Characterization of age-related changes in synaptic transmission onto F344 rat basal forebrain cholinergic neurons using a reduced synaptic preparation

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Griffith WH, DuBois DW, Fincher A, Peebles KA, Bizon JL, Murchison D. Characterization of age-related changes in synaptic transmission onto F344 rat basal forebrain cholinergic neurons using a reduced synaptic preparation. J Neurophysiol 111: 273–286, 2014. First published October 16, 2013; doi:10.1152/jn.00129.2013.—Basal forebrain (BF) cholinergic neurons participate in a number of cognitive processes that become impaired during aging. We previously found that age-related enhancement of Ca2+ buffering in rat cholinergic BF neurons was associated with impaired performance in the water maze spatial learning task (Murchison D, McDermott AN, Lasarge CL, Peebles KA, Bizon JL, and Griffith WH. J Neurophysiol 102: 2194–2207, 2009). One way that altered Ca2+ buffering could contribute to cognitive impairment involves synaptic function. In this report we show that synaptic transmission in the BF is altered with age and cognitive status. We have examined the properties of spontaneous postsynaptic currents (sPSCs) in cholinergic BF neurons that have been mechanically dissociated without enzymes from behaviorally characterized F344 rats. These isolated neurons retain functional presynaptic terminals on their somata and proximal dendrites. Using whole cell patch-clamp recording, we show that sPSCs and miniature PSCs are predominately GABAergic (bicuculline sensitive) and in all ways closely resemble PSCs recorded in a BF in vitro slice preparation. Adult (4–7 mo) and aged (22–24 mo) male rats were cognitively assessed using the water maze. Neuronal phenotype was identified post hoc using single-cell RT-PCR. The frequency of sPSCs was reduced during aging, and this was most pronounced in cognitively impaired subjects. This is the same population that demonstrated increased intracellular Ca2+ buffering. We also show that increasing Ca2+ buffering in the synaptic terminals of young BF neurons can mimic the reduced frequency of sPSCs observed in aged BF neurons.

basal forebrain; aging; synaptic transmission; GABA; behavior

DISCOVERY OF THE MECHANISMS responsible for age-related cognitive decline in humans remains among the most elusive goals in neuroscience. In contrast to the intuitive supposition that age-related cognitive impairment is the result of a general breakdown in neural systems, current evidence suggests that many neuronal properties are intact during aging and that a variety of subtle mechanisms are thought to contribute to age-related cognitive impairment (Burke and Barnes 2006; Toescu and Verkhratsky 2007). Regardless of exactly which pathways are affected, the ultimate endpoint is likely to be altered synaptic transmission. There is increasing evidence that disruption of synaptic function contributes to cognitive impair-
We have utilized a “reduced synaptic preparation” to study properties of spontaneous synaptic transmission during aging. This preparation consists of dissociated neurons with attached synaptic boutons (Akaike and Moorhouse 2003; Drew et al. 1988; Jun et al. 2011; Vorobjev 1991) and has been used previously to study presynaptic Ca\(^{2+}\) channels and GABAergic synaptic currents in the rat BF (Akaike et al. 1992; Rhee et al. 1999). We have extended these earlier studies that employed young animals to now include neurons from mature adults and aged individuals (up to 24 mo). Because disruption of the BF cholinergic system during aging is thought to be a critical determinant of age-related cognitive impairment, dementia, and pathology (Sarter and Bruno 2004), this preparation is an excellent model to study age-related changes in synaptic physiology and cognitive decline.

**METHODS**

**Animal subjects.** Male Fisher 344 rats were obtained from the NIA colony (Harlan, Indianapolis, IN) at 1–7 mo (young; \(n = 30\)) and 22–24 mo (aged, \(n = 26\)). Rats were housed in an AALAC-accredited vivarium that was maintained at a constant 25°C with a 12:12-h light-dark cycle and with free access to food and water. Some of the rats were used for behavioral characterization of cognitive status, and these were euthanized between 1 wk and 2 mo posttraining. The mean ages at death were 4.6 ± 0.4 mo for young rats and 23.7 ± 0.2 mo for aged rats. All animal procedures were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee.

