Investigation of Age-Related Cognitive Decline Using Mice as a Model System: Neurophysiological Correlates

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Objective: Learning and memory impairments without overt pathology often accompany advancing age. To gain a better understanding of the underlying neuronal substrates associated with this age-related cognitive decline, the authors have begun to use mice as an animal model system. As described in the companion paper, mice exhibit age-related impairments in cognition. Here, the authors explore the possibility that age-related changes in neuronal function may be the result of deregulation of cytosolic free calcium homeostasis. Methods: Calcium homeostasis in young and aged mice was examined by measuring the slow afterhyperpolarization (sAHP) in hippocampal neurons as well as assessing voltage-dependent calcium channel mediated long-term potentiation (vdccLTP). In addition, putative changes in phosphorylation of the L-type channel CaV1.2 by cAMP-dependent protein kinase were examined. Results: Both neurophysiological measures of calcium homeostasis indicated an increase in activity-dependent calcium influx. This increase was not the result of an age-related increase in phosphorylation of the L-type channel CaV1.2 by cAMP-dependent protein kinase. Conclusions: Like in other areas of biomedical research, mice have become an invaluable research tool in the investigation of learning and memory. It is expected that similar benefits can be realized by developing mouse models for age-related cognitive decline. (Am J Geriatr Psychiatry 2006; 14:1012–1021)

Key Words: Mice, learning and memory, aging, calcium, synaptic plasticity

The exact neuronal substrates the deficits in hippocampal-dependent learning and memory that often accompany aging are not currently known. Many age-related changes within the hippocampus have been documented, including alterations in mitochondrial function, oxidative stress, dysregulation of transcription factors, as well as alterations in glutamate transmission and synaptic plasticity. In addition to these well-documented changes, there is mounting evidence that a number of age-related...
changes in neuronal function within the hippocampus may be the result of deregulation of cytosolic free calcium homeostasis.8 Perhaps the most direct evidence for an age-related alteration in calcium homeostasis comes from calcium imaging studies that demonstrate a marked increase in intracellular calcium levels ([Ca\(^{2+}\)]\(_i\)) in neurons from aged rats in response to prolonged synaptic stimulation.9 It is likely that one of the key sources is calcium entering through L-type voltage-dependent calcium channels (L-VDCCs). The difference in [Ca\(^{2+}\)]\(_i\), between young and aged neurons becomes apparent only when synaptic stimulation is sufficiently strong to elicit action potentials in the soma.

An age-related increase in activity-dependent calcium influx would be expected to impact several aspects of neuronal function. Indeed, there are a number of age-related changes in neuronal function that appear to be a direct result of an increase in activity-dependent calcium influx. In the hippocampus, these changes include a pronounced decrease in neuronal excitability and alterations in the induction threshold for synaptic plasticity.

In many brain regions, including the hippocampus, bursts of action potentials are followed by a prolonged period of hyperpolarization that can last for several seconds.10 This hyperpolarization—known as the slow afterhyperpolarization (sAHP)—is the result of the activation of calcium-activated potassium channels.11 Enhanced neuronal excitability as manifested by a decrease in the sAHP accompanies several forms of hippocampal-dependent learning and memory, including trace eye blink conditioning,12–14 and the water maze.15 An age-related increase in cytosolic free calcium would be expected to enhance the sAHP. This is indeed the case. There are numerous reports documenting an age-related increase in the sAHP.13,16–19 It is important to note that this age-related increase in the sAHP correlates with decreased performance in the trace eye blink conditioning task20,21 and the Morris water maze.22 Furthermore, pharmacologic agents such as nimodipine (an L-type Ca\(^{2+}\) channel antagonist), which decrease the sAHP,17 also decrease age-related learning deficits in rabbits23,24 and rats.25,26

