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Differential effects and rates of normal aging in cerebellum and hippocampus

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Cognitive functions show many alternative outcomes and great individual variation during normal aging. We examined learning over the adult life span in CBA mice, along with morphological and electrophysiological substrates. Our aim was to compare cerebellum-dependent delay eyeblink classical conditioning and hippocampus-dependent contextual fear conditioning in the same animals using the same conditioned and unconditioned stimuli for eyeblink and fear conditioning. In a subset of the behaviorally tested mice, we used unbiased stereology to estimate the total number of Purkinje neurons in cerebellar cortex and pyramidal neurons in the hippocampus. Several forms of synaptic plasticity were assessed at different ages in CBA mice: long-term depression (LTD) in both cerebellum and hippocampus and NMDA-mediated long-term potentiation (LTP) and voltage-dependent calcium channel LTP in hippocampus. Forty-four CBA mice tested at one of five ages (4, 8, 12, 18, or 24 months) demonstrated statistically significant age differences in cerebellum-dependent delay eyeblink conditioning, with 24-month mice showing impairment in comparison with younger mice. These same CBA mice showed no significant differences in contextual or cued fear conditioning. Stereology indicated significant loss of Purkinje neurons in the 18- and 24-month groups, whereas pyramidal neuron numbers were stable across age. Slice electrophysiology recorded from an additional 48 CBA mice indicated significant deficits in LTD appearing in cerebellum between 4 and 8 months, whereas 4- to 12-month mice demonstrated similar hippocampal LTD and LTP values. Our results demonstrate that processes of aging impact brain structures and associated behaviors differentially, with cerebellum showing earlier senescence than hippocampus.

aging | cerebellum | hippocampus | behavior | synaptic plasticity

Processes of normal aging do not affect the CNS uniformly. There is stability in neuron number in most brain regions, including most regions of the hippocampus (reviewed in refs. 1 and 2), whereas significant loss of Purkinje neurons occurs in the cerebellum (3, 4). Stereological assessments of hippocampal pyramidal and granule neurons and cerebellar granule and Purkinje neurons in the same mice aged 12 or 28 months revealed stability in hippocampal neurons and cerebellar granule neurons and significant loss of Purkinje neurons (5). Learning and memory show many alternative outcomes and great individual variation during normal aging. Cerebellum-dependent learning is associated with Purkinje neuron number and is impaired by age-related decrements in morphology and function. Hippocampus-dependent learning is associated with reduced capacity for new learning in pyramidal neurons in the perforant pathway in normal aging (6). Data over the adult life span in human (7) and nonhuman mammals (8) suggest that cerebellum-essential tasks show age-related deficits at earlier ages than do hippocampus-essential tasks.

Traditionally, cerebellar and hippocampal substrates of learning, memory, and aging have been studied independently. We investigated structural and functional changes and associated mechanisms in the cerebellum and hippocampus over the adult life span in CBA mice—a mouse strain that retains auditory capacity into late life in contrast to C57BL/6 mice that are severely impaired in auditory acuity by 18 months (*SI Supplement 2*). Our aim was to compare cerebellum- and hippocampus-dependent learning and stereological counts of neuron numbers. We also compared neural/synaptic plasticity at three ages in genetically identical animals of the same strain in electrophysiological recordings from slices to determine long-term depression (LTD) in cerebellar cortex and LTD and long-term potentiation (LTP) in hippocampus.

For a cerebellum-dependent form of learning we used delay eyeblink classical conditioning, and for hippocampus-dependent learning we used contextual fear conditioning. The neural circuitry is well delineated for both model systems, and the behavioral and neurobiological parallels in these two forms of associative learning extend to all mammals that have been studied, including humans. To make classical eyeblink and fear conditioning as similar as possible, we used identical physical stimuli for the two paradigms: the conditioned stimulus (CS) was a 1-kHz, 85-dB tone, and the unconditioned stimulus (US) was stimulation to the eye (orbicularis oculi) muscles.

For both eyeblink and fear conditioning, we used the delay procedure with the tone CS presented before the onset of the US stimulation to the eye muscles. Mice learned to respond to the CS before the onset of the US. The learned response is called the conditioned response (CR). In the case of eyeblink conditioning that engages central nervous system brain circuitry and a somatic muscle response (an eyeblink), the CS-US interval is shorter (500 ms). In the case of fear conditioning that engages central nervous system brain circuitry and an autonomic system response (freezing), the CS-US interval is longer (1,000 ms). We hypothesized that over the life span of CBA mice there would be an age-related deficit in eyeblink conditioning that would appear in middle age, whereas fear conditioning would show little or no age-related deficit. On the basis of previous research, we also predicted that the eyeblink conditioning deficit would be associated with Purkinje neuron loss (9) and paralleling the stability in fear conditioning to late life would be stability in hippocampal pyramidal neuron number (10). Previous work with the C57BL/6 mouse strain demonstrated stability in delay eyeblink conditioning and Purkinje neuron number between the ages of 4 and 12 months (9). These results led us to predict that eyeblink and fear conditioning behavior, neuron number, and slice electrophysiology in hippocampus and cerebellum would be stable between 4 and 12 months in the CBA mouse strain tested in these studies.