**Behavioral characterization.** Rats were trained to perform a spatial memory task in a water maze consisting of a large circular tank (diameter 183 cm, wall height 58 cm) painted white and filled with water (27°C) made opaque by the addition of nontoxic paint. A retractable escape platform (diameter 12 cm; HVS Image, Buckingham, UK) was submerged 2 cm under the surface. External cues were visible in the form of large white shapes on black curtains. A video tracking system with camera mounted above the maze was used to record the behavioral data (Water 2020; HVS Image). The white F344 rats had a black patch dyed on their backs so they could be resolved by the motion-capture software.

The training protocol has been described in detail previously (Bizon et al. 2009; Gallagher et al. 1993). Rats received 3 training trials per day for 8 consecutive days. On each training trial, rats were placed in the water and allowed to swim until finding the platform or for 90 s, at which time they were moved to the platform by the experimenter. Rats remained on the platform for 30 s before the trial was ended and the animal was removed from the maze. The platform remained in a constant location while the swimming start position was changed for each trial. Every sixth trial was a probe trial, with the platform unavailable at the bottom of the pool for the first 30 s of the trial. Training and probe trials assess acquisition and search strategy, respectively (Bizon et al. 2009).

**Solutions and drugs.** The internal pipette solution contained (in mM) 124 CsCl, 20 HEPES, 10 TEA, 4 ATP, 10 EGTA, and 2 MgCl\(_2\), prepared with RNase-free water under sterile conditions, and contained 50 µl/mL RNase inhibitor (10 U/µl; Life Technologies, Carlsbad, CA), with pH adjusted to 7.2 using CsOH. Cells were continually perfused after dispersal with a recording solution containing (in mM) 140 NaCl, 3 KCl, 2.5 CaCl\(_2\), 1.2 MgCl\(_2\), 10 HEPES, and 33 n-glucose (pH 7.4 with NaOH, 310–330 mosM). The elevated [K\(^+\)] solution was identical except that it contained 10 mM KCl. The initial holding solution was slightly modified to contain only 1 mM CaCl\(_2\) and 2.7 mM KCl and was continuously oxygenated with 100% O\(_2\). The recording solution for brain slices contained (in mM) 124 NaCl, 3 KCl, 2 CaCl\(_2\), 1.2 MgCl\(_2\), 26 NaHCO\(_3\), 1.2 NaHPO\(_4\), and 10 n-glucose. This solution was bubbled with 5% CO\(_2\)-95% O\(_2\). Tetradotoxin (TTX; 500 or 1,000 nM; Calbiochem, La Jolla, CA) was present in the recording solution for recording miniature PSCs (mPSCs) and was included in the holding solution at 100 nM. Isolated inhibitory PSCs (iPSCs) were recorded in the presence of (-)-2-amino-5-phosphonovaleric acid (APV; 40 µM) and 6,7-dimethyl-2-nitrosoxamine-2,3-dione (DNOX; 20 µM). Bicuculline (10 µM) was added to the recording solution to block the iPSCs. Baclofen (10 µM) was dissolved in the recording solution. In a few experiments, BAFTA-AM was loaded into dissociated neurons at a final concentration of 3 µM in the recording solution with the same concentration of pluronic acid (Life Technologies). Loading time was 150 s, after which the solution was exchanged with normal recording solution and the cells were allowed to sit for 30 min to permit de-esterification of...
the BAPTA-AM. All chemicals were obtained from Sigma (St. Louis, MO) unless indicated otherwise.

Single-cell RT-PCR. Neurons were aspirated into the recording pipette, and the contents were ejected into a sterile nuclease-free 0.6-mL tube containing the initial RT solution (Griffith et al. 2006; Han et al. 2005). The tube was frozen in dry ice and then stored overnight (−80°C). Standard RT was performed with SuperScript II reverse transcriptase (Life Technologies).

