Carbachol-induced long-term synaptic depression is enhanced during senescence at hippocampal CA3-CA1 synapses

Ashok Kumar
Department of Neuroscience, McKnight Brain Institute, University of Florida,
Gainesville, FL 32610

Total number pages: 34
Number of figures: 7
Number of tables: 0
Word in the abstract: 167
Word in the introduction: 609
Word in the discussion: 959

Running title: Carbachol induced synaptic depression during aging

Corresponding Author:
Ashok Kumar, Ph.D.
Department of Neuroscience, McKnight Brain Institute
University of Florida
PO Box 100244
Gainesville, FL 32610-0244, USA.
Phone (352) 392-4085
Fax (352) 392-8347
Kash@mbi.ufl.edu
ABSTRACT:

Dysregulation of the cholinergic transmitter system is a hallmark of Alzheimer's disease and contributes to an age-associated decline in memory performance. The current study examined the influence of carbachol, a cholinergic receptor agonist, on synaptic transmission over the course of aging. Extracellular excitatory postsynaptic field potentials were recorded from CA3-CA1 synapses in acute hippocampal slices obtained from young adult (5-8 months) and aged (22-24 months) male Fischer 344 rats. Bath application of carbachol elicited a transient depression of synaptic transmission which was followed by a long-lasting depression (CCh-LTD) observed 90 min after carbachol cessation in both age groups. However, the magnitude of CCh-LTD was significantly larger in senescent animals and was attenuated by NMDA receptor blockade in aged animals. Blockade of L-type Ca$^{2+}$ channels inhibited CCh-LTD to a greater extent in aged animals compared to young adults. Finally, the expression of CCh-LTD was dependent upon protein synthesis. The results indicate that altered Ca$^{2+}$ homeostasis or muscarinic activation of Ca$^{2+}$ signaling contribute to the enhanced CCh-LTD during senescence.

Key Words: Aging, hippocampus, brain slices, electrophysiology, carbachol, cholinergic receptor, synaptic plasticity, long-term depression, protein synthesis
INTRODUCTION

Pronounced dysregulation of cholinergic neurotransmission is a characteristic of Alzheimer's disease, and it is believed to be partially responsible for the cognitive impairment associated with the disease (Collerton 1986; Kasa, et al. 1997; McKinney and Jacksonville 2005; Schliebs 2005). Additionally, the decline in memory performance during “normal” aging is associated with deficits in the cholinergic system, which includes a loss of cholinergic neurons, a marked decline in acetylcholine synthesis, a decreased expression of cholinergic muscarinic receptors, and an increase in the enzyme acetylcholine esterase, which is responsible for the hydrolysis and breakdown of acetylcholine (Amenta, et al. 1995; Bartus, et al. 1982; Blaker, et al. 1988; Decker 1987; Frotscher and Leranth 1985; Gallagher and Colombo 1995; Mesulam 2004; Strong, et al. 1980; Tayebati, et al. 2002).

The hippocampus, a brain region which plays an important role in learning and memory, receives cholinergic projections from the medial septal nucleus and diagonal band of Broca (Amaral and Kurz 1985; Frotscher and Leranth 1985; Gaykema, et al. 1990; Lewis, et al. 1967). Previous studies suggest that intact cholinergic innervation is necessary for certain forms of hippocampal-dependent memory processing (Bartus, et al. 1982; Callahan, et al. 1993; Dekker, et al. 1991; Drachman and Leavitt 1974; Fibiger 1991; Hasselmo 1999). Acetylcholine mediates its effects both through the nicotinic acetylcholine receptors and the G-protein-coupled muscarinic acetylcholine receptors (mAChRs). A family of five muscarinic receptor genes encoding functionally five distinct mAChRs (M₁-M₅) is expressed throughout the central nervous system, including the hippocampus (Bonner, et al. 1987; Buckley, et al. 1988; Caulfield 1993; Vilaro, et al.

3
Thus, activation of mAChRs could influence memory by regulating synaptic plasticity, which is altered with advanced age and hypothesized to contribute to the age-associated impairments in memory function (Burke and Barnes 2006; 2010; Foster 2007; 1999; Giocomo and Hasselmo 2007; Rosenzweig and Barnes 2003).