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The authors declare no conflict of interest.

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Results

Cerebellum-Dependent Versus Hippocampus-Dependent Learning. Eyeblink and fear classical conditioning stimuli were physically comparable with the primary differences being (*a*) the duration of the CS and (*b*) the intensity of the US. Control assessments demonstrated that robust fear conditioning could be carried out with eye muscle shock US electrodes that were used for eyeblink conditioning (*SI Supplement 1*, Figs S1 and S2). Assessments were also made using the acoustic startle response on age differences in startle magnitude to auditory stimuli of the intensity used in eyeblink and fear conditioning to document that CBA mice at all ages could hear and respond to the tone CS (*SI Supplement 2*, Fig S3).

Delay eyeblink conditioning. Cerebellum-dependent 500-ms delay eyeblink conditioning was compared over the five age groups in a 5- (age group) by-10 (training sessions) repeated measures ANOVA with percentage of CRs as the dependent measure. There were statistically significant effects of age group [$F(4, 39) = 2.86$, $P = 0.036$] and training sessions [$F(7, 273) = 23.14$, $P < 0.001$]. The interaction between age group and training sessions attained significance at the 0.10 but not the 0.05 level [$F(28, 273) = 1.47$, $P = 0.064$] (Fig. 1*A*). Post hoc comparisons of the statistically significant age effect using the Tukey honestly significant difference (HSD) test indicated that percentages of CRs in 24-month mice were significantly lower than in 4-month mice (Fig. 1*B*). Analyses of unconditioned response (UR) amplitudes were conducted on the first 10 trials of session 1 as a control for age differences in performance effects. Later trials were not analyzed for UR amplitude due to the possibility of contamination of UR topography via CR-UR summation effects. There were no age differences in UR amplitude.

Delay fear conditioning. Cued and contextual fear conditioning was compared in 38 of the same 44 CBA mice in five age groups in a 5- (age group) by-15 (training session) repeated measures ANOVA on percentage of time freezing. The effect of training sessions was significant, $F(14, 462) = 11.71$, indicating significantly greater freezing to the CS after shock in the initial session, greater freezing to context, and greater freezing to the CS in a new context (Fig. 2). Neither the effect of age group nor the interaction effect was significant, $F(4, 33) = 2.62$, $P = 0.073$ and $F(56, 462) = 0.964$, $P = 0.551$, respectively. Neither cued nor contextual fear conditioning was different across age groups. Hippocampus-dependent contextual fear conditioning was also compared among the five age groups of CBA mice in a one-way ANOVA with percentage of freezing responses over the 5-min contextual conditioning period as the dependent measure. The effect of age was not significant.

Stereological Neuron Counts. Unbiased stereology was used to estimate the total number of Purkinje neurons in cerebellum and pyramidal neurons in hippocampus in a subset of mice in each of the five age groups tested in classical eyeblink and fear conditioning.

Cerebellum: age differences in Purkinje neurons. There were six mice in each of the five age groups. A one-way ANOVA was used to compare unbiased estimates of total Purkinje neuron number in 30 cerebella of CBA mice spanning the age range of 4–24 months. There was a statistically significant effect of age, $F(4, 29) = 6.14$, $P < 0.001$. Post hoc comparisons of the significant age effect using the Tukey HSD test indicated significantly fewer Purkinje neurons in the age groups of 18 and 24 months in comparison with the age groups of 4, 8, and 12 months (Fig. 3*A*).

Hippocampus: age differences in pyramidal neuron number. The total number of pyramidal neurons in the hippocampus for two mice in each age group was estimated. A one-way ANOVA comparing number of neurons counted by age group was not significant, $F(4, 9) = 0.629$, $P > 0.05$. Pyramidal neuron number remained stable over the adult life span in CBA mice (Fig. 3*B*).

Age Differences in Long-Term Potentiation and Long-Term Depression in CBA Mice. Electrophysiological examination of the effects of age on synaptic plasticity and memory in cerebellum and hippocampus was carried out using slice preparations from CBA mice.