Alterations in calcium homeostasis may also account for age-related changes in the threshold for synaptic modification. In particular, alterations in calcium homeostasis appear to impact the induction threshold for long-term potentiation (LTP), a form of synaptic plasticity thought to be involved in learning and memory.27 How might an increase in calcium lead to an increase in the induction threshold for LTP? It seems likely that this is a direct consequence of the enhanced sAHP.28 In addition to regulating spike frequency by accommodation,10 the sAHP can act as a shunt, capable of reducing the amplitude of subsequent depolarizing events that fall within the duration of the sAHP.29 This is consistent with numerous reports that fail to find age-related differences in LTP using “standard” (e.g., 100 Hz for 1 second) induction protocols but demonstrate age-related differences when minimal stimulation protocols are invoked.30 Finally, we have recently demonstrated that genetic manipulations of neuronal excitability that functionally oppose the age-related decrease in neuronal excitability and enhance minimal stimulation LTP and learning in aged animals.31

The findings outlined here regarding the increase in VDCC-mediated calcium influx and alterations in LTP induction threshold are also consistent with LTP experiments using very strong stimulation protocols consisting of bouts of 200-Hz stimulation at intensities sufficient to elicit action potentials in the soma. Unlike LTP induced by weaker stimulations parameters, LTP induced by strong stimulations protocols does not require activation of N-methyl-D-aspartate receptors (NMDA-Rs) and appears to be mediated by Ca\(^{2+}\) influx through VDCCs and therefore has been called vdccLTP.32 In support of the idea that there is an age-related increase in Ca\(^{2+}\) influx through VDCCs, it has been previously demonstrated that vdccLTP is enhanced in aged rats when compared with young rats.33 Although it seems unlikely that this strong induction protocol resembles physiological events that occur during learning, it does support the notion that there is an age-related increase in activity-dependent calcium influx, and that this calcium has functional access to the same subcellular machinery that is activated by calcium influx through the NMDA receptors.

Much of the data reviewed here comes from experiments using rats as a model system for studying age-related changes in neuronal function and behavior. Although there is a growing body of literature regarding age-related impairments in spatial learning in mice, there are fewer reports examining the neurophysiological changes that occur during nor-
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Mal aging in mice. We present data from experiments designed to better parameterize age-related changes in calcium-dependent neuronal function in mice and examine one potential source for the increase in cytosolic calcium.

METHODS

Mice

All mice used in these experiments were either raised within our own colony or obtained from the National Institutes on Aging colony at Harlan Sprague Dawley (Indianapolis, IN). Mice raised in our own colony were 8–10 generations backcrossed into the C57Bl/6 background and were used to measure the sAHP. C57BL/6Nia mice obtained from NIA were used in all other experiments. Young animals were 4–6 months of age at the start of the experiments and aged animals were 22–24 months of age.

Slice Preparation

Animals were placed under deep halothane anesthesia before decapitation. Whole brain (minus the cerebellum) sagittal sections (400 μm) were made and rapid microdissection of the hippocampi was completed in ice cold artificial cerebrospinal fluid (aCSF) that was saturated with a mixture of 95% O2 and 5% CO2 and contained the following (in mM): 120 NaCl, 20 NaHCO3, 3.5 KCl, 1.25 NaH2PO4, 2.5 CaCl2, 1.3 MgSO4, 10 D-glucose.

Hippocampal slices were then transferred to a holding chamber to rest at room temperature for at least 1 hour before the start of the experiment. All recordings were made in a submerged chamber perfused continuously with oxygenated aCSF at a rate of approximately 2 mL/min at 31°C.

Slow Afterhyperpolarization Measurements

Current clamp recordings were made in CA1 pyramidal neurons using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) in bridge mode using the blind whole-cell technique. Whole-cell patch electrodes (4–6 MΩ) were used that contained 120 mM potassium methyl sulfate, 20 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 2 mM MgCl2, 4 mM NaATP, 0.3 mM Tris GTP, 7 mM phosphocreatine. After break in, cells were allowed to rest for at least 10 minutes during which time the input resistance and membrane potentials were monitored. After this resting period, cells were held at 5 mV below action potential threshold and the postburst AHP was measured 200 msec after the termination of a 50-msec current pulse of sufficient amplitude to elicit only five spikes. Statistical comparisons were made using the number of animals. When multiple cells within an animal were recorded from, they were averaged to a single value for each animal. Statistical comparisons were made between groups using a one-way analysis of variance (ANOVA).