Real-time PCR was performed with an ABI Prism 7700 or an ABI 7500 Fast sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan (Applied Biosystems) detection probes (Han et al. 2005). Primer Express 1.5 (Applied Biosystems) was used to design forward and reverse primers and TaqMan probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), choline acetyltransferase (ChAT), and glutamic acid decarboxylase 67 (GAD). Positive controls (whole brain cDNA) were run on each reaction plate, and negative controls included PCR without cDNA template, aspirated bath solution, and cDNA template without RT. Data were expressed as an amplification plot, and detection of a transcript was confirmed if the linear phase of the amplification plot (parallel to the positive marker GAPDH) crossed the threshold fluorescence intensity (CT; set above the fluorescent noise) before reaching the 36th amplification cycle. Nonparallel amplifications or plots crossing the CT after the 36th cycle were considered not detected. This limit was imposed because false positive signals from GAPDH (in negative controls) were occasionally observed to cross the CT after the 36th cycle.

Electrophysiological analyses. Analysis of the electrophysiological data was conducted blind with respect to cognitive status of the subject and to PCR results. Mini Analysis (6.0.7; Synaptosoft) and Clampfit 10 (Molecular Devices) software were employed for the analysis. Cumulative probability curves were compared with a Kolmogorov-Smirnov (K-S) test, whereas mean data were compared using a one-way ANOVA with Dunnett’s test for multiple comparisons or a Kruskal-Wallis rank test with a Dunn’s method multiple comparison for nonparametrically distributed data, as appropriate, with significance determined by $P < 0.05$ (SigmaPlot 12; SPSS, Chicago, IL). Values are means ± SE.

Identification of spontaneous postsynaptic currents. Most data are reported as spontaneous postsynaptic currents (sPSCs), which were recorded with no drugs in the recording solution. We specify sPSCs when the currents were recorded in APV/DNQX or when the currents were visually identified by decay kinetics (see RESULTS). Similarly, excitatory PSCs (ePSCs) are specified if currents were recorded in bicuculline or were visually identified. We refer to mPSCs if the currents were recorded in TTX.

RESULTS

The enzyme-free, acutely dissociated neuron reduced synaptic preparation. To investigate cell type-specific properties of synaptic physiology during aging with greater efficiency than that which can be attained in an aged slice preparation, we utilized the reduced synaptic preparation. This reduced preparation consists of acutely dissociated neurons that are not exposed to the usual proteolytic enzyme treatment and therefore retain functional presynaptic inputs on somas and proximal dendrites (Akaike and Moorhouse 2003). Figure 1 presents two methods of visualizing these regions of synaptic contact with fluorescence microscopy. Acutely dissociated BF neurons (Fig. 1A) can be treated with the fluorescent dye FM1-43 under conditions that encourage the uptake of this dye by cycling (endo- and exocytotic) membranes, such as those present at presynaptic terminals. After the loading of the terminals, the neurons are switched to a chilled low-[K+]o, low-[Ca2+]i medium that reduces spontaneous membrane activity and tends to retain the dye in the terminals. Under these conditions, most of the fluorescent signal is observed as moderately bright puncta or regions around the cell membrane that are likely to be presynaptic contacts (Fig. 1B). Some of these labeled regions appear to be associated with small structures visible in the differential interference contrast (DIC) image shown in Fig. 1A. Another method employs immunofluorescence techniques on fixed neurons with antibodies directed at presynaptic markers, such as synaptotagmin. Figure 1C shows an acutely dissociated BF neuron from a transgenic mouse that has expression of enhanced green fluorescent protein (eGFP) tied to the ChAT promoter so that cholinergic neurons fluoresce green. The red signal originates from regions of immunofluorescence for the presynaptic marker synaptotagmin (shown separately in Fig. 1D). Many of these regions of apparent synaptic contacts are located on proximal dendrites, with fewer observed in somatic areas. Both of these visualization methods demonstrate the utility of this preparation to investigate synaptic mechanisms.