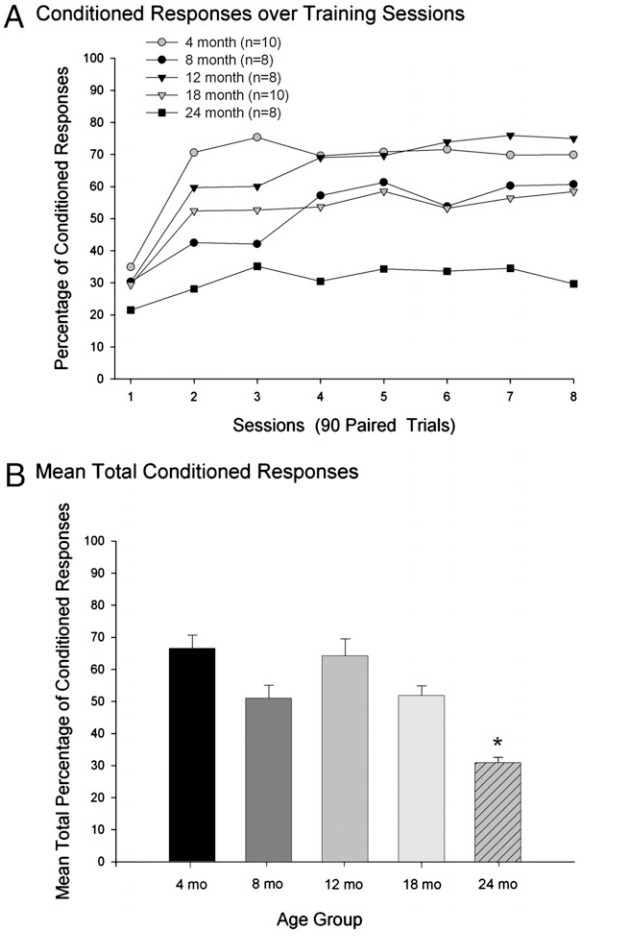

Fig. 1. Eyeblink classical conditioning at five ages in CBA mice. (*A*) Percentage of conditioned responses (CRs) over 10 sessions of 90 paired trials in 8–10 male CBA mice per age group. The 24-month group was significantly impaired in the acquisition of CRs. (*B*) Mean total percentage of CRs in the five age groups of CBA mice in *A* above. The 24-month group produced significantly fewer CRs. An asterisk indicates a significant difference (*$P < 0.05$). Error bars are SE of the mean.

Cerebellum. Cerebellar LTD was measured as slope of the intracellularly recorded excitatory postsynaptic potentials (EPSPs) of Purkinje neurons in the cerebellar cortex (vermis) of slices taken from CBA male mice at 4, 8, and 12 months of age. LTD in the 4-month age group was quite robust and much greater than that observed in the 8- and 12-month groups (Fig. 4). Twenty minutes following stimulation to induce cerebellar LTD, paired-samples *t* tests showed statistically significant changes in cerebellar LTD in 4-month mice compared with 8-month mice, $t(20) = 2.09$, $P < 0.0001$, and in 4-month mice compared with 12-month mice, $t(17) = 2.11$, $P < 0.0001$. There were no significant differences in LTD induction between the 8- and 12-month mice, $t(12) = 2.18$, $P = 0.74$. Interestingly, in terms of basal synaptic transmission, although the EPSP amplitudes were not different for cells in the three age groups (see *Methods*), the EPSP rise time (EPSP slope) for the cells from the 4-month mice was significantly faster than for the cells from the 8- and 12-month mice, the latter two groups not differing from each other [age groups $F_{(2,100)} = 14.4$, $P < 0.001$; post hoc Tukey tests, 4 vs. 8 months, $P < 0.001$, 4 vs. 12 months, $P < 0.001$, and 8 vs. 12 months (not significant), $P > 0.05$].

Hippocampus. Comparable hippocampal LTD and LTP were found between 12-month C57BL/6 and CBA mice (*SI Supple-*

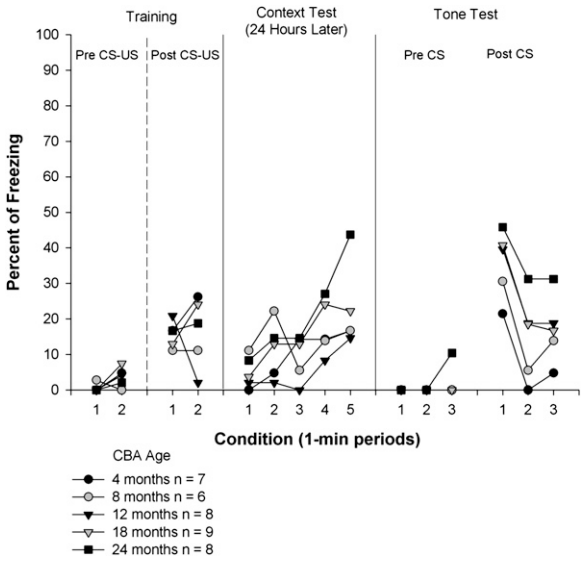

Fig. 2. Fear conditioning at five ages in CBA mice. Performance was assessed by percentage of time freezing in 1-min time intervals in cued and contextual fear conditioning. There were 8–10 male CBA/J mice tested in three sessions. Session 1 consisted of a 2-min pre-conditioned stimulus (pre-CS) baseline period and a 2-min period after pairing of the CS and unconditioned stimulus (US). Twenty-four hours later, the context test was carried out in session 2. Mice were placed for 5 min in the same chamber where they experienced the CS-US pairing. Session 3 occurred 1 h later in a different chamber distinguished by novel auditory, olfactory, tactile, and visual cues. There was a 3-min pre-CS period of no stimulation and a 3-min post-CS period after the tone CS sounded. There were no age differences in freezing.