MATERIALS AND METHODS

Subjects

Procedures involving animal subjects have been reviewed and approved by the Institutional Animal Care and Use Committee, and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Male Fischer 344 rats, young adult (5-8 months) and aged (22-24 months), were group housed (2 per cage), maintained on a 12:12 hr light schedule, and provided ad lib access to food and water.

Hippocampal slice preparation

The methods for hippocampal slice preparation have been published previously (Bodhinathan, et al. 2010; Kumar and Foster 2004; 2007; Kumar, et al. 2007). Briefly, rats were anesthetized with isoflurane (Halocarbon Laboratories, River Edge, NJ) and swiftly decapitated. The brains were rapidly removed and the hippocampi were dissected. Hippocampal slices (~ 400 µm) were cut parallel to the alvear fibers using a tissue chopper. The slices were incubated in a holding chamber (room temperature) containing standard artificial cerebrospinal fluid (ACSF) (in mM): NaCl 124, KCl 2, KH₂PO₄ 1.25, MgSO₄ 2, CaCl₂ 2, NaHCO₃ 26, and glucose 10. Thirty to sixty min before recording, 2-3
slices were transferred to a standard interface recording chamber (Harvard Apparatus, Boston, MA); the chamber was continuously perfused with standard oxygenated (95% O₂, 5% CO₂) ACSF at a flow rate of 2 ml/min. The pH and temperature were maintained at 7.4 and 30 ± 0.5°C, respectively. Humidified air (95% O₂, 5% CO₂) was continuously blown over the slices.

**Electrophysiological recordings**

Extracellular synaptic field potentials from CA3-CA1 synaptic contacts were recorded with glass micropipettes (4-6 MΩ) filled with recording medium (ACSF). Two concentric bipolar stimulating electrodes (outer pole: stainless steel, 200 µm diameter; inner pole: Platinum/Iridium, 25 µm diameter, FHC, Bowdoinham, ME) were positioned approximately 1 mm from either side of the recording electrode localized in the middle of the stratum radiatum. A single diphasic stimulus pulse of 100 µsec was passed via stimulators (SD9 Stimulator, Grass Instrument Co, West Warwick, RI) to the Schaffer collateral commissural pathway, in order to evoke field potentials at 0.033 Hz. A response baseline of the excitatory postsynaptic potential (EPSP, ~ 1.0 mV) was collected for at least 10 min prior to experimental manipulations (drug application) and for ~90 min following drug washout.

The signals were amplified, filtered between 1 Hz and 1 kHz, and stored for off-line analysis. Two cursors were placed around the initial descending phase of the waveform, and the maximum slope (mV/ms) of the EPSP was determined by a computer algorithm that found the maximum change across all sets of 20 consecutively recorded points (20 kHz sampling rate) between the two cursors. Changes in transmission properties induced by application of drug were calculated as the percent change from the
averaged baseline responses collected. For paired-pulse, a 50 msec inter-pulse interval was used. The paired pulses ratio was calculated by dividing the slope of the second synaptic response by the slope of the first response. To determine the effects of carbachol on the paired pulses ratio, the paired pulses ratio for each response was normalized by the average ratio calculated for the baseline recording.

**Drugs**

All drugs were bath applied by the addition of ACSF. AP-5 was obtained from Tocris (Tocris Bioscience, Ellisville, MO). Carbachol (carbamylcholine chloride), atropine, pirenzepine, nifedipine, and anisomycin were obtained from Sigma-Aldrich. Carbachol (50 µM), atropine (1 µM), pirenzepine (1 µM), and AP-5 (100 µM) were dissolved directly in ACSF. Nifedipine (10 µM) was initially dissolved in a small amount of dimethyl sulfoxide (DMSO) and diluted further by ACSF to a final DMSO concentration of 0.01%. Anisomycin (20 µM) was initially dissolved in a small amount of ethyl alcohol and diluted further by the ACSF. All antagonists were bath applied for 20-40 min before application of carbachol and had no noticeable effect on baseline synaptic transmission (data not shown).