Postsynaptic currents in the reduced synaptic preparation. Standard whole cell voltage clamp was used to record sPSCs of BF neurons. A comparison of sPSCs between a thin brain slice preparation and the reduced synaptic preparation is shown in Fig. 2. The properties of the sPSCs in the two preparations are quite similar, and the synaptic inputs to both preparations are dominated (>95%) by GABAergic IPSCs as judged by bicuculline sensitivity. Although there are enough EPSCs to generate an average EPSC in these examples in Fig. 2, it was common that no EPSCs were observed in the presence of bicuculline in the reduced synaptic preparation. In bicuculline (10 µM) alone, the mean sPSC frequency was 0.016 ± 0.011 Hz (n = 7) in the reduced synaptic preparation. For this reason we concentrated our investigation on the IPSCs. In this example, mean EPSCs and mean IPSCs are similar in the two preparations with the most obvious difference being slower decay kinetics of the IPSC in the slice relative to the reduced synaptic preparation. This may be due to a location of some synaptic inputs in the slice that are more distal than any in the reduced preparation. The great advantage of the reduced synaptic preparation is that it enables an abundance of data to be collected from aged subjects. Brain slice recordings from aged rats are difficult to obtain because of visualization issues and extensive extracellular matrix with age, thereby effectively limiting data from each animal. This shortcoming can be overcome with the isolated preparation.

Properties of IPSCs in the BF neuron reduced synaptic preparation are detailed in Fig. 3. In this example from an aged rat, sPSCs in control recording solution are shown with the superimposed sPSCs below (Fig. 3A). When TTX (500 nM) is added to the bath, mPSCs are observed (Fig. 3B). When the glutamate receptor antagonists APV (40 µM) and DNQX (10 µM) are included with TTX, as in Fig. 3C, mIPSCs are isolated. As expected for neurons with a synaptic input profile dominated by GABAergic transmission, the GABA_A receptor antagonist bicuculline (10 µM) blocks mIPSCs (Fig. 3D) and frequency is reduced to zero. During 3 or more minutes of recording mIPSCs in bicuculline, 9/11 neurons displayed zero or one mEPSC and the greatest frequency was 0.038 Hz. The scRT-PCR amplification plot and a DIC image of this neuron are shown in Fig. 3E. Sequences of cDNA specific for the endogenous positive marker GAPDH and the cholinergic neu-
ron marker ChAT were detected above threshold as shown. Sequence specific to the GABAergic neuronal marker GAD67 was not detected, identifying this neuron as cholinergic.

In a different cholinergic BF neuron, the holding potential was varied between $-60$ and $+60$ mV and the mean IPSCs at each potential were plotted superimposed, as shown in Fig. 3F, and the current-voltage ($I-V$) relation for these data is presented in Fig. 3G. The reversal potential is 0 mV, consistent with a chloride conductance and GABAergic IPSCs in a chloride-loaded ($120$ mM) cell. Additionally, the IPSC frequency was not attenuated by rundown over time. The frequency during an 8-min window starting after 24 min of recording was $126 \pm 39\%$ of the frequency recorded during the initial 8 min ($n = 3$; not shown).

