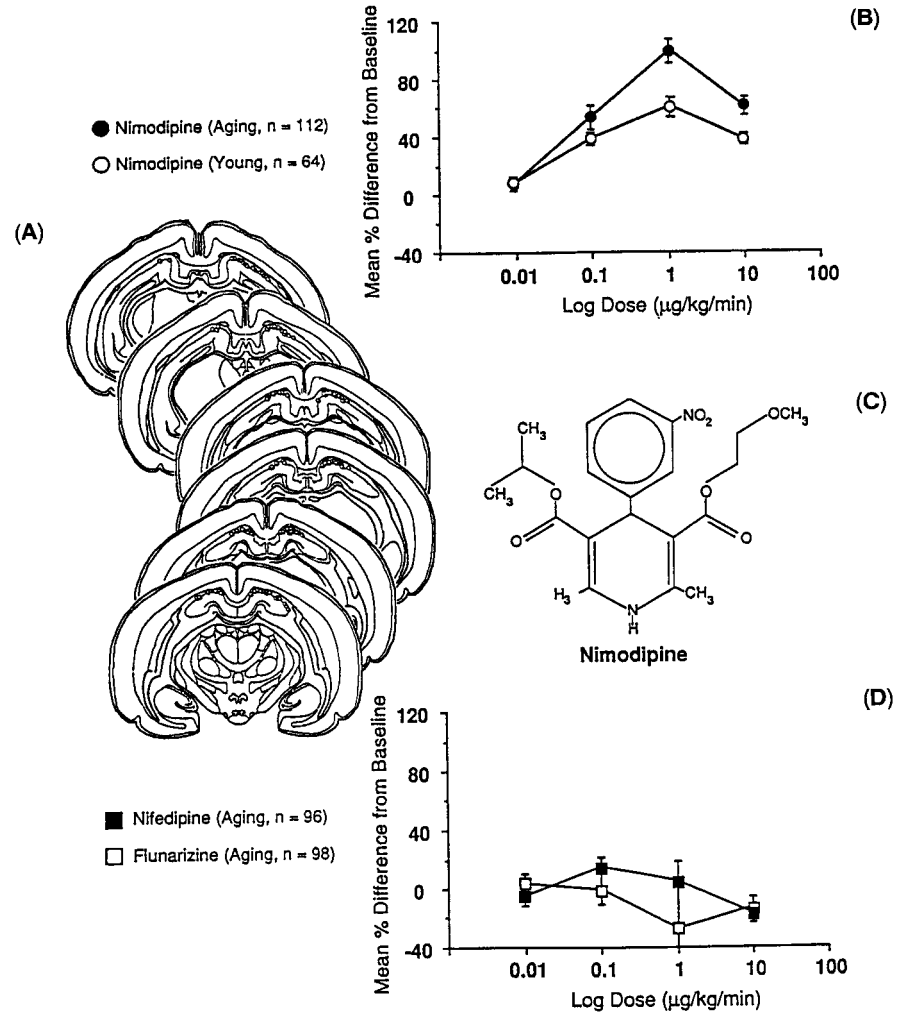

FIG. 4. Nimodipine increased excitability of hippocampal neurons recorded in vivo in conscious rabbits. **A:** The position of recording sites in CA1 of hippocampus. **B:** Dose-dependent effects of nimodipine on spontaneous activity of single hippocampal CA1 neurons recorded in vivo in aging (●) and young (○) rabbits. Nimodipine had a maximal effect at the dose that facilitated learning in aging rabbits (1.0 μg/kg/min). At all doses tested, the effect was larger in aging rabbits. **C:** The chemical structure of nimodipine. **D:** The same range of doses of nifedipine and flunarizine had no effect on CA1 pyramidal neuron firing rate, indicating that the nimodipine effect was mediated by direct actions on neurons, not via changes in cerebral blood flow. (Adapted from ref. 35.)

when measured both in vivo and in vitro in a variety of preparations designed to demonstrate that nimodipine acts directly on hippocampal neurons.