**Statistical analysis**

All statistical analyses were performed using StatView 5.0 (SAS Institute Inc, NC). Student’s *t*-tests, with a significance set at *p*<0.05, were employed to determine any changes in synaptic response induced by carbachol, as well as any possible differences between baseline and PPF ratio. Analysis of variance (ANOVA) was used to examine the interaction of age and drug treatment, and follow up ANOVAs were employed to localize age differences. Post hoc comparisons of the effects of pharmacological treatment
relative to the control condition on the level of CCh-LTD were performed using Fisher’s PLSD with a significance set at p < 0.05.

RESULTS

Synaptic depression induced by muscarinic M₁ activity is enhanced during senescence

The application of the nonselective cholinergic receptor agonist, carbachol (50 µM for 10 min), induces LTD in visual and perirhinal cortices, as well as the hippocampus of young animals, (Kirkwood, et al. 1999; Massey, et al. 2001; McCoy, et al. 2008; McCoy and McMahon 2007; McCutchen, et al. 2006; Scheiderer, et al. 2006; Scheiderer, et al. 2008; Volk, et al. 2007). Here, in hippocampal slices obtained from young adult (5-8 months) and aged (22-24 months) animals, I show that bath application of carbachol (50 µM for 10 min) rapidly decreased synaptic responses to a similar magnitude in young adult (22.02 ± 1.68 % of baseline, n = 23) and aged (23.83 ± 1.59, n = 29) animals. However, age differences began to emerge ~30 min following the start of washout, such that the synaptic responses were significantly decreased for aged animals, relative to young adults, at 30 min [F (1, 50) = 14.18, p < 0.0004], 60 min [F(1,49) = 22.82, p < 0.0001], and 90 min F(1, 23) = 6.02, p < 0.02] following cessation of carbachol application (Fig 1).

Pre-incubation (30-40 min) of the slices with atropine (1 µM) completely blocked the carbachol-induced synaptic depression in young adult (n = 5) [F (1, 16) = 143.99, p < 0.0001] and aged (n = 6) [F (1, 16) = 197.27, p < 0.0001] animals compared to respective control groups (young n = 12, aged n = 14) (Fig 2A&B). Furthermore, pre-incubation of
slices with the M₁ muscarinic receptor selective antagonist, pirenzepine (1 µM, 20-30 min) reduced the transient depression, such that the response recovered to baseline within 30 min of carbachol cessation in both age groups (Fig 2C&D). A higher dose of pirenzepine (10 µM) completely blocked the transient phase of carbachol-induced synaptic depression (data not shown).

**Mechanism for enhanced synaptic depression in aged animals**

To determine whether presynaptic mechanisms mediate the age-related difference in carbachol-induced synaptic depression, paired pulses facilitation was examined. In general, a change in the paired-pulse ratio suggests a presynaptic locus of expression, whereas no change indicates involvement of postsynaptic mechanisms (Dobrunz and Stevens 1997). For young adults, carbachol significantly increased the paired-pulse ratio relative to baseline during agonist application (p < 0.005, n = 15), and at 15 min (p < 0.0002, n = 15), 30 min (p < 0.0002, n = 15), and 60 min (p < 0.008, n = 14) following drug washout. No change in the paired-pulse ratio was observed 90 min after carbachol cessation. Similarly, in the case of aged rats, the paired-pulse ratio was significantly increased above baseline during agonist application (p < 0.007, n = 17), and at 15 min (p < 0.0002, n = 17), 30 min (p < 0.02, n = 17), and 60 min (p < 0.02, n = 17) following cessation of carbachol. Furthermore, there was no difference in the paired-pulse ratio between baseline and at 90 min after carbachol washout (Fig 3B and C). Finally, young adult rats exhibited a larger increase in the paired-pulse ratio than senescent animals at 15 min [F (1, 30) = 12.01, p < 0.002] and 30 min [F (1, 30) = 6.64, p < 0.02], following carbachol cessation (Fig 3C). The results suggest that reduced transient presynaptic transmitter release contributes to the early phase of carbachol-induced synaptic
depression in young adult and aged animals, which is consistent with previous results reported in cortex (Kimura and Baughman 1997; McCoy and McMahon 2007; Mrzljak, et al. 1993). However, the absence of a shift in paired-pulse ratio at ~60-90 min, following the start of drug washout, suggests that a shift in transmitter release does not mediate the age-difference in long-term synaptic depression induced by carbachol.