**Age-related changes in PSC properties.** We next compared the amplitude and frequency of sPSCs in cholinergic neurons across age groups. The most obvious difference between young and aged rats is the reduced frequency of sPSCs in aged BF cholinergic neurons. Figure 4 shows cumulative probability plots of the amplitudes and inter-event intervals of sPSCs and mPSCs for young and aged BF cholinergic neurons. The *inset* bar graphs show the mean values and standard errors for PSC amplitudes and frequencies. All of the cumulative probability curves are significantly different between young and aged when a K-S test (Mini Analysis 6.07) is performed with all events taken together, but the mean amplitudes within cells are not significantly different when compared by one-way ANOVA. The sPSC amplitude data were $60.6 \pm 5.0$ pA for young and $65.1 \pm 3.6$ pA for aged rats. For mPSC amplitudes, the data were $59.6 \pm 6.3$ pA for young and $46.6 \pm 4.3$ pA for aged rats ($P = 0.13$). The mean frequencies for both sPSCs and mPSCs are significantly reduced in aged rats. For sPSCs, the values were $0.79 \pm 0.1$ Hz in young and $0.50 \pm 0.07$ Hz in aged rats ($P = 0.01$, 1-way ANOVA); for mPSCs, the values were $0.29 \pm 0.05$ Hz in young and $0.13 \pm 0.02$ Hz in aged rats ($P < 0.01$). The whole cell capacitances ($C_m$) of the BF cholinergic neurons in these data sets were not significantly different, suggesting that young and aged cells were of similar size. The $C_m$ values in cells for sPSC analysis were $14.7 \pm 0.6$ pF for young and $14.1 \pm 0.4$ pF for aged rats. The $C_m$ values in cells for mPSC analysis were $15.4 \pm 4.9$ pF for young and $13.4 \pm 3.5$ pF for aged rats. The input resistance of whole cell patch-clamped acutely dissociated BF neurons is unchanged with age (Murchison et al. 2009).
Modulation of PSC frequency by elevated potassium. One way to estimate whether the reduced synaptic frequency with age was due to reduced function of aged synaptic terminals, as opposed to simply fewer synaptic contacts, would be to depolarize the presynaptic terminals while voltage-clamping the postsynaptic neuron. Exposing the voltage-clamped enzyme-free dissociated BF neurons to a recording solution containing 10 mM K+/H11001 increases the frequency of spontaneously occurring PSCs, presumably by depolarizing the presynaptic terminals. In the example from an aged rat shown in Fig. 5A, consecutive voltage-clamp records covering 24 continuous seconds are displayed stacked for each of the control, test, and wash conditions. Data collected over a 3-min span in each of the conditions (separated by 2 min to allow for bath exchange) show that the control frequency was a very low 0.08 Hz. This frequency increased to 0.58 Hz in elevated [K+] solution and declined to 0.18 Hz after washout. This property was not attenuated by rundown, because application of 10 mM K+/H11001 saline after 36 min of continuous recording was able to increase the frequency to 165 ± 18% of control (n = 3; not shown).

Fig. 2. Spontaneous postsynaptic currents (sPSCs) in the reduced synaptic preparation resemble those recorded from BF slices. A: a 2-min whole cell voltage-clamp recording from a BF slice showing sPSCs from a young rat. B: expanded section of recording in A. C: superimposed averages of spontaneous excitatory postsynaptic currents (sEPSC; (-)-2-amino-5-phosphonovaleric acid (APV)/6,7-dinitroquinoxaline-2,3-dione (DNQX) sensitive) and spontaneous inhibitory postsynaptic currents (sIPSC; bicuculline sensitive). D–F: recordings similar to those in A–C from a mechanically dissociated neuron showing spontaneous activity.

Fig. 3. Properties of sIPSCs using the reduced synaptic preparation. A: control whole cell voltage-clamp recordings (holding potential \(V_h = -60\) mV; top) and superimposed IPSCs with the average IPSC shown in black (bottom). B: same cell as in A, but in the presence of tetrodotoxin (TTX; 500 nM) to reveal miniature IPSCs (mIPSCs). C: same neuron as in A in the presence of TTX plus APV (40 \(\mu\)M) and DNQX (10 \(\mu\)M) to block EPSCs. D: finally, spontaneous mIPSCs are blocked by bicuculline (10 \(\mu\)M).