First, single CA1 pyramidal neurons were recorded in vivo in awake young and aging rabbits, prepared as for eyeblink conditioning (35). Intravenous administration of nimodipine caused an age- and concentration-dependent enhancement of the spontaneous firing rate of pyramidal neurons in these animals. Nimodipine caused a

greater effect on neuronal excitability in aging than in young rabbits (see Fig. 4). The dose used in our earlier study to enhance learning rates in aging rabbits (1 μg/kg/min) (32) was most effective in enhancing neuronal firing rate. Nifedipine, a calcium-channel antagonist that does not cross the blood–brain barrier as readily as nimodipine (31), caused no significant change in spontaneous firing rate. Similarly, the piperizine-type calcium antagonist flunarizine caused no alteration in firing rate at a wide range of doses, sufficient to control for the possibility that enhanced blood flow was the primary contributor to the excitability changes produced by nimodipine.

The blood flow issue is an important consideration in interpreting the behavioral effects of nimodipine, which is known to enhance blood flow (36) at doses that also enhance the learning rate. This is especially true because many "nootropics" are thought to be cerebral vasodilators (37) and because of the long-established effect of the dihydropyridines as peripheral vasodilators in the control of hypertension. Therefore, in our final series of experiments, we used the hippocampal slice preparation to determine if nimodipine increases CA1 pyramidal neuron excitability in vitro, a preparation where by definition blood flow could have no possible contribution to effects observed (17). The postburst AHP and accommodation (or spike frequency adaptation) have been used as measures of excitability in these studies. We demonstrated that the AHP was greater in both peak amplitude and integrated area in aging rabbit CA1 hippocampal neurons (see Fig. 5). High concentrations of nimodipine decreased the slow AHP, especially in the aging neurons. It was particularly interest-

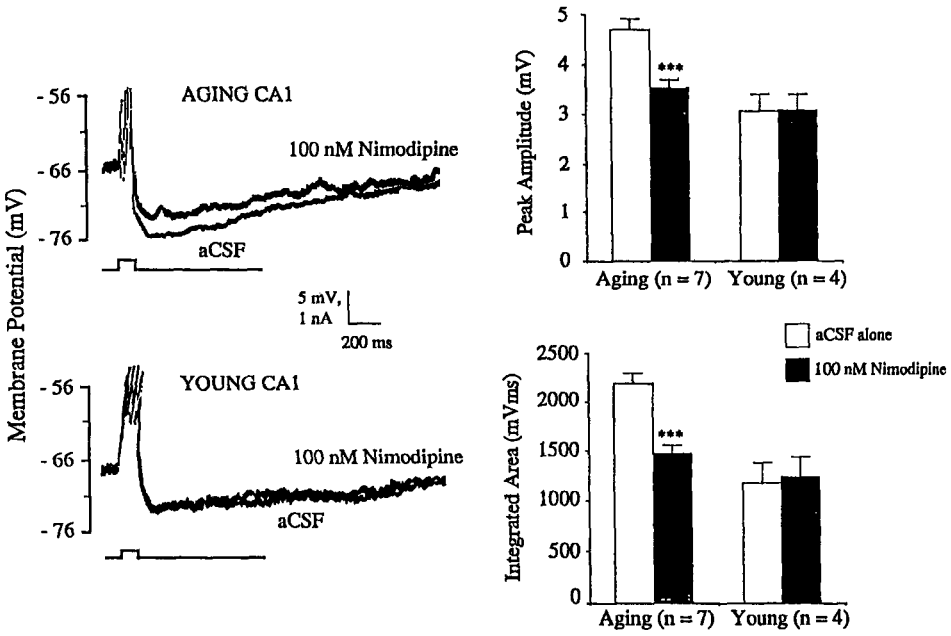

FIG. 5. Nimodipine at a concentration of 100 n*M* caused a significant reduction in the postburst afterhyperpolarization in CA1 neurons from aging but not young rabbits. The reduction was present both in the integrated area of the afterhyperpolarization (a reflection of increased duration) as well as in the peak amplitude of the response. Scale in left panel, 5 mV, 1 nA, and 200 ms. (Adapted from ref. 17.)

ing that concentrations of nimodipine as low as 100 n*M* caused a significant reduction in the postburst AHP in aging but not in young CA1 neurons (see Fig. 5). Both the peak amplitude and the integrated area of the AHP were reduced. It should be noted that nimodipine reduced the AHP in aging neurons to a size comparable to that observed in young neurons.