Activation of metabotropic glutamate or muscarinic receptors induces a form of long-term depression that requires protein synthesis (Huber, et al. 2000; Kumar and Foster 2007; Massey, et al. 2001; McCoy and McMahon 2007; Nosyreva and Huber 2005; Volk, et al. 2007). To determine the role of protein synthesis in CCh-LTD, slices were pre-incubated (30-40 min) in the protein synthesis inhibitor, anisomycin (20 µM). Anisomycin significantly attenuated the long-lasting synaptic depression in young adult [F (1, 15) = 4.60, p < 0.04] compared to respective interleaved vehicle control (n = 10, Fig 4A). Pre-incubation of slices obtained from aged rats in anisomycin, significantly reduced the CCh-LTD [F (1, 23) = 5.54, p < 0.02] compared to vehicle control (n = 10, Fig 4B). These results suggest that the expression of synaptic depression induced by muscarinic agonist is dependent upon protein synthesis in young adult and aged animals; however, the dependence of carbachol-induced synaptic depression on protein synthesis probably wanes with aging.

Another form of synaptic depression requires Ca^{2+} influx from N-methyl-D-aspartate (NMDA) receptors and/or L-type voltage gated Ca^{2+} channels, and is protein synthesis independent (Christie, et al. 1997; Norris, et al. 1998; Oliet, et al. 1997; Thiels, et al. 1994). In order to determine whether CCh-LTD requires Ca^{2+} from these sources, I pre-applied (30-40 min) the NMDA receptor blocker, AP-5, or the L-type Ca^{2+} channel...
antagonist, nifedipine, to the bath solution of slices obtained from young adult and aged
animals. Pre-incubation of slices with AP-5 (100 µM), failed to block the CCh-LTD in
slices obtained from young adults (n = 5), but significantly reduced CCh-LTD in aged
rats (p < 0.008, n = 5) compared to respective interleaved control groups (young, n = 12,
aged, n = 14) (Fig 5 A&B). Prior application of nifedipine (10 µM) reduced the long-
lasting synaptic depression in young adult animals (p < 0.007, n = 5) compared to control
(n = 12), and blocked the CCh-LTD in aged animals (p < 0.0001, n = 5) compared to
control (n = 14) (Fig 5 C&D). However, nifedipine failed to attenuate the transient phase
of CCh-LTD in either group.

Synaptic depression induced by carbachol does not require synaptic activation

The reduction of CCh-LTD by the NMDA receptor antagonist, AP-5 suggests a
role for synaptic activity in induction processes. In order to elucidate the role of synaptic
activation in mediating the enhanced synaptic depression induced by carbachol during
senescence, I performed experiments wherein carbachol was applied without concurrent
synaptic stimulation. The baseline synaptic activation was turned off to the second path
during carbachol application and 10 min following carbachol cessation. No difference
was observed in the level of synaptic depression induced by carbachol with and without
synaptic stimulation in young adult (Fig 6) and aged (Fig 7A & C) animals. To control
for the possibility that carbachol was inducing activity in CA3 cells (Bianchi and Wong
1994), we examined the effects of afferent stimulation during carbachol application in
slices wherein the CA3 region was surgically removed. Compared to intact slices, there
was no difference in the magnitude of CCh-LTD in slices in which the CA3 region was
removed, regardless of whether stimulation occurred during carbachol application (Fig
The results are consistent with earlier studies, demonstrating that CCh-LTD is independent of synaptic activation in younger animals (Massey, et al. 2001; Volk, et al. 2007).