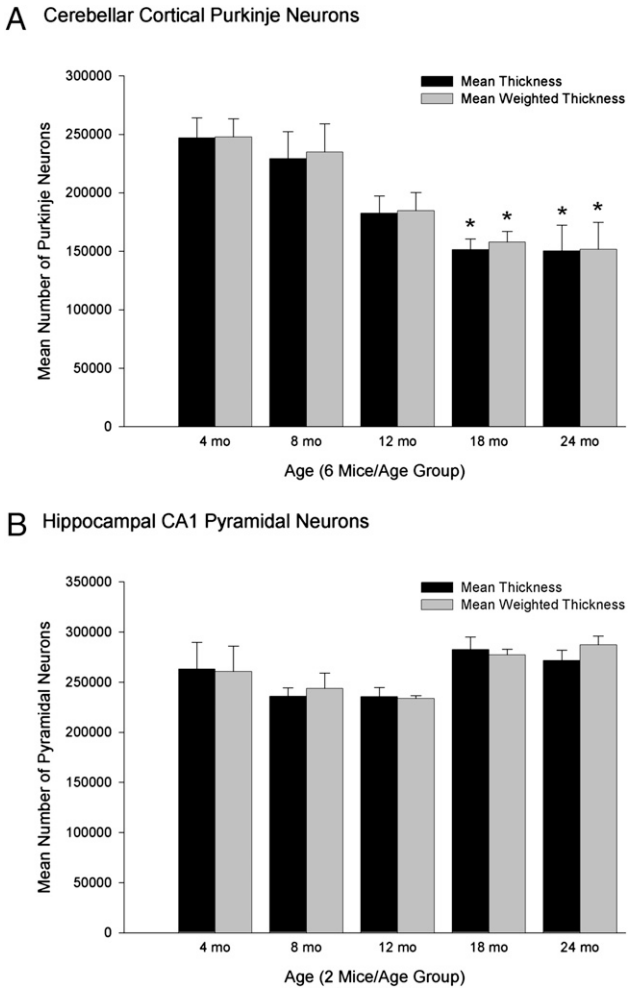

Fig. 3. Stereological neuron counts in cerebellum (*A*) and hippocampus (*B*). (*A*) Mean estimated number of Purkinje neurons in the entire cerebellar cortex of CBA mice aged 4, 8, 12, 18, and 24 months. Stereological counts were carried out on six mouse cerebella per age group. (*B*) Mean estimated number of pyramidal neurons in the hippocampus of CBA mice aged 4, 8, 12, 18 and 24 months. Stereological counts were made on two mouse hippocampi per age group. Bars represent SE of the mean.

ment 3, Fig S4). Our main aim was to examine hippocampal LTD at two different age points (4 and 12 months) in CBA mice, using the standard paradigm of 1 Hz for 15 min (900 pulses). Hippocampal LTP was also examined in the two different age groups across three different stimulation protocols for inducing LTP: 25, 100, and 200 Hz. Twenty minutes following hippocampal LTD-inducing stimulation, there was a significant induction of LTD from baseline recordings across both age groups ($P < 0.0001$). When 4- and 12-month age groups were compared, there were no significant differences (Fig. 5). Thirty minutes following all three LTP-inducing stimulation protocols, LTP was significantly induced from baseline recordings in both 4- and 12-month age groups ($P < 0.0001$), but again, when extracellular field fEPSP waveforms from 4- and 12-month groups were compared following the specific LTP-inducing stimulation, no group differences were detected (Fig. 5). Following low-frequency stimulation, hippocampal LTD appeared to be quite robust in both the 4- and 12-month CBA age groups. Furthermore, both the 4- and 12-month CBA mouse groups were capable of demonstrating strong levels of LTP following each of the stimulation protocols that were tested, with the 25-Hz protocol showing the greatest effect (presumed NMDA-dependent activation).

Discussion

In this study we examined age-related differences in cerebellum-dependent delay eyeblink classical conditioning and hippocampus-dependent contextual fear conditioning in CBA mice tested at one of five ages: 4, 8, 12, 18, or 24 months. There were statistically significant age differences in cerebellum-dependent delay eyeblink conditioning, with 24-month mice showing impairment in comparison with younger mice. These same mice showed stability over the five age groups in hippocampus-dependent contextual fear conditioning and also in cued fear conditioning. Cerebellum-dependent eyeblink conditioning was impaired in older mice, whereas hippocampus-dependent contextual fear conditioning was stable.

Stereological counts of Purkinje neurons in cerebellar cortex and pyramidal neurons in hippocampus in a subset of the mice that had been tested behaviorally indicated significant loss of Purkinje neurons in 18- and 24-month mice and stability in the same mice in pyramidal neuron number. These results with age differences in Purkinje and hippocampal pyramidal neurons measured along with behavior in the same mice replicate previous studies in which age differences in these variables have been assessed independently, such as delay eyeblink classical conditioning (8, 9), contextual fear conditioning (11), Purkinje neurons (9), and hippocampal pyramidal neurons (12). We also evaluated age differences in electrophysiological responses in cerebellar and hippocampal slices in CBA mice. Surprisingly, results with cerebellar LTD indicate that neural plasticity is more robust in 4-month mice and showed deficits as early as 8 months of age. We had not predicted this early decline in cerebellar plasticity on the basis of our previous work with behavior and morphology. Hippocampal LTP and LTD were stable between 4 and 12 months.