E: the single-cell RT-PCR (scRT-PCR) amplification plot for this neuron is shown at left, and a DIC image of the neuron is shown at right. GAPDH is the positive control, and ChAT is the cholinergic marker. ARs is the change in reporter fluorescence. F: averaged sIPSCs from holding potentials of 60, 40, 20, -20, -40, and -60 mV (top to bottom). G: plot of the data in F shows the reversal potential near 0 mV in the high-chloride pipette solution.
Interestingly, the increase in sPSC frequency by 10 mM K⁺ was significantly greater in BF cholinergic neurons from aged rats. The frequency in young rats increased by 16.4%, whereas that in aged rats increased by 93.8%. As shown in the graph in Fig. 5B, the age-related difference in sPSC frequency in control recording solution (3 mM K⁺) is eliminated in 10 mM K⁺ solution because of the greater increase in aged neurons. The mean frequencies in 3 mM K⁺ were 0.95 ± 0.09 Hz in young and 0.58 ± 0.07 Hz in aged rats (P < 0.001). The mean frequencies in 10 mM K⁺ were 1.11 ± 0.09 Hz in young and 1.12 ± 0.13 Hz in aged rats (P > 0.50). The frequency of sPSCs was sensitive to TTX, as depicted in Fig. 5C. Control frequencies in 3 and 10 mM K⁺ were compared with frequencies in the presence of 1 μM TTX. The K⁺ stimulated increase in frequency in aged rats was significantly more sensitive to TTX than that in young rats, although there was no difference in percentage of inhibition of sPSC frequency in control [K⁺]. In 3 mM K⁺, TTX reduced the sPSC frequency by 53.0 ± 9.5% in young and by 52.2 ± 5.9% in aged rats. The inhibition by TTX in 10 mM K⁺ was 45.1 ± 7.7% in young and 70.0 ± 5.1% in aged rats (P < 0.05).

Changes in sPSC frequency are related to cognitive status in aged rats. These data suggest that local BF synaptic activity is changed with age, but it is unknown whether these changes could contribute to cognitive impairment during aging. We next tested whether spontaneous events on cholinergic neurons correlated with cognitive function. Young and aged rats were tested for cognitive impairment using a standard water maze paradigm (see METHODS). We found that the decrease in sPSC frequency in control recording solution (3 mM K⁺) is significantly greater in cognitively impaired rats. The frequency in aged unimpaired is not significantly different from the frequency in young rats. As shown in Fig. 6A, the mean frequencies of sPSCs from cholinergic BF neurons of behaviorally characterized rats in 3 mM K⁺ solution were 0.86 ± 0.12 Hz for young, 0.71 ± 0.16 Hz for aged unimpaired, and 0.48 ± 0.09 Hz for aged impaired rats. This relationship is maintained if the frequency values for each neuron are averaged within each individual subject, as shown in Fig. 6B. The mean frequencies by subject were: 0.87 ± 0.18 Hz for young, 0.73 ± 0.19 Hz for aged unimpaired, and 0.46 ± 0.07 Hz for aged impaired rats. In depolarizing recording solutions (10 mM K⁺), the sPSC frequencies of aged impaired and unimpaired rats were both increased such that they were not different from that of young rats (Fig. 6C). The values in 10 mM K⁺ were 0.96 ± 0.10 Hz in young, 1.06 ± 0.17 Hz in aged unimpaired, and 1.08 ± 0.23 Hz in aged impaired rats. These latter data show that aged synapses (both aged impaired and aged unimpaired) are capable of activity levels comparable to those of young synapses when stimulated. The distribution of the spatial LIs for young and aged subjects are shown in Fig. 6D. Aged subjects with LIs above the range of young (~270) were categorized as cognitively impaired (AI).

Additional properties of sPSCs and cognitive status. Because we believe that increased Ca²⁺ buffering with age contributes to physiological dysfunction in the BF and cognitive impairment, we wanted to test the possibility that increased buffering at the synaptic terminals of young rats would mimic the excitability changes seen in synaptic transmission in aged rats. We applied the Ca²⁺ chelator BAPTA-AM (3 μM) to load exogenous rapid buffering capacity into the terminals of BF neurons from young rats using the reduced synaptic preparation. This extra buffer remains in the adherent terminals but is diluted from the postsynaptic neuron by the contents of the patch electrode. As shown in the bar graphs in Fig. 7A, the added buffer significantly reduced the spontaneous frequency of PSCs in control (3 mM K⁺), but this difference was overcome in elevated [K⁺] solution. This parallels the results from aged impaired rats, although the frequency reduction is larger. Loading the young terminals with twice as much exog-
enous buffer reduced the frequency to near zero (data not shown).

