Available online at www.sciencedirect.com

SCIENCE DIRECT®

Experimental Neurology 197 (2006) 291 – 300

Experimental Neurology

www.elsevier.com/locate/yexnr

Regular Article

Calcium chelation improves spatial learning and synaptic plasticity in aged rats

Alexander Tonkikh[a,1], Christopher Janus[b,1], Hossam El-Beheiry[a], Peter S. Pennefather[c], Marina Samoilova[a], Patrick McDonald[a], Aviv Ouanounou[a], Peter L. Carlen[a,d,*]

[a]Toronto Western Research Institute, University Health Network, Toronto, Ontario, Canada
[b]Centre for Research in Neurodegenerative Disease, University of Toronto, Toronto, Ontario, Canada
[c]Faculty of Pharmacy, University of Toronto, Canada
[d]Departments of Medicine (Neurology) and Physiology, University of Toronto, Toronto, Ontario, Canada

Received 15 February 2005; revised 8 June 2005; accepted 23 June 2005
Available online 22 July 2005

Abstract

Impaired regulation of intracellular calcium is thought to adversely affect synaptic plasticity and cognition in the aged brain. Comparing young (2–3 months) and aged (23–26 months) Fisher 344 rats, stratum radiatum-evoked CA1 field EPSPs were smaller and long-term potentiation (LTP) was diminished in aged hippocampal slices. Resting calcium, in presynaptic axonal terminals in the CA1 stratum radiatum area, was elevated in aged slices. Loading the slice with the calcium chelator, BAPTA-AM, depressed LTP in young slices, but enhanced this plasticity in old slices. Forty-five minutes following LTP-inducing high frequency stimulation, resting calcium levels were significantly increased in both young and old presynaptic terminals, and significantly reduced by pretreatment with BAPTA-AM. In vivo, intraperitoneal administration of BAPTA-AM prior to training in the reference memory version of the Morris water maze test, significantly improved the acquisition of spatial learning in aged animals, without a significant effect in young rats. These results support the hypothesis that increasing intracellular neuronal buffering power for calcium in aged rats ameliorates age-related impaired synaptic plasticity and learning.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Aging; Calcium regulation; Synaptic plasticity; Spatial learning

Introduction

Cognitive decline is a common and debilitating problem in aged populations. Impaired calcium regulation in neurons and raised intracellular calcium levels are considered key factors in brain aging (Khachaturian, 1994; Foster and Kumar, 2002; Mattson and Chan, 2003; Toescu and Verkhratsky, 2003; Toescu et al., 2004; Whalley et al., 2004). Both aged rats and humans show spatial learning deficits, highly correlated to hippocampal damage (Rosenzweig et al., 1997) Also, links have been made between impaired calcium homeostasis and learning deficits in aged animals (Wu and Disterhoft, 2002).

There are several possible mechanisms underlying calcium related dysfunction in aged neurons, including altered calcium extrusion, buffering, or uptake (Martinez-Serrano et al., 1992), impaired mitochondrial function (Mattson and Liu, 2002), and increased calcium influx through voltage-gated calcium channels as measured in aged CA1 neurons (Campbell et al., 1996; Thibault et al., 2001). In addition to overt neuronal loss, this age-dependent cognitive dysfunction could be caused by subtle, progressive deterioration in neuronal function secondary to calcium dysregulation and impaired calcium dynamics (Toescu and Verkhratsky, 2003; Lott and Head, 2005), which may be reversible with the appropriate therapy.

* Corresponding author. Toronto Western Research Institute, Toronto Western Hospital, 399 Bathurst St., Room Mc12-413, Toronto, Ontario, Canada M5T 2S8. Fax: +1 416 6035745.
E-mail address: carlen@uhnres.utoronto.ca (P.L. Carlen).
[1] Equal contributions.

0014-4886/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2005.06.014

Long-term potentiation (LTP) is described as a long-lasting increase in synaptic efficacy which follows high frequency stimulation of afferent fibers (for reviews, see Malenka and Nicoll, 1999; Pang and Lu, 2004). It is thought to be a cellular correlate of learning and memory in the mammalian brain (for discussion, see Martin et al., 2000; Geinisman et al., 2004). The effects of aging on LTP and other forms of synaptic plasticity are complex (Rosenzweig and Barnes, 2003). High level tetanic stimulation to the stratum radiatum in the CA1 area of the hippocampus in aged animals does not usually show any deficit in LTP induction or decay compared to younger animals both in vivo (Landfield et al., 1978) and in vitro (Landfield and Lynch, 1977; Deupree et al., 1993; Moore et al., 1993; Barnes et al., 1996; Norris et al., 1996; Shankar et al., 1998). However, when lower intensity LTP-producing stimuli are used, impaired induction of LTP is clearly evident in the CA1 region of old animals (Deupree et al., 1993; Moore et al., 1993; Rosenzweig et al., 1997; Watson et al., 2002). Reduced hippocampal LTP has been found to be associated with impaired spatial learning in aged Fisher 344 rats (Lanahan et al., 1997).

We have previously shown that BAPTA-AM administration to hippocampal slices from young mature rats, decreased intracellularly recorded excitatory postsynaptic potentials (EPSPs) (Niesen et al., 1991) in dentate granule neurons and field EPSPs (fEPSPs) (Ouanounou et al., 1996, 1999) in the CA1 region. BAPTA-AM (bis(*O*-AMinophenoxy)-ethane-*N*,*N*,*N*,*N*-tetraacetic acid) is a membrane permeant mobile calcium chelator (Tsien, 1980) with a dissociation constant (K_D) of ~0.2 μM, which is in the physiological range. Since this membrane permeable prodrug is hydrolyzed intracellularly to the membrane impermeable calcium chelator, BAPTA, this treatment increases the calcium buffering power of the neuronal cytoplasm. Hence, it appears that increasing the intracellular calcium buffering power of presynaptic terminals in a young slice depresses evoked neurotransmitter release. This effect was shown to depend on the calcium chelator having fast binding kinetics, since EGTA-AM, a precursor of EGTA, which has a similar calcium binding affinity as BAPTA but much slower binding kinetics, was ineffective in diminishing the fEPSP in the CA1 region of young slices (Ouanounou et al., 1999). On the other hand, in the aged slices, we showed that both EGTA-AM and BAPTA-AM increased the fEPSPs, suggesting that, in the case of the aged CA1 synapses, it is not the speed of calcium chelation which is important, but rather the buffer power itself. From these data, we concluded that intracellular calcium regulation is impaired and calcium is raised in aged neurons.

Based on the above, we hypothesize that if one could buffer the poorly regulated intracellular calcium levels in aged synapses, age-impaired LTP (Rosenzweig and Barnes, 2003) and its correlated spatial reference learning and memory will be enhanced. The objectives of the following experiments were: (i) to test if increasing the intracellular calcium buffering power in aged hippocampal slices by administering the membrane permeant calcium chelator, BAPTA-AM, would improve LTP in the CA1 region, (ii) to test the hypothesis that resting intracellular calcium, particularly in stratum radiatum CA1 presynaptic terminals, is higher in aged animals and is decreased by BAPTA-AM perfusion, and (iii) to test if spatial learning in aged rats would also be improved by the administration of the membrane permeant calcium chelator, BAPTA-AM.