Long-Term Potentiation Experiments

Extracellular recordings of field excitatory postsynaptic potentials (fEPSP) were made in the stratum radiatum of CA1 with Pt/Ir electrodes (FHC, Bowdoinham, ME) with resistance of approximately 2 MΩ. Field EPSPs were evoked by stimulating the Schaffer afferent fibers with bipolar platinum electrodes. Test stimuli (100 μsec in duration) were delivered once every two minutes at one-half to two-thirds maximal stimulation strength. After 10 minutes of baseline recording, long-term potentiation was induced by four one-half second trains of 200-Hz stimulation with an interstimulus interval of 15 seconds at a stimulation strength sufficient to produce a population spike (measured in the cell body layer with a second recording electrode). This was done to ensure activation of L-type calcium channels. After the tetanus, the stimulation strength was reset to the pretetanus level and test stimuli were delivered once every two minutes for at least one hour after the tetanus. Changes in synaptic strength were measured as the change in slope of the fEPSP, normalized to the 10 minutes of baseline recorded before the tetanus. Long-term potentiation was examined in slices prepared from young and aged animals in normal aCSF. In addition, LTP was induced in slices prepared from the same animals but in the presence of 50 μmol/L D-2 amino-5-phosphonopentanoate (APV), which was made fresh daily and added directly to the circulating aCSF. Statistical comparisons were made using the number of slices. However, each animal contributed at most...
one slice to either condition (aCSF or aCSF plus APV); therefore, the number of slices in a given condition is equivalent to the number of animals. Within-group comparisons (i.e., pre- versus posttetanus fEPSP slope values) were made using the average fEPSP slope of the last 10 minutes in a single-group t-test with the hypothesized mean set as 100. Statistical comparisons between age groups were made using a one-way ANOVA on the last 10 minutes of data (averaged).

**Western Blot Analysis**

The FP1-antibody was produced in rabbits against a peptide covering residues 818–835 of CaV1.2 and recognizes both the full-length protein (long form) and the calpain-truncated protein (short form). The anti-CH3P antibody, which specifically binds to CaV1.2 when phosphorylated at serine 1928, was raised against a peptide that was derived from residues 1923–1932 and phosphorylated by PKA. All brain tissue for Western blot analysis was obtained from mice used in the 200-Hz LTP experiments. Brain tissue was harvested as described previously; however, one-half of the brain was used for the LTP experiments and the other half was further dissected in ice cold aCSF to isolate the hippocampus and cortex, snap frozen on liquid nitrogen, and stored at −80°C. Subsequently, 50 mg hippocampal tissue was homogenized in 0.5 mL 1% Triton X-100, 10 mM Tris-Cl, pH 7.4, 20 mM EDTA, 10 mM EGTA containing protease inhibitors (1 μg/mL pepstatin A, 10 μg/mL leupeptin, 20 μg/mL aprotinin, 200 nM phenyl-methane-sulfonyl fluoride, 8 μg/mL calpain inhibitor I, and 8 μg/mL calpain inhibitor II), as detailed elsewhere. Triton X-100-insoluble material was sedimented by ultracentrifugation. CaV1.2 was immunoprecipitated from cleared Triton X-100 extracts with a saturating concentration of anti-FP1 (10 μg in 500 μL before immunoblotting with anti-CH3P and subsequently with anti-FP1). All blots were exposed for increasing amounts of time (e.g., 30, 60, 120 seconds) and only those signals were used that doubled with twice as long exposure times to ensure that quantifications were performed with signals in the linear range only. Over time, the enhanced chemiluminescence (ECL) signals decay; although exposure times were typically quite short and did usually not show any substantial decay in signal during the periods during which film was exposed, short exposures were always taken first and long ones last; in reverse order, the time-dependent decay could reduce the short exposure signals, thereby potentially masking partial saturation of the longer exposures.