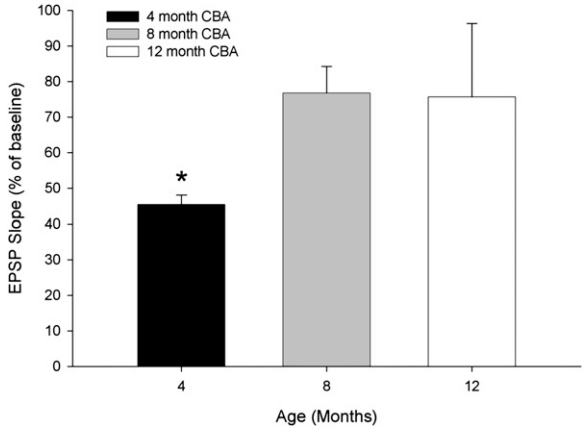

Fig. 4. Cerebellar LTD recorded from 4-, 8-, and 12-month CBA mouse slices. A histogram is shown of EPSP slope from 4-, 8-, and 12-month CBA male mouse slices following low-frequency stimulation designed to induce cerebellar LTD. The increased amount of LTD in the 4-month group is statistically significant ($P < 0.0001$).

Classical Conditioning. CBA mice become blind early in development, but vision is not essential for auditory cued eyeblink or cued or contextual fear conditioning. Vision is less of a primary sense in normal intact mice that rely more on olfactory, tactile, and auditory senses. In mice with vision impaired early in life, dependence on other senses is even greater. The effects of vision on fear conditioning in mice were examined by comparing C3H mice with retinal degeneration to C3.BLiA-+Pde6b congenics without retinal degeneration (13). Results indicated that vision played a minimal role in responsiveness in fear conditioning. In the present study, cues differentiating the boxes used for cued and contextual fear tests were olfactory, tactile, and auditory. CBA mice had several more salient contextual cues apart from visual cues.

Behavioral testing was carried out 5 days per week and was completed for eyeblink and fear conditioning in 10 days. The order of behavioral testing was consistently eight daily sessions of eyeblink conditioning using a 100-ms 0.3-mA shock US to eye muscles followed by two daily sessions of fear conditioning with a 2-s 1.5-mA shock US to eye muscles. We considered the possibility that the eight eyeblink conditioning sessions involving a total of 720 presentations of the mild shock US might have habituated mice to shock to eye muscles. However, the shock US in fear conditioning was 20 times the duration and 5 times the magnitude of the shock used in eyeblink conditioning. This magnitude of shock US to the eye muscles evoked significantly greater freezing than foot shock (*SI Supplement 1*). Previous fear conditioning studies of normal aging have found stability in cued and contextual fear conditioning 24 h after initial training (11, 14). The low intensity shock US used for eyeblink conditioning did not alter the pattern of results reported in previous studies for age differences in fear conditioning.

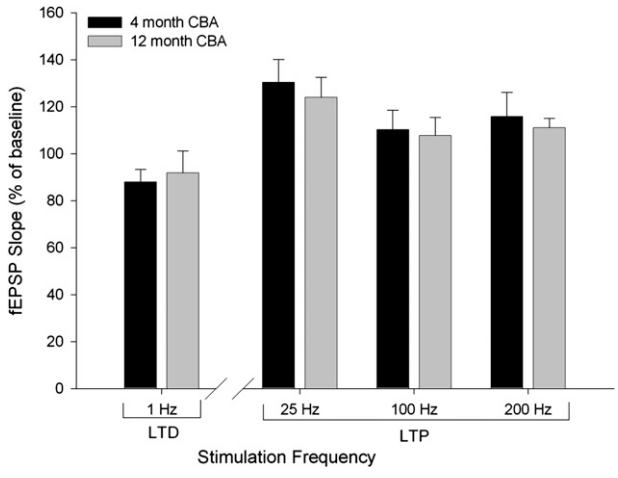

Fig. 5. Varying stimulation frequencies (25, 100, and 200 Hz) used to induce hippocampal LTP (and LTD) in 4- and 12-month male CBA mouse slices. A histogram is shown of fEPSP slope values (percentage of baseline) from 4- and 12-month CBA male mouse slices following low-frequency stimulation designed to induce hippocampal long-term depression (LTD) and long-term potentiation (LTP). Although there were appreciable amounts of both hippocampal LTD and hippocampal LTP (compared to baseline) across all stimulation frequencies tested, there were no significant differences found between the 4- and 12-month age groups in any of the stimulation conditions that were tested.