Materials and methods

Hippocampal slice preparation and electrophysiological recordings

Tissue preparation

Brain slices were obtained from young-adult (2–4 months) and aged (24–27 months) Fischer 344 rats. Rats were anesthetized with Halothane (Halocarbon Laboratories, River Edge, NJ) and decapitated, and the brain was quickly removed, hemisected, and placed in ice-cold (4°C) artificial CSF (ACSF) for ~3 min. Although the skulls of aged animals are somewhat thicker than those of young animals, the period required to remove the brain was not substantially longer, and we have not observed consistent differences in the viability of slices from aged and young animals. Brain slices were cut to 350–400 μm thickness with a Vibratome (Series 1000; Technical Products, Inc., St. Louis, MO) and incubated in ACSF at room temperature for a minimum of 1 h before being transferred to an interface-like chamber where oxygenated aCSF continuously perfused the slice at a rate of 1–2 ml/min. ACSF contained (in mM): 120 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 NaHCO, and 10 D-glucose, pH 7.4, continuously bubbled with 95% O_2–5%. Experiments were conducted at 34°C.

Electrophysiological recordings

Using a microelectrode filled with 150 mM NaCl, fEPSPs were recorded in the stratum radiatum of the hippocampal CA1 region, in response to stimulation of the Schaffer collateral/commissural pathway. Extracellular field recordings were measured in isolated, intact hippocampal tissue. Only hippocampal specimens whose field response amplitude was > = 0.5 mV were included for further study. Field responses of all tissue included for further study were measured initially for > = 10 min to ensure stability before continuing. Stimuli were applied through a bipolar electrode using current pulses at intensity sufficient to evoke 50% of the maximal fEPSP, so as to increase the chance of seeing impaired age-related posttetanic synaptic enhancement by avoiding maximal stimulation. After recording a stable baseline response for 15 min, five high frequency stim-

ulation (HFS) (100 Hz, for 200 ms each) trains of tetanic stimulation with 5-s intervals were delivered to induce LTP. Following HFS, recordings continued for 45 min. In the first experimental group, the perfusate contained aCSF with or without 50 μM BAPTA-AM for the entire experiment. In the second experimental group, wherein Fura-2 fluorescence imaging of calcium was also performed, the perfusate contained aCSF with or without 10 μM BAPTA-AM and 1 μM probenecid for the time of recording. Probenecid was used to prevent the extrusion of the BAPTA salt and the fluorescent probe from the intracellular compartment via anion pumps (Ouanounou et al., 1996). We performed series of control experiments studying effect of probenecid on fEPSPs (n = 5), and did not find any significant difference in the shape, timing, and amplitude of response. The evoked fEPSPs were averaged over each 5 min period, expressed as a percent change from the baseline response, and the differences in posttetanic synaptic plasticity between experimental groups were analyzed.

Calcium measurements

Fluorescence intensities from images of calibrated solutions of known calcium ion concentrations and constant Fura-2 concentrations were obtained, prior to performing calcium ion concentration measurements in hippocampal slices. For a Fura-2 experiment, the calcium ion concentration was the intensity of the emission fluorescence with excitation at 380 nm in zero calcium solution, divided by the emission intensity measured using the same excitation wavelength in saturating calcium (e.g. 1 mM). The autofluorescence component was subtracted automatically before estimating the ratioed pair values.

Presynaptic structures were filled with the fluorescent calcium indicator, Fura-2, as described previously (Regehr and Tank, 1991; Wu et al., 1994). In brief, a small amount of the membrane-permeant form of Fura-2 (50 μg of Fura-2 AM, 5–10 μl of DMSO with 25% pluronic acid, and 50 μl of extracellular solution buffered to pH 7.3 with 10 mM HEPES) is pressure-injected into the stratum radiatum using a Picospritzer II (General Valve, Fairfield, NJ) via a pipette of 2–3 μm tip diameter. After being taken up by axons, intracellular esterases cleave the AM form leaving the membrane impermeant indicator, which diffuses down the axon into the presynaptic terminals. Thirty minutes after injection, brain slices are illuminated at 340/380 nm and separate images are taken in a small spot in the striatum radiatum area, 300–500 μm away from the injection site, to avoid contamination of the optical recordings by accidental postsynaptic indicator loading. A digital CCD camera (SenSys, Photometrics, Tucson, AZ, USA) monitored changes in fluorescence emission, and images were stored and analyzed using the Axon Imaging Workbench (Axon Instruments, Foster City, CA, USA). A longpass blue set filter block XF02-2 (Omega Optical) with a 330 nm wide-band exciter was used to visualize Fura-2 emission. All images were taken through a 60× water immersion objective Nikon, N.A. 1.0 (Fig. 3B). Neutral density filters were used to reduce photobleaching. Only slices with stable background fluorescence were used for the experiments, which occurred in approximately 60% of the loadings. Recordings of the fluorescent signal and field potential were synchronized using the trigger function of the Axon Imaging Workbench program (Axon Instruments, Inc).

In our experiments, we measured resting Ca concentration during the 45 min period after HFS and discovered that even 30 min after the HFS, there was marked and variable fluctuations in the measured intracellular Ca in both young and old slices. The time course of recovery following a single stimulus was also a very variable parameter using Fura-2, and did not reveal any difference between the two age groups.

We performed control series of experiments loading old and young slices with the less photosensitive and more time stable Ca Green-AM fluorescent probe in order to test the local axonal loading technique and to confirm localization of the Ca fluorescent indicator to axons by confocal imaging. Here, we used the same local loading protocol as described above for Fura-2 AM. Images were acquired using a Biorad MRC600 laser scanning confocal microscope using a 60×-water immersion objective (Fig. 3C).

Morris water maze test

The effect of BAPTA-AM on spatial learning was evaluated in the water maze test (Morris, 1984), which is frequently used for characterization of hippocampal-dependent spatial memory in aged rats (Barnes et al., 1996; Byrnes et al., 2004; Geinisman et al., 2004). This test requires a rat to locate a submerged escape platform using distant cues in the environment. Experimentally naive 4 months (n = 13) and 22 months (n = 17) old Fisher 344 male rats were used. BAPTA-AM (5 mg/kg), dissolved in dimethyl sulfoxide (DMSO), was administered by intraperitoneal (ip) injection in the presence of probenecid (25 mg/kg). There is much evidence supporting the hypothesis that systemically administered BAPTA enters the brain tissue and has direct CNS actions. IV BAPTA-AM given 3 h prior to the induction of focal cerebral ischemia in rats was significantly neuroprotective (Tymianski et al., 1993). Using the same protocol, our group showed that 3 h following in vivo ip injection of Calcium Crimson-AM, a fluorescent analogue of BAPTA-AM, dye fluorescence was clearly visible in hippocampal and cerebral cortical neurons. Furthermore, in slices removed from such BAPTA-AM treated animals, whole cell recordings showed depressed calcium-dependent long-lasting afterhyperpolarizations. Recently, Bartnik et al. (2005) have shown that IV administered APTRA-AM, a molecule similar to BAPTA-AM, but with lower affinity for calcium, was also markedly neuroprotective when administered postfocal cerebral ischemia. In addition, a recent publication about the pharmaco-

kinetics of neuroprotective substances (Satoh et al., 2001), showed that IV and IP injections provide almost the same effect, and higher plasma concentrations are measured following IP drug administration.