A relationship between the postburst AHP and accommodation has been suggested (38), i.e., neurons with large AHPs have difficulty firing at high rates to a long depolarizing pulse because of the braking action of the AHP on the firing rate (18,19,21). Accommodation or spike frequency adaptation, another index of excitability, was also studied in aging and young neurons. Aging neurons fired fewer action potentials during a long depolarizing pulse, i.e., they showed more accommodation. Nimodipine at relatively high doses (10 μM) reduced accommodation in both young and aging neurons. In addition, nimodipine was effective in significantly reducing the amount of accommodation in concentrations as low as 10 n*M* only in aging neurons.

It is clear that nimodipine's effects on the postburst AHP and on accommodation could well be secondary consequences of modulation of calcium influx via voltage-gated L-type calcium channels. In voltage-clamped dissociated hippocampal pyramidal neurons from young adult guinea pigs, nimodipine blocked a significant proportion of inward calcium currents, whereas Bay K 8644 enhanced inward currents (39). In addition, and more directly to the point, nimodipine in concentrations as low as 100 n*M* reduced the depolarizing plateau of the calcium action potential in CA1 neurons from aging rabbit hippocampal slices (40) (see Fig. 6). This depolarizing plateau was markedly enhanced in aging CA1 neurons compared to young cells. Further experiments will be necessary to address this point, but these experiments suggest that the L-type calcium current may be selectively enhanced in aging CA1 neurons. Furthermore, they suggest that the behavioral effects we and others have observed with nimodipine administration may be at least partially mediated by direct antagonism of L-type calcium channels in hippocampal neuron membranes.

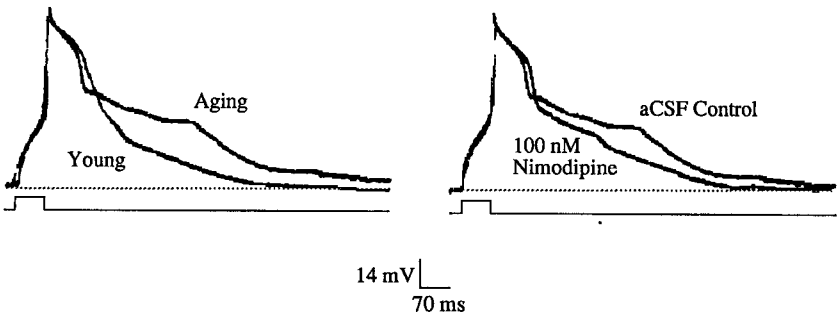

FIG. 6. Calcium action potentials are prolonged in aging neurons and reduced by low concentrations of nimodipine. **Left panel:** Overlay records of calcium action potentials from an aging and a young CA1 hippocampal neuron. Note the biphasic characteristic of the calcium action potential with only the later "plateau" phase enlarged in the aging neuron. **Right panel:** 100 n*M* nimodipine reduces the size of the plateau phase of the calcium action potential in aging neurons. (Adapted from ref. 40.)

COMMENTS AND CONCLUSIONS

The series of studies we have summarized on learning in young and aging brain have been designed to begin to address the "calcium hypothesis of aging" (1,2) with the use of a powerful model behavioral system combined with a cellular neurobiological analysis. Eyeblink conditioning, the behavioral task we have used in our work, is a relatively simple and extremely well-controlled model of associative learning (10). The variant of the paradigm we have studied, trace eyeblink conditioning, is dependent on the hippocampus for its successful acquisition (11,41). The hippocampus is critical for learning in mammalian brain (22,23,42) and is known to be affected at the cellular neurophysiological and neuroanatomical levels by the alterations that occur during normal aging and in disease states most common in aging brain such as Alzheimer disease (12–14). Thus, we have chosen to concentrate our experimental efforts on this temporal lobe structure.

Eyeblink conditioning was originally designed as a paradigm to be used to analyze laws of human learning and only subsequently was adapted for use in the rabbit (10,43). Because of parallel changes in the ease of learning the eyeblink paradigm that occur in rabbit and human across the life span, it has been argued that this behavioral task should be an especially powerful behavioral model system for use in analyzing alterations that occur in learning capacities in aging brain (43–45). In our own experiments, we have shown that trace eyeblink conditioning is acquired less robustly in normal aging compared to normal young control subjects (15). Parallel changes that occur during the aging process in the hippocampus may well be one reason for the behavioral parallels that are observed across species.