**DISCUSSION**

The principal finding of the present study is that the carbachol-induced synaptic depression at CA3-CA1 hippocampal synapses is enhanced in aged animals relative to young adults. At least two major forms of LTD can be observed in the CA1 hippocampal region. One form can be observed following activation of metabotropic glutamate receptors, which is dependent upon protein synthesis (Huber, et al. 2000; Kumar and Foster 2007; Massey, et al. 2001; McCoy and McMahon 2007; Nosyreva and Huber 2005; Volk, et al. 2007). The other form is protein-synthesis independent and requires membrane depolarization, as well as a rise in postsynaptic Ca$^{2+}$ influx through NMDA receptors and L-channels (Bolshakov and Siegelbaum 1994; Christie, et al. 1997; Dudek and Bear 1992; Mulkey and Malenka 1992; Norris, et al. 1998; Oliet, et al. 1997; Thiels, et al. 1994). It is now apparent that the susceptibility and expression of these forms of LTD change across the lifespan (Dudek and Bear 1993; Kemp, et al. 2000; Kumar and Foster 2007; Norris, et al. 1996; Nosyreva and Huber 2005). The results of the current study reveal that CCh-LTD, like stimulation induced LTD (Foster 2002; Foster and Kumar 2002; Norris, et al. 1998; Norris, et al. 1996) and metabotropic receptor activation induced LTD (Kumar and Foster 2007), is enhanced during senescence. In each case, there is an essential role for L-channels in mediating age differences, supporting the idea that altered Ca$^{2+}$ homeostasis contributes to age differences in synaptic depression (Foster
288 2007). However, it should be noted that the metabotropic receptors induced LTD and
289 CCh-LTD during senescence, also involve a significant contribution of NMDA receptors.
290 NMAD receptor and L-channel activity depend upon the membrane potential, and
291 cholinergic activity can increase excitation of hippocampal neurons (Benson, et al. 1988;
293 Muller and Misgeld 1986). Previous researchers have suggested that an increase in Ca$^{2+}$
294 signaling following M$_1$ muscarinic receptor activation, is likely due to the inhibition of
295 the afterhyperpolarization and enhancement of a slow onset depolarization (Egorov, et al.
296 1999; Wu, et al. 2004). Moreover, muscarinic activity can have direct effects that
297 increase NMDA receptor currents (Markram and Segal 1990) and enhance the Ca$^{2+}$
298 signaling associated with backpropagating action potentials (Cho, et al. 2008), which
299 augment NMDA receptor activity and increase Ca$^{2+}$ influx due to increased cell discharge
300 activity. A reduction in synaptic depression by NMDA receptor blockade suggests that
301 CCh-LTD depends upon synaptic activity. However, we observed that carbachol induces
302 significant synaptic depression both in stimulated and non-stimulated pathways in the
303 same slice, suggesting another source of glutamate. Recent work indicates that CCh can
304 act on muscarinic receptors on astrocytes, increasing intracellular Ca$^{2+}$ and triggering the
306 Parpura, et al. 1994; Shelton and McCarthy 2000). The results are consistent with earlier
307 studies, demonstrating that CCh-LTD is independent of synaptic activation in younger
308 animals (Massey, et al. 2001; Volk, et al. 2007). In contrast, other studies have shown
309 that CCh-LTD in the visual cortex (Kirkwood, et al. 1999) and in the hippocampus
310 (Scheiderer, et al. 2006) of juvenile animals depends upon synaptic activation, suggesting
the possibility of a developmental shift in the induction mechanism as observed during metabotropic glutamate receptor activation-induced synaptic depression (Dumas and Foster 1997; Kumar and Foster 2007; Luscher and Huber; Nosyreva and Huber 2005).

Previous studies have demonstrated that M₁ muscarinic cholinergic receptors contribute to the regulation of synaptic plasticity in young rats (Kirkwood, et al. 1999; Scheiderer, et al. 2006). Further, a recent study indicates that M₁ receptors are required for another form of long-term synaptic depression induced by metabotropic glutamate receptor agonist (s)-3, 5-dihydroxyphenylglycine (Kamsler, et al. 2010). Results of the current study that pirenzepine, a selective M₁ cholinergic receptor antagonist significantly attenuated the carbachol-LTD in both young adult and aged rats, extends previous findings to aged animals, demonstrating that M₁ cholinergic receptors play a role in modulating synaptic plasticity in the aging hippocampus. Expression of CCh-LTD likely involves presynaptic mechanisms and is protein-synthesis dependent that is consistent with earlier findings, which show that the expression of various forms of LTD requires protein synthesis (Huber, et al. 2000; Kumar and Foster 2007; Massey, et al. 2001; McCoy and McMahon 2007; Nosyreva and Huber 2005; Sajikumar and Frey 2003; Volk, et al. 2007).