The DMSO alone has no effect on rats locomotor activity, swimming capabilities, or learning performance in the water maze (Saucier et al., 1996). Probenecid, an anion transport inhibitor used clinically (Shionoiri, 1993), blocks extrusion of BAPTA-AM from neurons, lowering the effective dose without affecting the final effect of BAPTA-AM on fEPSPs (Saucier et al., 1996; Ouanounou et al., 1998, 1999). Each day, 1.5 h before testing, all rats were injected (ip) with probenecid followed, 1/2 h later, by the injection of BAPTA-AM to the experimental group, and DMSO to the control group. To familiarize rats with the task, spatial training was preceded by non-spatial pretraining with the pool surrounded by a curtain, thus making extra-maze spatial cues inaccessible (Cain, 1997). During spatial training, the curtain was removed, and rats were released from pseudo-randomly chosen points along the wall of the pool in the middle of quadrants not containing the platform (NE, SE, and SW). The trial ended when a rat climbed the platform placed in the center of the NW quadrant (the target quadrant, TQ), or in 60 s, whichever came first. A rat was allowed to stay on the platform for 10 s. The following day after the last training session, all rats were tested for the development of spatial bias in a 60 s probe trial with the escape platform removed from the pool. The behavior of a rat in the pool was recorded by a video tracking system (HVS Image Advanced Tracker VP200, HVS Image, Buckingham, UK).

Methods for statistical analysis

The evoked fEPSPs were averaged over each 5 min period, normalized to initial response, and expressed as a percent change from the baseline response. The differences in posttetanic synaptic plasticity between experimental groups were analyzed.

A region of interest (ROI) was selected after the loading procedure and observation of the SR region under high magnification. The zone of fluorescense was defined by a ROI draw function in the AIW program (Axon Instruments, Foster City, CA, USA). The mean value of the ROI was measured throughout the image sequence. After that, the data were normalized for statistical analysis.

We realize that the K_D of calcium indicators can increase three- to fourfold when loaded into some types of cells (Brenowitz and Regehr, 2003), and because we do not know the intracellular buffering capacity, the accuracy of the reported values is useful mainly for comparing the relative values between aged and young neurons. Taking into account that calculation of this parameter depends on many variables and can be controversial, we decided to evaluate calcium ions level using the ratiometric technique, which directly reflects intracellular calcium concentration and is relatively not affected by photobleaching. We compared it with other measurements in this series of experiments using the ratio value in young, untreated slices as a standard 100% point.

In electrophysiological and Ca measurements, statistical significance was calculated using a two-population independent t test (significance <0.05).

For behavioral studies, data were analyzed using analysis of variance (ANOVA) with groups as between, and training sessions as within-subject factors. The young rats group was used as a point of reference related to the drug effect. Therefore, the results are reported separately for the two age cohorts. All plotted and reported data are shown as means ± SEM.

Results

BAPTA-AM effects on LTP

The baseline control fEPSPs were significantly smaller in aged compared to younger slices (aged, −0.98 ± 0.31 mV, n = 16; young, −1.96 ± 0.27 mV, n = 15, $P < 0.05$). All later comparisons were normalized to the initial control values and percentage changes were then measured. In the first experimental group, wherein 50 μM of BAPTA-AM was perfused onto the slice and no fluorescent dye was used, we found that in the CA1 region of hippocampal slices from aged rats, LTP was significantly increased in the BAPTA group ($F(1,10) = 11.01$, $P < 0.05$) when compared to age-matched controls (Fig. 1); the magnitude of LTP at the end of 45 min recording was 226.9 ± 23.6% and 135.2 ± 5.5% for aged BAPTA-treated and control groups, respectively. There was no significant interaction between the experimental groups and time ($F(2,40) = 1.08$, $P > 0.05$), and the LTP did not change over time ($F(2,40) = 0.60$, $P > 0.05$). In contrast, in slices taken from young rats, LTP was reduced, but not significantly ($F(1,8) = 3.86$, $P > 0.05$) in the presence of BAPTA-AM (Fig. 1B). The magnitude of LTP at the end of 45 min recording was 127.4 ± 9.0% and 169.9 ± 13.7% for young BAPTA and control groups, respectively. Similarly, when grouped by time interaction, there were no significant differences ($F(1,32) = 1.31$, $P > 0.05$), and the response was stable over time ($F(1,64) = 1.19$, $P > 0.05$). Additional comparison of responses within the control (aCSF) aged and young groups (Figs. 1A and B, closed squares) revealed that the slices from aged rats had reduced LTP; however, the difference was not significant ($F(1,9) = 3.01$, $P > 0.05$). Initial baseline responses were similar for BAPTA-AM and aCSF conditions ($F(1,8) = 0.75$, $P > 0.05$; $F(1,10) = 2.21$, $P > 0.05$, for young and aged rats, respectively).

In the second experimental group, we also studied LTP using an identical HFS protocol, simultaneously measuring presynaptic intracellular calcium with axonally-injected

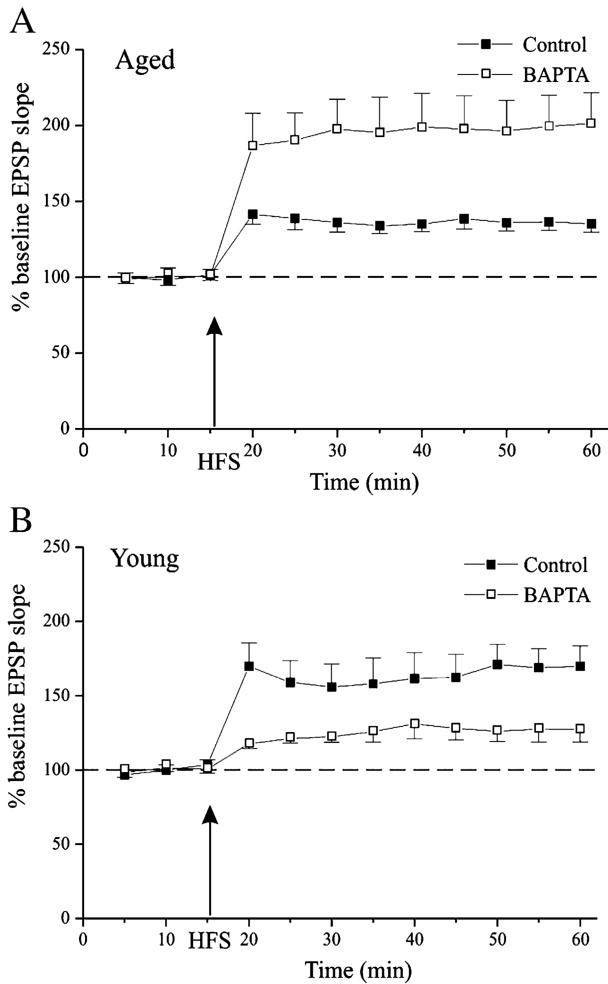

Fig. 1. The effect of BAPTA-AM (50 μM) on LTP recorded in the striatum radiatum of the hippocampal CA1 region. (A) In slices from aged (25 months, n = 12) Fisher 344 rats, LTP was significantly increased in the presence of BAPTA-AM when compared to age-matched control (aCSF) slices (226.9 ± 23.6% and 135.2 ± 5.5%, respectively, 45 min after HFS). (B) Slices from young (2–3 months, n = 10) rats, LTP was significantly decreased by BAPTA-AM when compared to age-matched control (aCSF) slices (127.4 ± 9.0% and 169.9 ± 13.7%, respectively, 45 min after HFS). Arrows denote the time of high frequency stimulation (HFS).