Phosphorylation data are presented as the average ratio of phosphorylated CaV1.2 at serine 1928 over the amount of protein as determined by immunostaining the same plot with the anti-FP1 antibody. Statistical comparisons were made between groups using a one-way ANOVA on data normalized to young adult values using the number of animals as the sample size (N).

**RESULTS**

**Measurements of the Slow Afterhyperpolarization and L-Type Voltage-Dependent Calcium Channel-Dependent Long-Term Potentiation**

To determine the extent of the age-related increase in activity-dependent calcium influx, we measured the sAHP in neurons in the CA1 region of the hippocampus in slices from young (seven mice/15 neurons) and aged (four mice/11 neurons) C57BL/6 mice. As illustrated in Figure 1, the sAHP recorded from an aged mouse pyramidal neuron (bottom panel A2) is significantly larger in amplitude at its maximum and persists longer than the sAHP recorded from CA1 pyramidal neurons typically found in the young mouse hippocampus (top panel A1). This is also true for the group data plotted in Figure 1B with the aged mice (6.2 ± 1.2 mV; mean ± standard deviation [SD]) exhibiting a significant increase in mean sAHP amplitude when compared with young mice (4.7 ± 0.92) (F1,9 = 7.1, p = 0.0262; one-way ANOVA). These results are consistent with previous reports using rats demonstrating an age-related increase in the sAHP.
less (F1,9 = 6.7, p = 0.0285; one-way ANOVA) than the mean input resistance measure in young mice (59.5 ± 0.7 MΩ; mean ± SD). It is interesting to note that most studies using rabbits17,19 and rats9 have not reported a difference in input resistance; however, a recent report found that aged animals that are impaired in the Morris water maze exhibited an increase in input resistance.22

In addition to measuring the sAHP, we examined the amount of LTP that was sustained in the absence of calcium contributed through the NMDA receptor channel complex. For these experiments, we used an induction protocol that was sufficient to ensure action potentials in the soma (see “Methods”) thereby activating L-type calcium channels. Induction of LTP was examined in hippocampal slices from young and aged animals either in normal aCSF or in aCSF that contained 50 μmol/L APV. Representative fEPSPs are presented in Figure 2A for each of the four conditions before and after (black arrows) induction of LTP. In normal aCSF, the repeated 200-Hz stimulation resulted in substantial LTP in slices from young (209% ± 28.9%; mean ± SD; N = 13) and aged animals (231.5% ± 40.1%; mean ± SD; N = 11), which was significantly different from the pretetanus baseline (t12 = 13.3, p < 0.0001 and t10 = 9.6, p < 0.0001, respectively) as determined by a single group t-test with a hypothesized mean of 100. There was no significant difference in the amount of LTP recorded in young and aged slices during the last 10 minutes of the experiment (F1,22 = 0.002, p = 0.9633; one-way ANOVA). When the experiments were carried out identical as before, but in the presence of the NMDA receptor antagonist APV, the 200-Hz stimulation produced significant LTP in slices from young (124.6% ± 13.4%; mean ± SD; N = 11) and aged animals (151.9% ± 36.1%; mean ± SD; N = 13), which was significantly different from the pretetanus baseline (t10 = 6.1, p = 0.0001 and t12 = 5.2, p = 0.0002, respectively) as determined by a single-group t-test with a hypothesized mean of 100. However, in the presence of APV, the 200-Hz stimulation resulted in significantly more LTP in aged slices when compared with slices prepared from young animals during the last 10 minutes of the experiment (F1,22 = 5.6, p = 0.0273; one-way ANOVA). These results are, to our knowledge, the first verification in mice of an age-related increase in NMDA receptor independent LTP and are consistent with previous experiments using rats.33 These results, combined with our sAHP experiments described here, are suggestive of an activity-dependent calcium influx by L-type channels that is enhanced in aged mice.
Regulation of Activity-Dependent Calcium Influx by Modulation of CaV1.2