We have summarized a series of studies in which we used a combined behavioral, pharmacological, and neurophysiological approach in examining the calcium hypothesis of aging. The task we have used, trace eyeblink conditioning, depends on the hippocampus for its successful acquisition. We know that hippocampal neurons show evidence for increased excitability as learning takes place in this task. In vivo, they fire more action potentials to the tone CS as it gains behavioral control (46,47); in vitro, they show reduced postburst AHPs, reflecting a reduction in a calcium-activated outward potassium current (24–28,48). This same AHP is increased in aging hippocampal neurons, apparently reflecting increased calcium levels in aging neurons and possibly contributing to the learning impairments seen in many tasks in aging mammals (16,17). Attempts to pharmacologically reduce these increased calcium levels with nimodipine, a dihydropyridine calcium-channel antagonist, have shown positive behavioral results. Aging rabbits learn the eyeblink task at about the same rate as their young controls. Neurophysiological measures show that nimodipine increases hippocampal neuronal excitability in an age- and concentration-dependent manner, i.e., single neurons show higher baseline firing rates in conscious aging rabbits; the AHP and spike frequency adaptation are both reduced in hippocampal slices by nimodipine concentrations in a physiologically meaningful range (10–100 n*M*); the depolarizing plateau potentials of calcium action potentials (which are selectively increased in aging neurons) are also decreased by 100 n*M* nimodipine in the aging hippocampal brain slice. These data indicate that nimodipine acts directly on aging hippocampal neurons to increase their excitability. We hypothesize

that nimodipine may be exerting an important portion of its behaviorally enhancing effects through direct actions on the hippocampus in aging brain. These data also suggest that nimodipine may be acting to reverse the consequences of elevated intraneuronal calcium levels as posited by the calcium hypothesis of aging.

Our own experiments have concentrated on using eyeblink conditioning in rabbit as a behavioral model combined with appropriate in vivo and in vitro neurophysiological and biophysical cellular analyses of substrates of change. Our observations that nimodipine facilitates learning in aging brain have also been paralleled by work in other behavioral model systems. For example, delayed matching to sample performance has been shown to be facilitated in aging primates (49), and swimming and radial maze performance has been enhanced in aging rats (50,51). A cardinal feature of these experiments has been that behavioral facilitation has been observed in normal aging but not young animals. A second feature of these studies is that the behavioral tasks that have been utilized have been hippocampally dependent. Both of these features are important for attempts to generalize work in animal models to the human. Less specifically "cognitive" tasks also have been reported to show improved performance after nimodipine treatment: gait pattern in aging rats (30,52), a variety of skilled motor tasks such as rope climbing and walking on narrow beams in aging rats (51), and open field performance in aging rabbits (53). Improved sensorimotor performance in aging humans, who often suffer from impaired performance on skilled motor tasks, certainly would be a welcome generalization of these animal behavioral models to the human condition.

Other important behavioral work of Finger and LeVere showed that nimodipine is very useful in facilitating functional recovery from brain damage and/or stroke (54,55). Learning of higher-level operant tasks is improved after lesions if nimodipine is administered. The connection between this work and the calcium hypothesis of aging is likely to be at a more theoretical level. The cellular mechanisms for the behavioral recovery of function observed is likely to involve antagonism of excess calcium influx in injured neural tissue at or near the lesion focus of damage, thus facilitating adaptive changes in the system after injury. Given that there are likely to be some common substrates with cellular changes occurring, albeit over a rather longer time period, during the course of aging, this functional work should be considered in the context of our current discussion.

Eyeblink conditioning is also impaired in Alzheimer patients (56,57) known to have hippocampal degeneration (13). We have demonstrated that normal aging individuals demonstrate less robust eyeblink conditioning than do their younger counterparts (15). These data suggest that eyeblink conditioning in rabbits should be an excellent animal model with which to evaluate the mechanisms of, and therapeutic interventions for, learning deficits in the aging human population as well as in Alzheimer patients (6,8,43,45). Given that aging individuals generally have excess calcium levels in neurons that can lead to impaired neural function and to difficulties forming new associations, this model system should be an excellent one to evaluate compounds such as nimodipine whose therapeutic action is mediated by L-type calcium-channel antagonism. Our goal is to use a powerful animal model of associative learning, combined with appropriate cellular neurophysiological and pharmacological approaches, to determine how compounds such as nimodipine may be acting

to enhance learning in aging brain. Information gained from this approach should be useful in maximizing the use of nimodipine for this application and in designing new and/or improved compounds to compensate for negative behavioral effects of brain aging on learning.

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