fluorescent Fura-2 AM and bath applied probenecid, and recording the fEPSP (Fig. 2). In the aged group normalized to the initial response, the peak fEPSP amplitude increased up to 154 ± 8% during the first 5 min after HFS, with a slow decline over the ensuing 45 min (Fig. 2A). This effect was registered in 5 of 8 slices in the aged group. The other 3 slices lost their responses entirely several minutes following HFS, presumably due to synaptic/neuronal damage or death. Application of 10 μM BAPTA-AM caused a 167 ± 11% increase in the fEPSP during the first 5 min after HFS, and the ensuing fEPSPs were significantly larger (P < 0.05) 5 and 10 min after HFS, compared to the fEPSPs in the non-BAPTA treated aged control slices (n = 8). With the BAPTA-AM pretreatment, no aged slices lost their responses. In young slices, as expected, HFS caused a significant and persistent increase in the fEPSPs over 45 min (Fig. 2B). However, application of BAPTA-AM reduced fEPSPs to 60% of the control levels after HFS (n = 5, P < 0.05). The difference between this experiment and the previous experiment include the perfusion of probenecid, and the presynaptic axonal loading of Fura-2, which is also a calcium chelator like BAPTA, in addition to its fluorescent properties, presumably leading to more pronounced chelation of intracellular calcium.

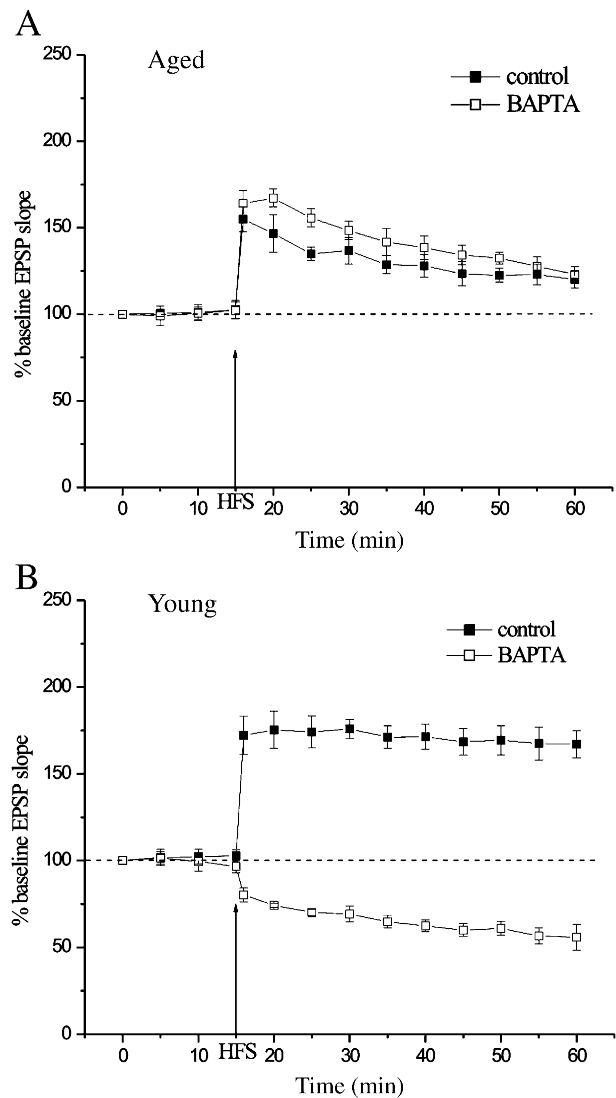

Fig. 2. The effect of BAPTA-AM (10 μM) in the presence of 1 μM probenecid and presynaptically-loaded Fura-2 AM (for intracellular calcium measurements) on LTP recorded in the striatum radiatum of the hippocampal CA1 region. (A) In slices from aged (24 months, n = 8) Fisher 344 rats, the peak fEPSP amplitude, increased up to 167 ± 5.1% in the presence of BAPTA-AM during the first 5 min after HFS, with a slow decline over the ensuing 45 min compared to age-matched controls (n = 8) (no added BAPTA-AM) slices (146 ± 10.6%), respectively. The fEPSPs were significantly larger (P < 0.05) 5 and 10 min after HFS. (B) In the presence of BAPTA-AM, slices from young (2–3 months, n = 8) rats showed significantly depressed fEPSPs following HFS when compared to age-matched control slices (n = 7) (169.3 ± 7.7% and 55.8 ± 7.3%, respectively, 45 min after HFS). Arrows denote the time of HFS.

Intracellular calcium

The second group of experiments was also designed to measure changes in the resting intracellular calcium from the CA1-stratum radiatum region. The whole slice loading technique was employed initially to assess resting calcium levels in the CA1 stratum radiatum area. We discovered that the mean resting Ca level in aged slices (n = 5) was 124.2 ± 7.3% higher than in young slices (n = 6); however, because of low resolution and poor cellular specificity (e.g. dye filling of other cell types such as glia), this method was replaced by the local axonal injection technique. For better cellular specificity, we then focussed on measurements of presynaptic intracellular calcium. Resting Ca levels in old slices (n = 16) were significantly higher 120.5 ± 6.6% than in young slices (n = 15) ($P < 0.05$; Fig. 3A). The intracellular calcium was also measured 45 min following the series of 5 HFS after 15 min of stable baseline recording. Control experiments, performed without BAPTA-AM application in separate groups for young (n = 7) and old (n = 8) rats, showed that 45 min after the HFS, the resting calcium levels were significantly higher in both groups. In the aged group, the resting calcium rose to 131.5 ± 8.2% (n = 8) ($P < 0.05$ compared to the value before HFS). In the young group, the resting calcium rose to 113.8 ± 7.4% (n = 7) ($P < 0.05$ compared to the value before HFS). The addition of BAPTA-AM to the perfusate significantly decreased the resting calcium levels in both the old and young slices compared to the non-BAPTA treated group 45 min after HFS. In the old slices, BAPTA-AM caused a significant ($P < 0.05$) decrease from baseline 13.1 ± 3.9% (n = 8). In the young slices, BAPTA-AM decreased the resting calcium by 9.8 ± 2.1% (n = 8) ($P < 0.05$).

Confocal imaging of slices loaded with the Ca Green-AM fluorescent probe revealed similar image intensity and loading pattern in young and old groups (e.g. Fig. 3C). Here, the presynaptic axon is clearly filled with Ca Green-AM loaded into SR axons.