Stereological Neuron Counts. Neuron counts of Purkinje neurons in C57BL/6 mice in Mariani's laboratory used conventional profile-based or 2D counting methods (15–18) and stability was observed to the age of 18 months with decline by 24 months. Our results using design-based stereological methods, more age groups, and more mice per group suggested that age-related loss of Purkinje neurons in CBA mice occurs earlier in the life span, between 12 and 18 months. We reported a similar result for C57BL/6 mice (9).

Stability of pyramidal neuron number in hippocampus was one of the initial demonstrations of unbiased stereology that reversed long-held assumptions about neuron loss in normal aging. Loss of hippocampal neurons and synapses had previously been considered a hallmark of normal aging. This neuron loss was thought to be a substrate of age-related learning and memory deficits. Stereological studies in humans were the first to demonstrate that only a relatively minor neuron loss occurs with aging and that this loss is restricted to specific brain regions, including hippocampal subregions (19). Similar results were reported for rats, even for aged rats that demonstrated the poorest performance in a water maze task (20). The absence of age-related changes in pyramidal neurons in the CA fields and granule neurons in dentate gyrus were extended C57BL/6J mice, one of the most commonly used laboratory mouse strains. No age-related decline was observed in number of CA1 pyramidal neurons or dentate gyrus granule neurons (12). Results of stereological hippocampal pyramidal neuron counts in CBA mice aged 4–24 months extend this result to another mouse strain.

Electrophysiology. In this study, we examined processes of LTD in cerebellar cortical slices from 4–8- and 12-month CBA mice, and LTD and LTP in hippocampal slices from 4- and 12-month CBA mice. Cerebellar LTD is much greater in the 4-month group than the 8- and 12-month groups, indicating that the older groups exhibit less synaptic plasticity. Indeed, there was no difference between the two older groups as to their levels of LTD. However, it is important to note that there still was an appreciable amount of LTD in both the older groups; it just was not as strong as that of the youngest group tested. Although the LTD data are expressed as percentage of baseline EPSP slope, the fact that the basal rise time for cells from the 4-month mice was faster may have contributed to the greater LTD seen for this age group. It is interesting that the age-related impairment in cerebellar LTD develops between 4 and 8 months in CBA mice, but the impairment in eyeblink conditioning does not appear until somewhere between 18 and 24 months. The neural deficit appears earlier than the behavioral deficit, a not uncommon circumstance, as, for example, in Parkinson's disease (21). Similarly, hippocampal LTP was found in both age groups and stimulation protocols tested, with the greatest amount occurring following the 25-Hz protocol (NMDA-dependent LTP). Collectively, these results suggest that changes in

synaptic plasticity and learning associated with aging appear to occur earlier in the cerebellum compared with the hippocampus.

Cognitive functions show many alternative outcomes and great individual variation during normal aging. Brain memory systems and associated brain structures differ in the magnitude of age-related decline. Aging in hippocampus-dependent learning and memory is associated with reduced functional capacity of pyramidal neurons in the CA fields, but neuron number is stable. Cerebellum-dependent learning and memory are associated with Purkinje neuron loss and age-related impairment in morphology as well as function. We had demonstrated in human aging that in the same individuals hippocampus-dependent measures such as word-list retrieval showed smaller deficits in comparison with young adults than did cerebellum-dependent delay eyeblink conditioning (7). In the present study within the 2-year period that covers most of the mouse life span, we used measures with parallels to human aging and demonstrated some of the cellular and electrophysiological mechanisms underlying normal aging in hippocampal and cerebellar substrates of learning and memory.

Methods

Behavior. ***Animals.*** Fifty-one CBA mice were tested at the age of 4, 8, 12, 18, or 24 months; 44 mice completed all assessments. Mice were purchased from the NIA colony at Jackson Laboratory and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facility. Mice had ad libitum access to food and water and were housed in groups of four in polycarbonate microisolator filtered-top cages in a temperature- and humidity-controlled room that was ventilated using a dedicated system. Room lights were timed on a 12/12-h light/dark schedule. Temple University's Institutional Animal Care and Use Committee (IACUC) reviewed and approved all procedures.

Eyeblink classical conditioning. Standard surgery and eyeblink conditioning techniques were published previously (9, 22), and electrode implantation is described in *SI Supplement 1*. Briefly, mice were anesthetized and four Teflon-coated stainless steel wires were implanted in the orbicularis oculi muscles of the left eyelid. The two wires most rostral recorded differential electromyography (EMG), and the two most caudal delivered the shock US. A recovery period of 4–5 days occurred before behavioral testing began.

Each paired classical conditioning trial consisted of a 600-ms 1-kHz 85-dB SPL tone CS that coterminated with a 100-ms 0.3-mA DC pulse US generated by an IsostimStimulator/Isolator. The intertrial interval was 25 ± 5 s. A total of 100 delay eyeblink classical conditioning trials were presented per session. Each of the 10 blocks of trials consisted of 9 paired CS-US trials and 1 CS-only test trial. Analyses are reported for the 90 paired trials per session for eight daily sessions.