The CCh-LTD imitates endogenous and cholinergic-dependent synaptic depression of glutamate transmission, and studies have established that cholinergic innervations to the hippocampus are pivotal to normal learning and memory functions (Bartus, et al. 1982; Callahan, et al. 1993; Dekker, et al. 1991; Drachman and Leavitt 1974; Fibiger 1991; Hasselmo 1999). Alterations in cholinergic transmission due to aging or pathological conditions could contribute to a decline in cognitive functions. The results
indicate that CCh-LTD is one of the few forms of synaptic plasticity that can alter during senescence (Foster 2002), and may contribute to an age-related reduction in synaptic transmission in the CA1 region (Barnes, et al. 1992; Jouveneau, et al. 1998; Landfield, et al. 1986); thus causing a memory decline in aged animals (Barnes, et al. 2000; Deupree, et al. 1993).

Aging is associated with a marked decrease in the cholinergic system, including a decline in acetylcholine synthesis, release, presence in the synapse. Furthermore, the cholinergic hypothesis of aging states that serious loss of function of the cholinergic neurons arising from the basal forebrain and terminating in the cortex and hippocampus contributes to impairment in cognitive function associated with advanced age and neurodegenerative diseases (Bartus 2000; Bartus, et al. 1982). Despite years of research, enhancing cholinergic neurotransmission is the primary pharmacological intervention for treating and forestalling the cognitive deficit associated with neurodegenerative diseases. The results of the current study demonstrate that even in the face of less cholinergic tone in the hippocampus with advanced age, the senescent CA3-CA1 synapses are capable of cholinergic modulation. In addition, the higher concentration of muscarinic agonist used in this study, as well as several previous studies (Kirkwood, et al. 1999; Massey, et al. 2001; McCoy, et al. 2008; McCoy and McMahon 2007; McCutchen, et al. 2006; Scheiderer, et al. 2006; Scheiderer, et al. 2008; Volk, et al. 2007), may not represent the in vivo condition, but emphasize the functional significance of modulating cholinergic transmission exogenously. Future studies using a lower concentration of muscarinic agonist could possibly represent such a condition in vivo and may provide an explanation about both the waning cholinergic function with advanced age and neurodegenerative
diseases, as well as the intrinsic mechanisms that contribute to enhanced synaptic depression.

In conclusion, the present study demonstrates that CCh-LTD is enhanced in senescent when compared to young adults. The data provide evidence for a role of L-channels in the induction of carbachol-LTD during aging as reported previously in the visual cortex (McCoy and McMahon 2007). The finding of age-related differences in carbachol-induced synaptic depression, including a role of NMDA receptors and L-channels, provides an avenue for investigating the role of modulating cholinergic transmission in learning and memory during aging. Future studies will better elucidate the signaling cascades involved in the enhanced carbachol-induced synaptic depression at CA3-CA1 hippocampal synapses, as well as influence of cholinergic modulation on other forms of synaptic plasticity and cognitive function during senescence.

Acknowledgements:

The author is extremely grateful to Prof. Thomas C. Foster, who let me use his laboratory resources to conduct the required experiments, and for providing encouraging comments and suggestions. This work was supported by, National Institutes of Health Grant AG014979, MH059891 and the Evelyn F. McKnight Brain Research Grant. Special thanks Dr. Lori McMahon for valuable discussion and providing important insights and to Kace William King and Jose E. Herrera for editorial assistance.


Araque A, Sanzgiri RP, Parpura V, and Haydon PG. Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J Neurosci* 18: 6822-6829, 1998b.

Auerbach JM, and Segal M. Muscarinic receptors mediating depression and long-term potentiation in rat hippocampus. *J Physiol* 492 (Pt 2): 479-493, 1996.