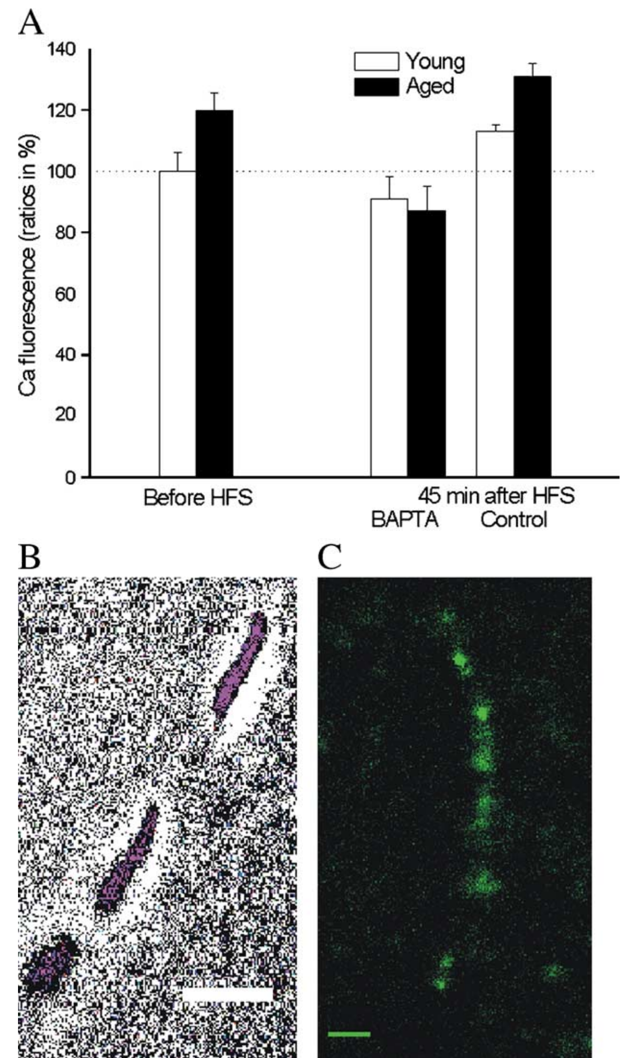

Fig. 3. Presynaptic intracellular calcium fluorescence measurement from the CA1-stratum radiatum region. (A) Presynaptic intracellular calcium ratiometric fluorescence measurement from the CA1-stratum radiatum region following local application of Fura-2 AM before and 45 min after HFS. Initial ratio value in the young slices used as a standard 100% point. Forty-five minutes after HFS, without the perfusion of BAPTA-AM, there was a significant rise of the intracellular calcium (n = 8). These levels were significantly reduced following 45 min of BAPTA-AM exposure (n = 8). Forty-five minutes after HFS, without the perfusion of BAPTA-AM, the resting calcium significantly rose in the nerve terminals (n = 7). All three groups were significantly different from each other with each type of treatment. (B) High power magnification image of a presynaptic terminal in the CA1 field. Image taken 30 min after selective pressure loading with Fura-2 AM. Water immersion ×60, long working distance, 1.00 N/A objective. Digital subtraction of autofluorescence was performed using AIW2.2 software. (C) Confocal image of an axon in an old hippocampal slice loaded locally with Ca Green-AM using similar technique. Scale bars = 5 μm.

Spatial learning

The results show that both experimental (BAPTA-AM + DMSO + probenecid) and control (DMSO + probenecid) aged rats significantly improved their escape latencies during the 8 days of training ($F(3,77) = 5.62$, $P < 0.05$, day factor; Fig. 4A). However, the administration of BAPTA-AM before the training significantly improved learning acquisition of platform spatial location in this group of rats ($F(1,11) = 5.48$, $P < 0.05$, group factor; Fig. 2A). This positive effect of BAPTA-AM was further substantiated by shorter search paths of the BAPTA treated rats ($F(1,11) = 4.52$, $P = 0.05$). This result was expected since BAPTA-AM treated rats did not differ from their control counterparts in their swim speed or thigmotaxic (wall hugging) behavior (data not shown). The results of the probe trial administered at the end of training revealed that both BAPTA and control rats showed similar average number of crosses of the platform site (1.3 ± 0.4 and 1.4 ± 0.5, respectively), indicating a comparable memory for the platform position. This result is not surprising after the examination of the groups' learning curves during training. Although BAPTA-AM treated rats showed a significantly

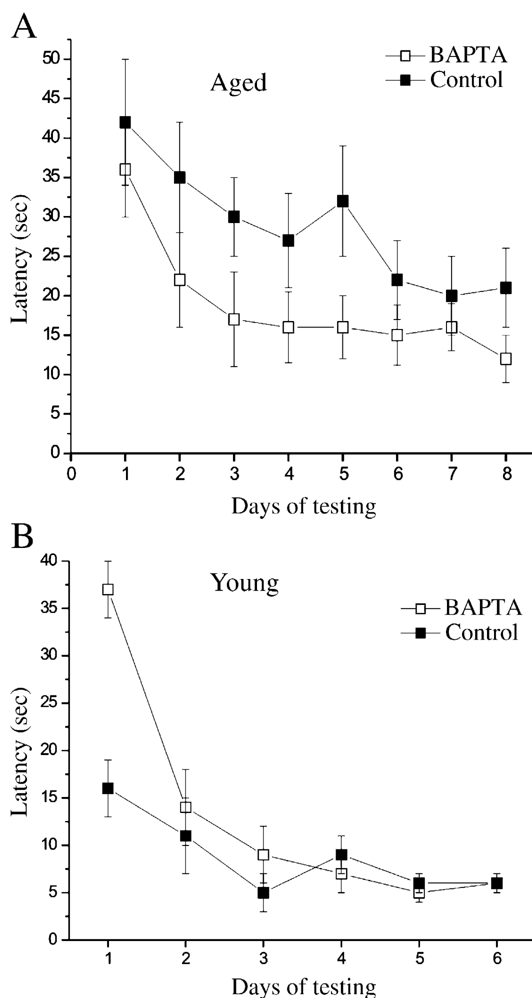

Fig. 4. The average (means of 3 trials per day) escape latencies to reach a submerged escape platform of (A) aged (n = 17) and (B) young (n = 13) BAPTA-AM treated and control Fisher 344 rats during place discrimination training in the water maze test. Since the young rats showed rapid acquisition of spatial information in the test, they were trained over 6 days only.

faster spatial learning, the control rats showed comparable escape latencies in the last days (days 6–8) of the training (Fig. 4A). Thus, it is likely that at the end of training rats of both groups obtained a comparable knowledge of the escape platform location. Similarly to aged rats, both the BAPTA-AM treated and control groups of young, 4 months old, rats significantly improved their performance over training ($F(3,55)$ = 28.87, P < 0.05, days factor; Fig. 4B), but BAPTA-AM had no significant effect on learning in this age cohort of rats. However, BAPTA-AM significantly changed the rats initial performance in the water maze test ($F(3,55)$ = 6.39, P < 0.01, group × day interaction; Fig. 4B), significantly increasing escape latencies of BAPTA-treated rats ($t(11)$ = −4.26, P < 0.05) during the first day of training. Removing the scores of day 1 from the analysis eliminated the interaction effect, indicating a transient initial effect of BAPTA-AM on young rats' performance in the water maze test. Also, young rats of both groups showed similar performance during the probe trial (2.7 ± 0.21 and 3.3 ± 0.61, for BAPTA and control rats, respectively), indicating a comparable spatial memory after training.