Because phosphorylation of CaV1.2 by cAMP-dependent protein kinase (PKA) strongly enhances its activity, and CaV1.2 is the prevailing L-type Ca^2+ channel in the forebrain, we hypothesized that an increase in CaV1.2 phosphorylation may account for a substantial portion of the age-related rise in activity-dependent neuronal Ca^2+ influx. To examine this possibility, we quantified the amount of phosphorylation of CaV1.2 using an antibody that specifically binds to CaV1.2 when phosphorylated at serine 1928.

FIGURE 2. Voltage-Dependent Calcium Channel Long-Term Potentiation Is Enhanced in Hippocampal Slices Prepared From Aged Animals

(A) Representative extracellular recordings from the CA1 region of the hippocampus from young (left) and aged (right) mice in the presence (bottom) or absence (top) of the NMDA receptor antagonist amino-5-phosphonopentanoate (APV) (50 μmol/L). The traces are superimposed for each group with representative traces before and after (marked by arrows) induction of LTP. (B) Average slope of the field excitatory postsynaptic potential (fEPSP) is plotted for each of the four groups for the duration of the experiment. Slices from aged mice exhibited significantly more long-term potentiation in the presence of APV when compared with slices taken from young animals. Data are represented as the mean ± standard error of mean.
the main phosphorylation site of PKA \(^{36,37}\) and PKC.\(^ {40}\) Figure 3A presents a representative blot with samples from three different young and aged animals. Immunosignals for anti-CH1923-1932P (upper panel labeled CH3P) and anti-CNC1 (which recognizes the loop between domains II and III of Ca\(_v\)1.2; labeled FP1 in the lower panel) were quantified by densitometry and the signals for anti-CH1923-1932P corrected for differences in relative amounts of Ca\(_v\)1.2 based on the CNC1 signals. FP1 recognizes the full-length Ca\(_v\)1.2 subunit as well as a shorter isoform that is created by calpain-mediated proteolytic processing. This processing can be induced in the hippocampus by Ca\(^{2+}\) influx through NMDA-R.\(^ {41}\) The group data are presented in Figure 3B as the average ratio of phosphorylation signal and FP1 signal of the upper of the two bands protein for young (\(N = 12\)) and aged (\(N = 13\)) mice. Surprisingly, we found no substantial difference in the relative phosphorylation of Ca\(_v\)1.2 (\(F_{1,23} = 0.32, p = 0.5766\); one-way ANOVA), suggesting that in mice, unlike rats,\(^ {42}\) the enhanced calcium influx is not the result of an increase in the phosphorylation of this specific calcium channel, and therefore alternate sources for the enhanced calcium must be considered. Truncation of the C-terminus of Ca\(_v\)1.2 can also lead to an increase in activation,\(^ {43}\) and calpain has been implicated in the cognitive impairments associated with Alzheimer disease.\(^ {44,45}\) However, we did not detect any change in the ratio of long to short forms of Ca\(_v\)1.2 (\(F_{1,23} = 0.32, p = 0.5766\); one-way ANOVA), nor did we see any age-related increase in the short (\(F_{1,23} = 0.052, p = 0.8213\); one-way ANOVA) or long (\(F_{1,23} = 1.3, p = 0.2577\); one-way ANOVA) form (Figure 3C).