Kamsler A, McHugh TJ, Gerber D, Huang SY, and Tonegawa S. Presynaptic m1 muscarinic receptors are necessary for mGluR long-term depression in the hippocampus. *Proc Natl Acad Sci U S A* 107: 1618-1623, 2010.


Kremin T, Gerber D, Giocomo LM, Huang SY, Tonegawa S, and Hasselmo ME. Muscarinic suppression in stratum radiatum of CA1 shows dependence on presynaptic M1 receptors and is not dependent on effects at GABA(B) receptors. *Neurobiol Learn Mem* 85: 153-163, 2006.


Oh MM, Power JM, Thompson LT, Moriearty PL, and Disterhoft JF. Metrifonate increases neuronal excitability in CA1 pyramidal neurons from both young and aging rabbit hippocampus. *J Neurosci* 19: 1814-1823, 1999.


Valentino RJ, and Dingledine R. Presynaptic inhibitory effect of acetylcholine in the hippocampus. J Neurosci 1: 784-792, 1981.


Wu WW, Chan CS, and Disterhoft JF. Slow afterhyperpolarization governs the development of NMDA receptor-dependent afterdepolarization in CA1 pyramidal neurons during synaptic stimulation. *J Neurophysiol* 92: 2346-2356, 2004.
Figure legends

**Figure 1.** Long-term synaptic depression induced by the selective muscarinic agonist carbachol is increased during senescence. (A) Time course of synaptic responses recorded from aged (filled circle, n = 15) and young adult (open circle, n = 10) rats, showing the 10 min baseline, a rapid decrease during carbachol (50 µM, 10 min) application (solid line), and recovery during a 90 min washout period. For the purpose of clarity, each data point represents the mean of four consecutive responses. (B) Representative traces of EPSP responses for the time points indicated in A for young adult (right) and aged (left) rats. (C) Distribution of normalized EPSP slopes for slices obtained from aged (filled circle) and young adult (open circle) rats following 30 (left panel), 60 (middle panel), and 90 (right panel) mins following carbachol washout. (D) Mean of carbachol-induced synaptic depression during carbachol application (CCh), 15, 30, 60, and 90 min following carbachol washout in aged (filled bar) and young adult (open bar) for all rats. The means are represented by the horizontal black bars. For this and all subsequent figures, error bars equal SEM. Asterisk represents a significance difference relative to baseline (dashed line). Pound sign represents a significant difference between the aged and young adult groups. The number above each bar indicates number of slices recorded for each group at each time point.

**Figure 2.** Synaptic depression induced by carbachol is dependent on activation of muscarinic receptors. Cholinergic receptor selective antagonist, atropine (1 µM) completely blocked CCh-LTD in young adult (n = 5) and aged (n = 6) rats. Time course of synaptic responses showing the 10 min baseline before carbachol application, during application (solid line), and following 30 min carbachol washout in presence (filled
Carbachol applied in presence of pirenzepine (1 µM) in slices obtained from young adult (C) and aged (D) rats reduced the carbachol mediated synaptic depression in young adult (n = 5) and aged (n = 5) rats. Representative traces of EPSP responses for the time points indicated in C and D for control (left panel) and pirenzepine (right panel) slices obtained from young-adult and aged rats.

**Figure 3.** Effect of carbachol on the paired-pulse ratio in young adult and aged rats. Time course of carbachol effects on the first (filled circle) and second (open circle) synaptic response and paired-pulse ratio (filled diamond) in slices obtained from young adult (A) and aged (B) rats. Each individual response was computed as a percent of the mean baseline response (dashed line) collected during the 10 min just prior to carbachol application. For the purpose of clarity, each data point represents the mean of four consecutive responses. C) Mean paired-pulse ratio during carbachol application (CCh), 15, 30, 60, and 90 min following carbachol washout in aged (filled bar) and young adult (open bar) rats. Error bars equal SEM. Asterisks represent a significance difference relative to baseline and pound sign represents a significant difference between the aged and young adult groups. The number above each bar indicates number of slices recorded for each group at each time point.