Discussion

The key findings of the in vitro studies are that resting intracellular and particularly presynaptic calcium levels were raised in aged compared to young slices, and that the administration of a membrane-permeable mobile calcium chelator (BAPTA-AM) decreased resting presynaptic intracellular calcium in both young and old slices, and enhanced LTP in the aged slices. Resting levels of ionic intracellular calcium ($[Ca^{2+}]_i$) are hypothesized to be raised in aged neurons and to be neurotoxic, but this measurement has been rarely made, and never in presynaptic terminals. Resting calcium was found not to be increased in old compared to young neurons (Hartmann et al., 1996; Murchison and Griffith, 1998) or raised in old neurons (Kirischuk and Verkhratsky, 1996) and aged synaptosomes (Martinez-Serrano et al., 1992). Aged cerebellar neurons had similar initial intracellular free calcium values as younger neurons, but significantly higher calcium values as a function of the time the slices were maintained in vitro (Xiong et al., 2004). Thibault et al. (2001) showed that resting $[Ca^{2+}]_i$ was not different in young and old neurons, but during repetitive action potentials, the calcium rise and the associated impairment of the EPSP frequency facilitation were greater in the older neurons. Ultrastructural analysis reveals a lower density of presynaptic terminals per unit length of postsynaptic membrane of labeled pyramidal neurons in the aged brain (Wong et al., 2000). Hence, in the aged slices, if this is a significant factor, we should expect lower Ca levels, contrary to our results. Toescu and Verkhratsky (2003) concluded that the overall duration of cytoplasmic calcium signals becomes longer in aged neurons and that "these prolonged calcium signals may exert a local excitotoxic effect, removing preferentially the most active synapses".

Calcium buffering had markedly different effects on synaptic transmission. In the young slices, as expected, the BAPTA-mediated calcium decrease was associated with decreased synaptic transmission both with control and posttetanic evoked responses. In contrast in the aged slices, intracellular calcium buffering was associated with increased control and posttetanic evoked fEPSPs. In the experiments designed to measure intracellular calcium, wherein slices were treated with BAPTA-AM, probenecid, and presynaptic loading with the fluorescent calcium chelator, Fura-2 AM, the posttetanic responses decayed faster than in the first set of experiments. Even so, in these aged slices, BAPTA-AM application, which decreased resting intracellular presynaptic calcium, enhanced post-tetanic synaptic plasticity. The differences between these experiments could be explained by increased buffering of

presynaptic intracellular calcium by the axonally-loaded Fura-2 and the decreased buffer extrusion by probenecid, and/or by phototoxicity from the Fura-2 per se.

It is unclear whether the important locus of calcium chelation is occurring pre- and/or postsynaptically. LTP per se is considered to be mediated by mainly postsynaptic elements (Malenka and Nicoll, 1999; Nicoll, 2003), although there is also evidence for a presynaptic component (Sokolov et al., 2002; Emptage et al., 2003). In slices from young animals, we showed that the BAPTA-AM effect of decreasing evoked EPSPs in CA1 neurons was a presynaptic action, because postsynaptic injection of the BAPTA salt did not diminish the EPSP (Niesen et al., 1991). We have already shown that a calcium chelator (EGTA) administered intracellularly, enhanced neuronal calcium currents in dentate granule neurons of hippocampal slices from aged (24–27 months), but not from young mature (24 months) Fischer 344 rats (Reynolds and Carlen, 1989). This result in aged neurons could be from buffering of raised intracellular calcium, diminishing calcium-mediated inactivation of inward calcium current. If this effect on calcium currents also operates presynaptically at the stratum radiatum-CA1 synapse, then increased synaptic transmission could result from an increased axonal action potential-evoked nerve terminal calcium current.

Given that the intracellular calcium is raised in the aged stratum radiatum CA1 presynaptic terminals as measured here, what are the implications for synaptic transmission? Thibault et al. (2001) showed that elevation of postsynaptic $[Ca^{2+}]_i$ by the selective L-type calcium channel agonist, Bay K8644, in young neurons, depressed frequency facilitation similar to what was noted in aged neurons. David and Barrett (2003) raised intracellular presynaptic calcium in mouse motor nerve terminals by inhibiting mitochondrial calcium uptake. This depressed both the amplitude and posttetanic potentiation of these postsynaptic endplate potentials. Depression of these postsynaptic potentials was delayed by nerve terminal loading of the calcium chelator, EGTA. Using the calyx of Held as a model of a glutamatergic synapse, Billups and Forsythe (2002) showed that mitochondrial depolarization raised the presynaptic cytoplasmic calcium concentration and reduced transmitter release after short EPSC trains (100 ms, 200 Hz), and this effect was reversed by raising mobile calcium buffering capacity with EGTA. Chan et al. (2002) reported that PC12 cells and primary hippocampal neurons expressing mutant presenilin-1 mutations were sensitized to cell death induced by DNA damage, which was correlated with increased intracellular calcium levels, induction of p53, upregulation of the calcium-dependent protease *m*-calpain, and mitochondrial membrane depolarization. This cell death was attenuated by intracellular calcium chelation by BAPTA-AM.

One possible factor contributing to raised intracellular calcium at rest or following stimulation in aged neurons, is the evidence for decreased intracellular calcium buffering in aged neurons (Bu et al., 2003). The addition of a membrane-permeable calcium buffer, BAPTA, as was done here, could replace both the lack of endogenous buffer and also, because of its mobility properties, enhance the effective diffusion of intracellular calcium away from foci of high calcium concentration (Nowycky and Pinter, 1993).

As expected, in young slices, BAPTA-AM was associated with a significant drop in the resting calcium signal and in evoked synaptic transmission both at rest (Ouanounou et al., 1999) and following HFS. Conversely in the aged slices, a similar degree of drop in intracellular resting calcium by BAPTA-AM application enhanced synaptic transmission and LTP. We hypothesize that this could be due to reversal of age-related elevation of resting and stimulus-evoked intracellular calcium to more normal levels, thereby removing the neurotoxic effects of the raised calcium. Foster et al. (2001) showed that the cytosolic expression and activity of the calcium-dependent protein phosphatase, calcineurin, increased in the hippocampus during aging, and this increase was negatively correlated with cognitive function measured by the Morris water escape task.

Other ways to decrease intracellular calcium have also resulted in improved synaptic plasticity properties in aged neurons. Hippocampal slices from aged rats have a lower long-term depression (LTD) induction threshold than younger slices (Norris et al., 1996), a finding which was reversed by bathing the aged slices in low-calcium aCSF, or by blocking L-type calcium channels by nifedipine, which prevented LTD and enabled synaptic enhancement in response to 5 Hz stimulation (Norris et al., 1998). We have demonstrated that frequency potentiation, which was impaired in slices from aged rats, was ameliorated by perfusing BAPTA-AM (Ouanounou et al., 1999). The result that BAPTA-AM enhanced in vitro LTP in hippocampal slices from aged rats led us to the in vivo spatial learning experiments. The approximately 20% changes in intracellular calcium could have very large effects on cellular mechanisms, given that many clinically effective drugs have similar or smaller magnitude cellular actions at relevant concentrations causing significant clinical effects.