Sessions were computer scored and also visually checked to confirm the accuracy of the automated scoring procedure. Whenever the EMG recorded from the orbicularis oculi exceeded 5 standard deviations above a baseline established in the 249-ms Pre-CS period, a response was considered to have occurred. A CR was scored if a response occurred after the 60-ms startle period and before the US onset (between 61 and 499 ms after CS onset). Percentage of CRs in the paired condition is the dependent measure. Data were analyzed with SPSS (version 16.0) software.

Classical fear conditioning. Detailed methods for fear conditioning using an orbicularis oculi stimulation US are presented in *SI Supplement 1*. Briefly, for session 1 involving cued fear, the mouse explored the box for 2 min, and an 85-dB, 1-kHz, 30-s tone CS sounded and coterminated with a 2-s 1.5-mA shock US to the orbicularis oculi muscles. After the shock terminated, freezing rate was scored at 10-s intervals for 2 min, with a subsequent pairing of CS and US. Session 2, testing for contextual fear, occurred 24 h later. The mouse was placed into the same box used in session 1 and freezing was scored for 5 min. Session 3, testing for cued fear, lasted for 6 min and began one h after session 2 in a different room in a box that had different tactile, auditory, and olfactory cues. The first 3 min were for exploration of the novel box. At the start of the second 3-min period, the tone CS sounded for 30 s.

Histology and stereology. Mice were deeply anesthetized with isoflurane and perfused through the heart with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. One day before sectioning, each brain was refrigerated and stored in distilled water. Sections were cooled to 1 °C and cut in the coronal plane through with a Vibratome 3000 Sectioning System at a thickness of 70 μm. Sections were mounted on glass slides and stained with thionin. Investigators carrying out the neuron counts were blind to mouse age-group membership.

Cerebellum. Stereological counts of Purkinje neurons for six brains in each of five age groups were carried out. Methods for cerebellar Purkinje neuron counts were presented previously (9). An optical fractionator stereological design (23) was used to make unbiased estimates of total Purkinje neuron number (Stereo Investigator software, Version 5.05.4 ©2002, MicroBrightField). The PC-controlled motor system on the microscope stage was composed of a 3-inch × 2-inch *XY* stepping stage (0.1 μm resolution, 6 μm accuracy), a *z*-axis motor (0.1-μm resolution), a joystick, a slide holder, and a 3-axis controller. A three-dimensional optical dissector counting probe (*X*, *Y*, and *Z* dimensions of 149 × 109 × 10 μm, respectively) was applied to a systematic random sample of sites in the cerebellar cortex over the entire cerebellum. A 60× oil immersion objective (numerical aperture = 1.4) was used for the neuron counts. The mean section thickness for these 30 cerebella was 27.98 μm (SD = 2.36), and there was not a significant age difference in section thickness. A border guard of 5 μm was set at the top and the bottom of the section, and each Purkinje neuron with a visible nucleus was counted as the experimenter focused down through 10 μm of tissue in each 149 × 109-μm counting frame. The total number of Purkinje neurons in the entire cerebellum for each mouse was estimated when the number of Purkinje neurons counted in the known fraction of the cerebellar cortex was multiplied by the inverse of the sampling fraction.

Hippocampus. Stereological counts of pyramidal neurons in hippocampus for two brains in each of five age groups were carried out. Sectioning began at the posterior portion of the brain and continued until the hippocampus was not visible for five consecutive slices. Each mouse yielded between 28 and 57 sections. Tracing began when the dentate gyrus first appeared in the posterior sections and ended with the disappearance of the dentate gyrus and CA fields as the lateral ventricles became more prominent. Each tracing included the hippocampus in one hemisphere.

An optical fractionator stereological design was used to make unbiased estimates of total pyramidal neuron number, using the same system as described for Purkinje neuron counts. A three-dimensional optical dissector counting probe (*x*, *y*, and *z* dimensions of 35 × 35 × 10 μm, respectively) was applied to a systematic random sample of sites over the entire hippocampus. Every third section was probed. For each hippocampus there were ≈10 sections outlined using the 4× objective. The outline of the section generated a path to select sampling sites using the designated step lengths (300 μm in the *x* direction and 200 μm in the *y* direction). This path completely covered the full extent of the outlined space. The mean number of sampling sites was 206.6 ± 44.30 SD for an entire hippocampus, and the range was 158–282. There were no significant age differences in the number of sampling sites. A 60× oil immersion objective was used for the neuron counts. The mean section thickness for all brains was 47.12 ± 11.35 (SD) μm, and there was not a significant age difference in section thickness. A border guard of 5 μm was set at the top and the bottom of the section, and each pyramidal neuron with a visible nucleus was counted as the experimenter focused down through 10 μm of tissue in each 35 × 35-μm counting frame.