**Figure 4.** Expression of carbachol induced synaptic depression is dependent on protein synthesis. Time course of the effects of pre-incubation of slices with protein synthesis inhibitor, anisomycin on carbachol induced synaptic depression. Pre-incubation of slices
with anisomycin (20 µM) obtained from (A) young adult (n = 7) or (B) aged (n = 10) animals significantly attenuated the CCh-LTD. Bar diagrams showing the mean of EPSP responses 90 min following wash out of carbachol recorded in slices obtained from young adult and aged rats in presence (filled bar) and absence of anisomycin (open bar). Relative to control (Cont), CCh-LTD was significantly reduced by pre-incubation with anisomycin (Aniso) in both groups. Asterisk indicates a significant depression from the control group. Representative traces of EPSP responses for the time points indicated in A and B of carbachol induced-LTD with and without (control) anisomycin from both age groups.

**Figure 5.** Age differences in CCh-LTD depend on activation of NMDA receptors and L-type Ca\(^{2+}\) channels. Time course of the effects of pre-incubation of slices with AP-5 and nifedipine on carbachol induced synaptic depression. Bath application of NMDA receptor inhibitor, AP-5 (100 µM, n = 5) did not reduce the CCh-LTD in (A) young adults (n = 5) but significantly attenuated it in (B) aged rats (n = 5). (C) Pre-incubation of slices with L-type Ca\(^{2+}\) channel blocker, nifedipine (10 µM, n = 5) reduced the late phase of CCh-LTD in (C) young adults (n = 5) and blocked LTD in (D) aged rats (n = 5) without affecting the transient synaptic depression. Bar diagrams showing the mean of EPSP responses 30 min following wash out of carbachol recorded in slices obtained from young adult (E) and aged (F) rats. Relative to control, CCh-LTD was impaired by pre-incubation with nifedipine (Nif) in both groups; AP-5 partially but significantly reduced the CCh-LTD in aged rats. Asterisks indicate significant depression from baseline (dotted line) and pounds
sign indicate significant difference from the control level of CCh-LTD; † indicates significant age difference.

**Figure 6.** Carbachol-induced synaptic depression does not require synaptic activation in young adults. (A) Time course of synaptic responses showing the 10 min baseline before carbachol application, a rapid decrease during application (solid line), and continued synaptic depression during a 60 min washout in absence (open circle) and in presence (filled circle) of synaptic activation for young adult rats. The basal stimulation was turned off during and 10 min following carbachol application. Carbachol induced significant synaptic depression in stimulated and un-stimulated paths. For the purpose of clarity, each data point represents the mean of four consecutive responses. The inserts show representative traces of EPSP responses for the time points indicated in A for stimulated (left panel) and un-stimulated (right panel) paths. (B) Bar diagrams show the level of synaptic depression following 30 and 60 min of carbachol washout in stimulated (filled bar) and un-stimulated (open bar) paths. Asterisks represent a significance difference relative to baseline (dotted line).

**Figure 7.** Carbachol-induced synaptic depression in intact and region CA3 removed slices obtained from aged animals. Time course of synaptic responses showing the 10 min baseline before carbachol application, a rapid decrease during application (solid line), and continued synaptic depression during a 60 min washout in absence (open circle) and in presence (filled circle) of synaptic activation for intact (A) and region CA3 removed (B) slices. The basal stimulation was turned off during and 10 min following
carbachol application. Carbachol induced significant synaptic depression in stimulated and un-stimulated paths in intact as well as CA3 removed slices. For the purpose of clarity, each data point represents the mean of four consecutive responses. Bar diagrams show the level of synaptic depression following 30 and 60 min of carbachol washout in stimulated (filled bar) and un-stimulated (open bar) paths for intact (C) and CA3 removed (D) slices. Asterisks represent a significance difference relative to baseline. Distribution of normalized EPSP slopes in all experiments in aged rats for stimulated (filled circle) and un-stimulated (open circle) paths following 30 and 60 min of carbachol cessation in intact (E) and region CA3 removed (F) slices. The mean responses are also shown (horizontal black bar). Representative traces of EPSP responses for the time points indicated in A and B for stimulated (upper panel) and un-stimulated (lower panel) paths for intact (left) and region CA3 removed (right) slices obtained from aged rats.