In this paper, we provide the first report, to our knowledge, that the intraperitoneal administration of a membrane-permeant calcium chelator (BAPTA-AM), enhances spatial learning in aged rats. Our findings are in agreement with the observation that age-related working memory impairment, which was correlated with increases of the L-type calcium channel protein in the CA1 area of the hippocampus, was ameliorated by administration of the calcium channel blocker, nimodipine (Veng et al., 2003), which also improved recent memory in aged rats tested in the radial maze (Levere and Walker, 1992).

Our data support the hypothesis that compromised intracellular calcium regulation and buffering in the aged brain, could underlie the age-related decline in cognitive abilities. Future in vitro experiments using different calcium buffers and studying different central synapses, and in vivo studies

addressing more precisely the effect of calcium chelation on learning acquisition and retention of information, should further elucidate the cellular mechanisms underlying cognitive impairment in aged animals. This study strongly suggests that chelating intracellular neuronal calcium with a membrane-permeant mobile calcium buffer could be a promising strategy for treating age-related learning deficits.

Acknowledgments

Supported by the Alzheimer's Association, Canadian Institutes of Health Research and the Heart and Stroke Foundation of Canada.

References

Barnes, C.A., Rao, G., McNaughton, B.L., 1996. Functional integrity of NMDA-dependent LTP induction mechanisms across the lifespan of F-344 rats. Learn. Mem. 3, 124–137.

Bartnik, B.L., Spigelman, I., Obenaus, A., 2005. Cell-permeant calcium buffer induced neuroprotection after cortical devascularization. Exp. Neurol. 192, 357–364.

Billups, B., Forsythe, I.D., 2002. Presynaptic mitochondrial calcium sequestration influences transmission at mammalian central synapses. J. Neurosci. 22, 5840–5847.

Brenowitz, S.D., Regehr, W.G., 2003. Calcium dependence of retrograde inhibition by endocannabinoids at synapses onto Purkinje cells. J. Neurosci. 23, 6373–6384.

Bu, J., Sathyendra, V., Nagykery, N., Geula, C., 2003. Age-related changes in calbindin-D28k, calretinin, and parvalbumin-immunoreactive neurons in the human cerebral cortex. Exp. Neurol. 182, 220–231.

Byrnes, M.L., Richardson, D.P., Brien, J.F., Reynolds, J.N., Dringenberg, H.C., 2004. Spatial acquisition in the Morris water maze and hippocampal long-term potentiation in the adult guinea pig following brain growth spurt-prenatal ethanol exposure. Neurotoxicol. Teratol. 26, 543–551.

Cain, D.P., 1997. Prior non-spatial pretraining eliminates sensorimotor disturbances and impairments in water maze learning caused by diazepam. Psychopharmacology (Berl.) 130, 313–319.

Campbell, L.W., Hao, S.Y., Thibault, O., Blalock, E.M., Landfield, P.W., 1996. Aging changes in voltage-gated calcium currents in hippocampal CA1 neurons. J. Neurosci. 16, 6286–6295.

Chan, S.L., Culmsee, C., Haughey, N., Klapper, W., Mattson, M.P., 2002. Presenilin-1 mutations sensitize neurons to DNA damage-induced death by a mechanism involving perturbed calcium homeostasis and activation of calpains and caspase-12. Neurobiol. Dis. 11, 2–19.

David, G., Barrett, E.F., 2003. Mitochondrial Ca^{2+} uptake prevents desynchronization of quantal release and minimizes depletion during repetitive stimulation of mouse motor nerve terminals. J. Physiol. (London) 548, 425–438.

Deupree, D.L., Bradley, J., Turner, D.A., 1993. Age-related alterations in potentiation in the CA1 region in F344 rats. Neurobiol. Aging 14, 249–258.

Emptage, N.J., Reid, C.A., Fine, A., Bliss, T.V., 2003. Optical quantal analysis reveals a presynaptic component of LTP at hippocampal Schaffer-associational synapses. Neuron 38, 797–804.

Foster, T.C., Kumar, A., 2002. Calcium dysregulation in the aging brain. Neuroscientist 8, 297–301.

Foster, T.C., Sharrow, K.M., Masse, J.R., Norris, C.M., Kumar, A., 2001. Calcineurin links Ca^{2+} dysregulation with brain aging. J. Neurosci. 21, 4066–4073.

Geinisman, Y., Ganeshina, O., Yoshida, R., Berry, R.W., Disterhoft, J.F., Gallagher, M., 2004. Aging, spatial learning, and total synapse number in the rat CA1 stratum radiatum. Neurobiol. Aging 25, 407–416.

Hartmann, H., Eckert, A., Velbinger, K., Rewsin, M., Muller, W.E., 1996. Down-regulation of free intracellular calcium in dissociated brain cells of aged mice and rats. Life Sci. 59, 435–449.

Khachaturian, Z.S., 1994. Calcium hypothesis of Alzheimer's disease and brain aging. Ann. N. Y. Acad. Sci. 747, 1–11.

Kirischuk, S., Verkhratsky, A., 1996. Calcium homeostasis in aged neurons. Life Sci. 59, 451–459.

Lanahan, A., Lyford, G., Stevenson, G.S., Worley, P.F., Barnes, C.A., 1997. Selective alteration of long-term potentiation-induced transcriptional response in hippocampus of aged, memory-impaired rats. Neuroscience 17, 2876–2885.

Landfield, P.W., Lynch, G., 1977. Impaired monosynaptic potentiation in in vitro hippocampal slices from aged, memory-deficient rats. J. Gerontol. 32, 523–533.

Landfield, P.W., McGaugh, J.L., Lynch, G., 1978. Impaired synaptic potentiation processes in the hippocampus of aged, memory-deficient rats. Brain Res. 150, 85–101.

Levere, T.E., Walker, A., 1992. Old age and cognition: enhancement of recent memory in aged rats by the calcium channel blocker nimodipine. Neurobiol. Aging 13, 63–66.

Lott, I.T., Head, E., 2005. Alzheimer disease and Down syndrome: factors in pathogenesis. Neurobiol. Aging 26, 383–389.

Malenka, R.C., Nicoll, R.A., 1999. Long-term potentiation—A decade of progress? Science 285, 1870–1874.

Martin, S.J., Grimwood, P.D., Morris, R.G., 2000. Synaptic plasticity and memory: an evaluation of the hypothesis. Annu. Rev. Neurosci. 23, 649–711.

Martinez-Serrano, A., Blanco, P., Satrustegui, J., 1992. Calcium binding to the cytosol and calcium extrusion mechanisms in intact synaptosomes and their alterations with aging. J. Biol. Chem. 267, 4672–4679.

Mattson, M.P., Chan, S.L., 2003. Neuronal and glial calcium signaling in Alzheimer's disease. Cell Calcium 34, 385–397.

Mattson, M.P., Liu, D., 2002. Energetics and oxidative stress in synaptic plasticity and neurodegenerative disorders. Neuromol. Med. 2, 215–231.

Moore, C.I., Browning, M.D., Rose, G.M., 1993. Hippocampal plasticity induced by primed burst, but not long-term potentiation, stimulation is impaired in area CA1 of aged Fischer 344 rats. Hippocampus 3, 57–66.

Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11, 47–60.