Slice Electrophysiology. ***Animals.*** A total of 48 CBA mice were tested in one of the age groups of 4, 8, and 12 months. Mice were purchased from the NIA colony at Jackson Laboratory and maintained in an AAALAC-approved animal facility. The housing room was temperature and humidity controlled and ventilated using a dedicated system. Room lighting was timed for a 12/12-h light/dark schedule. Mice were housed in groups of 4 in polycarbonate microisolator filtered-top cages. All mice had ad libitum access to food and water. The University of Southern California's IACUC reviewed and approved all procedures.

Cerebellum. Recordings from cerebellar cortical slice preparations (intracellular EPSPs from Purkinje neurons) were conducted to determine the properties of LTD as a function of age in 4-, 8-, and 12-month CBA mice. The recording chamber was perfused with oxygenated aCSF [1.25 mM NaH_2PO_4, 124 mM NaCl, 3 mM KCl, 2.4 mM $CaCl_2$-$2H_2O$, 1.3 mM $MgSO_4$-$7H_2O$, 26 mM $NaHCO_3$, 10 mM glucose (pH 7.3), 290 mOsm] and sucrose (225 mM sucrose, 3 mM KCl, 3.7 mM $MgSO_4$-$7H_2O$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM glucose) solution with picrotoxin (100 mM) to block GABA inhibition. Standard conditions were used to evoke cerebellar LTD by conjoint 1-Hz stimulation of parallel fibers and depolarization of Purkinje cell membranes (24).

Recognition of the Purkinje cell layer was done using a dissecting microscope. Whole-cell patch, voltage-clamped (−70 mV) configuration was achieved by the blind approach on Purkinje cells. Cells that required >500 pA holding current were rejected; otherwise, current-clamped configuration was used for all recordings. When filled with an internal solution [140 mM CsCl, 2 mM MgCl, 0.5 mM EGTA, 4 mM NaATP, 0.3 mM NaGTP, 10 mM Hepes, 5 mM QX314 (pH 7.3), 280 mOsm], the recording pipette (Corning no. 0010, PG10165-4, WPI) had a resistance of 1.5–2 MΩ. Membrane potential was

recorded (gain, 5; filter, 5 kHz; Axopatch 1D, Axon Instruments) at room temperature (24 °C; 100 μM picrotoxin). Data were acquired by pCLAMP 8 (Axon Instruments) at a sampling rate of 50 kHz.

Parallel fibers were stimulated (0.1 ms) by a platinum wire in a glass pipette (<1 MΩ, aCSF) that was placed at the top third of the molecular layer vertically above the recording pipette. The level of stimulation on the stimulus isolator was set so that the amplitude of the EPSP was between ≈10 and 15 mV. The stimulus values were approximately the same for all cells. Ten-minute baseline and 45-min poststimulus periods were recorded at 0.033 Hz. LTD was induced by 600 parallel fiber stimuli at 1 Hz with paired Purkinje cell depolarization (−70 to −20 mV, 200 ms). Cells that had an EPSP amplitude decreasing to 0 mV during the poststimulus period were rejected.

The relative slope of EPSPs was analyzed by Clampfit 8 (Axon Instruments) by setting the two cursors at the middle one-third of the left, rising slope. The mean, standard deviation, and standard error mean of the relative slope were calculated and graphed by Microsoft Excel. ANOVA was calculated between 15 and 20 min during the poststimulus period (levels were asymptotic). One-way ANOVAs and post hoc paired-sample *t* tests (two-tailed) were used to examine differences between age groups, with $P < 0.05$ considered as significant.

Hippocampus. Electrophysiological recordings [extracellular field excitatory postsynaptic potentials (fEPSPs) in the CA1 region in response to Schaffer collateral-commissural fiber stimulation] were conducted within hippocampal slice preparations from CBA male mice (4 and 12 months) to determine the properties of LTD and LTP as a function of age. Following analysis of input/output curves, no differences were found between 4- and 12-month mice in stimulus intensities used to elicit fEPSP waveforms or between baseline synaptic transmissions before induction of low-frequency stimulation (LFS) or high-frequency stimulation (HFS). A standard LTD induction protocol (900 pulses at 1 Hz for 15 min) was used on hippocampal slices in the CBA 4- and 12-month age groups (25). Three different stimulation protocols were used to induce hippocampal LTP: one train of 1 s at 25 Hz (selectively induces NMDA-mediated LTP), 1 s at 100 Hz (induces mixed LTP), and 0.5 s at 200 Hz [selectively induces NMDA-independent voltage-dependent calcium channel (VDCC) LTP] (26). Hippocampal slice preparation procedures were exactly the same as in ref. 25. A 15-min baseline period was established with aCSF before delivering LFS to induce LTD or HFS to induce LTP. Recording continued for 30 min following LFS and HFS. All baseline values were standardized by averaging the means of measurements taken during the 15-min period before stimulation protocols for each individual slice. One-way ANOVAs were calculated as planned comparisons to analyze the effects of frequency stimulation for each age group (4 months vs. 12 months) between 25 and 30 min during the poststimulus periods. Paired-sample *t* tests (two-tailed) were used to examine whether there were significant changes from baseline for the post-LFS/HFS periods and for 4-month vs. 12-month groups at each stimulation frequency.

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