**DISCUSSION**

Although there are numerous reports regarding age-related increases in the sAHP in rats (for a recent review, see \(^ {46}\)), much less is known about this phenomenon in mice.\(^ {31}\) Consistent with the rat literature, we find that there is an age-related increase in the sAHP. These data, together with our observation of an enhanced vdccLTP in aged mice, strongly suggest that there is an increase in activity-dependent cytosolic calcium concentration in neurons within the

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**FIGURE 3. Aging Is Not Associated With an Increase in Phosphorylation of Ca\(_v\)1.2 at Serine 1928 in C5\(^{7}\)BL/6 Mice**

(A) Representative immunostaining for phosphorylated serine 1928 (upper lanes; CH3P) and for relative channel protein levels of the \(\alpha_1\) subunit of Ca\(_v\)1.2 (bottom lanes; FP1) for hippocampal samples obtained from three young and three aged animals. (B) Immunosignals obtained by immunoblotting with CH3P and FP1 were quantified by densitometry. Signals for phosphorylated serine 1928 were corrected for differences in relative amounts of Ca\(_v\)1.2 based on the corresponding FP1 signals from the long form of Ca\(_v\)1.2 (the lower bands constitute a C-terminally cleaved form that is lacking serine 1928). Relative phosphorylation (ratio of CH3P:FP1) is plotted for samples taken from young and aged animals. (C) Densitometry of immunosignals from immunoblotting with FP1 for the long form, short form, as well as the ratio of long to short forms. There was no significant difference between protein samples taken from young and aged animals. Data in (B) and (C) are represented as the mean ± standard error of mean and are normalized to the values obtained from the young adult mice.
CA1 region of the hippocampus in mice. We should note here that in the companion paper,47 we examined the water maze performance in the same mice in which we examined the vdccLTP described previously. We failed to find any significant correlations between behavior and levels of vdccLTP (data not shown). This lack of correlation might be in large part the result of the extremely poor performance in the water maze that we observed in these animals (Figure 1B in the companion paper47). In light of our finding that C57BL/6 mice exhibit a less severe age-related phenotype in the win-shift version of the Olton 8-arm radial maze (Figure 2), it seems likely that this hippocampal-dependent learning and memory task may be better suited for these types of correlational studies in the future.

What is the source of the apparent increase in cytosolic calcium that was revealed in our neurophysiological experiments? One likely source is L-type voltage-dependent calcium channels. Previously, an age-related upregulation of PKA-mediated phosphorylation, which is thought to bring the channel from the functionally silent state to an active state thereby increasing channel availability, has been reported in Lobund-Wistar rats.42 This does not appear to be the case in C57BL/6 mice. When we examined the phosphorylation of CaV1.2 at serine 1928, we did not find any significant difference between young and aged animals (Figure 3). Therefore, it seems that in this regard, mice and rats are different. It is possible that in mice, the age-related increase in calcium channel availability is mediated by phosphorylation of another L-type calcium channel isoform (i.e., CaV1.3), which is also phosphorylated by PKA.48 It is also possible that the increase in calcium channel availability is the result of an age-related increase in L-type calcium channel expression as has been suggested by experiments using Fischer-344 rats.9,49,50 However, in light of the data presented here, it seems unlikely that the age-related increase in intracellular calcium is the result of an increase in CaV1.2 because we did not find an age-related increase in either the long or short form of CaV1.2 (Figure 3C). Similarly, it seems unlikely that the age-related increase in intracellular calcium is the result of a calpain-mediated increase in CaV1.2 activity through C-terminal cleavage given that there was no change in the ratio of the long and short forms of CaV1.2 (Figure 3C). One final alternate explanation is that there may be a deficit in mitochondrial calcium buffering as it has been reported in basal forebrain neurons from aged rats.51

Much of what we currently know regarding the neurobiologic substrates of age-related cognitive decline at the cellular and molecular level comes from rodent studies. Although the use of mouse models to study normal aging is in its infancy, transgenic mice have the potential to shed light on several of the central issues regarding calcium deregulation and age-related cognitive decline. Because many of these questions are currently centered on ion channel function, and many of these channel proteins (e.g., CaV1.3 and CaV1.2) have been cloned and sequenced, transgenic mouse models could be generated to examine these questions directly. Thus, transgenic mice will not only help to elucidate the mechanisms underlying neuronal aging and age-related cognitive decline, but they may also help identify potential cellular and molecular targets for therapeutic intervention.

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