Murchison, D., Griffith, W.H., 1998. Increased calcium buffering in basal forebrain neurons during aging. J. Neurophysiol. 80, 350–364.

Nicoll, R.A., 2003. Expression mechanisms underlying long-term potentiation: a postsynaptic view. Philos. Trans. R. Soc. London, B Biol. Sci. 358, 721–726.

Niesen, C., Charlton, M.P., Carlen, P.L., 1991. Postsynaptic and presynaptic effects of the calcium chelator BAPTA on synaptic transmission in rat hippocampal dentate granule neurons. Brain Res. 555, 319–325.

Norris, C.M., Korol, D.L., Foster, T.C., 1996. Increased susceptibility to induction of long-term depression and long-term potentiation reversal during aging. J. Neurosci. 16, 5382–5392.

Norris, C.M., Halpain, S., Foster, T.C., 1998. Reversal of age-related alterations in synaptic plasticity by blockade of L-type Ca^{2+} channels. J. Neurosci. 18, 3171–3179.

Nowycky, M.C., Pinter, M.J., 1993. Time courses of calcium and calcium-bound buffers following calcium influx in a model cell. Biophys. J. 64, 77–91.

Ouanounou, A., Zhang, L., Tymianski, M., Charlton, M.P., Wallace, M.C., Carlen, P.L., 1996. Accumulation and extrusion of permeant Ca^{2+} chelators in attenuation of synaptic transmission at hippocampal CA1 neurons. Neuroscience 75, 99–109.

Ouanounou, A., Carlen, P.L., El_Beheiry H., 1998. Enhanced isoflurane

suppression of excitatory synaptic transmission in the aged rat hippocampus. Br. J. Pharmacol. 124, 1075–1082.

Ouanounou, A., Zhang, L., Charlton, M.P., Carlen, P.L., 1999. Differential modulation of synaptic transmission by calcium chelators in young and aged hippocampal CA1 neurons: evidence for altered calcium homeostasis in aging. J. Neurosci. 19, 906–915.

Pang, P.T., Lu, B., 2004. Regulation of late-phase LTP and long-term memory in normal and aging hippocampus: role of secreted proteins tPA and BDNF. Ageing Res. Rev. 3, 407–430.

Regehr, W.G., Tank, D.W., 1991. Selective fura-2 loading of presynaptic terminals and nerve cell processes by local perfusion in mammalian brain slice. J. Neurosci. Methods 37, 111–119.

Reynolds, J.N., Carlen, P.L., 1989. Diminished calcium currents in aged hippocampal dentate gyrus granule neurones. Brain Res. 479, 384–390.

Rosenzweig, E.S., Barnes, C.A., 2003. Impact of aging on hippocampal function: plasticity, network dynamics, and cognition. Prog. Neurobiol. 69, 143–179.

Rosenzweig, E.S., Rao, G., McNaughton, B.L., Barnes, C.A., 1997. Role of temporal summation in age-related long-term potentiation-induction deficits. Hippocampus 7, 549–558.

Satoh, S.-i., Utsunomiya, T., Tsurui, K., Kobayashi, T., Ikegaki, I., Sasaki, Y., Asano, T., 2001. Pharmacological profile of hydroxy fasudil as a selective rho kinase inhibitor on ischemic brain damage. Life Sci. 69, 1441–1453.

Saucier, D., Hargreaves, E.L., Boon, F., Vanderwolf, C.H., Cain, D.P., 1996. Detailed behavioral analysis of water maze acquisition under systemic NMDA or muscarinic antagonism: nonspatial pretraining eliminates spatial learning deficits. Behav. Neurosci. 110, 103–116.

Shankar, S., Teyler, T.J., Robbins, N., 1998. Aging differentially alters forms of long-term potentiation in rat hippocampal area CA1. J. Neurophysiol. 79, 334–341.

Shionoiri, H., 1993. Pharmacokinetic drug interactions with ACE inhibitors. Clin. Pharmacokinet. 25, 20–58.

Sokolov, M.V., Rossokhin, A.V., Astrelin, A.V., Frey, J.U., Voronin, L.L., 2002. Quantal analysis suggests strong involvement of presynaptic mechanisms during the initial 3 h maintenance of long-term potentiation in rat hippocampal CA1 area in vitro. Brain Res. 957, 61–75.

Thibault, O., Hadley, R., Landfield, P.W., 2001. Elevated postsynaptic $[Ca^{2+}]_i$ and L-type calcium channel activity in aged hippocampal neurons: relationship to impaired synaptic plasticity. J. Neurosci. 21, 9744–9756.

Toescu, E.C., Verkhratsky, A., 2003. Neuronal ageing from an intraneuronal perspective: roles of endoplasmic reticulum and mitochondria. Cell Calcium 34, 311–323.

Toescu, E.C., Verkhratsky, A., Landfield, P.W., 2004. Ca^{2+} regulation and gene expression in normal brain aging. Trends Neurosci. 27, 614–620.

Tsien, R.Y., 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry 19, 2396–2404.

Tymianski, M., Wallace, M.C., Spigelman, I., Uno, M., Carlen, P.L., Tator, C.H., Charlton, M.P., 1993. Cell-permeant Ca^{2+} chelators reduce early excitotoxic and ischemic neuronal injury in vitro and in vivo. Neuron 11, 221–235.

Veng, L.M., Mesches, M.H., Browning, M.D., 2003. Age-related working memory impairment is correlated with increases in the L-type calcium channel protein alpha1D (Cav1.3) in area CA1 of the hippocampus and both are ameliorated by chronic nimodipine treatment. Brain Res. Mol. Brain Res. 110, 193–202.

Watson, J.B., Khorasani, H., Persson, A., Huang, K.P., Huang, F.L., O_Dell, T.J., 2002. Age-related deficits in long-term potentiation are insensitive to hydrogen peroxide: coincidence with enhanced autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II. J. Neurosci. Res. 70, 298–308.

Whalley, L.J., Deary, I.J., Appleton, C.L., Starr, J.M., 2004. Cognitive reserve and the neurobiology of cognitive aging. Ageing Res. Rev. 3, 369–382.

Wong, T.P., Marchese, G., Casu, M.A., Ribeiro-da-Silva, A., Cuello, A.C., De Koninck, Y., 2000. Loss of presynaptic and postsynaptic structures is accompanied by compensatory increase in action potential-dependent synaptic input to layer V neocortical pyramidal neurons in aged rats. J. Neurosci. 20, 8596–8606.

Wu, W.W., Disterhoft, J., 2002. Calcium homeostasis and learning deficits in aging. In: Mattson, M.P. (Ed.), Advances in Cell Aging and Gerontology, vol. 10. Elsevier, Amsterdam, pp. 67–89.

Wu, L.G., Saggau, P., Division of Neuroscience, B.C.o.M.H.T., 1994. Presynaptic calcium is increased during normal synaptic transmission and paired-pulse facilitation, but not in long-term potentiation in area CA1 of hippocampus. J. Neurosci. 14 (2), 645–654.

Xiong, J., Camello, P.J., Verkhratsky, A., Toescu, E.C., 2004. Mitochondrial polarisation status and $[Ca^{2+}]_i$ signaling in rat cerebellar granule neurons aged in vitro. Neurobiol. Aging 25, 349–359.