The Ca\(^{2+}\)/H11001 dependence of the spontaneous PSC frequency was further confirmed in BF neurons from young rats using the reduced synaptic preparation and recording solution containing different Ca\(^{2+}\)/H11001 concentrations ranging from 0.6 to 4.8 mM. In Fig. 7, the mean frequencies were normalized to the frequency in 1.2 mM external Ca\(^{2+}\)/H11001 (physiological concentration). The sPSC frequency is dependent on external [Ca\(^{2+}\)/H11001] with a Hill coefficient of 2.6, suggesting a rather high level of Ca\(^{2+}\)/H11001 binding cooperativity for transmitter release at these synapses.

We also measured the sPSC amplitudes in BF cholinergic neurons from young and aged behaviorally characterized rats. A trend toward increased amplitude in aged rats became significantly different from that in young rats when cognitive status was considered. The sPSC amplitude was increased in aged unimpaired rats in control (3 mM K\(^{+}\)/H11001) conditions, as shown in Fig. 8. The same trend was present in 10 mM K\(^{+}\) solution but did not reach significance (P = 0.29). The mean amplitudes in control [K\(^{+}\)] were 51.9 ± 4.8 pA for young, 67.4 ± 6.0 pA for aged unimpaired, and 57.1 ± 5.0 pA for aged impaired rats. The mean amplitudes in elevated [K\(^{+}\)] were 58.2 ± 5.7 pA for young, 73.6 ± 8.3 pA for aged unimpaired, and 66.0 ± 5.4 pA for aged impaired rats. There was no significant difference between the sPSC amplitudes in the two solutions.

Additionally, we analyzed the time constants of decay (\(\tau_d\)) of visually identified spontaneous IPSCs in behaviorally characterized rats. An average IPSC was obtained for each neuron and was best fit with a double-exponential decay curve. There were no significant differences between young and aged impaired or unimpaired rats. The values for \(\tau_1\) and \(\tau_2\), respectively, were 8.13 ± 0.92 and 38.28 ± 2.60 ms in young, 8.66 ± 0.91 and 46.84 ± 6.15 ms in aged unimpaired, and 9.15 ± 0.75 and 45.81 ± 4.65 ms in aged impaired (data not shown).

No change in GABAB\(_{A}\)-mediated modulation of synapses with age. Our results do not suggest a general deterioration of synaptic function with age because age-related frequency deficits could be overcome with mild K\(^{+}\) depolarization of the terminals. Further proof for the viability of aged synapses would be gained by comparing pharmacological modulation across aging. We tested the age-related status of G protein-coupled receptor modulation of synaptic function by applying the GABAB\(_{A}\) agonist baclofen (10 \(\mu\)M) to young and aged BF neurons in depolarizing solution (10 mM K\(^{+}\)), as shown in

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\(A\) Control \hspace{1cm} Elevated [K\(^{+}\)] (10 mM) \hspace{1cm} Wash

\(B\)

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Fig. 5. Modulation of sIPSC frequency can be accomplished by depolarizing the presynaptic terminals with an elevated [K\(^{+}\)] solution. \(A\): whole cell voltage-clamp recordings (−60 mV) in control solution (3 mM K\(^{+}\)), in 10 mM K\(^{+}\), and after washout. In this example from an aged ChAT\(^{+}\) neuron, elevated [K\(^{+}\)] increased the PSC frequency to 0.58 Hz, from 0.08 Hz in control. \(B\): summary graph shows frequency of sPSCs in both control (3 mM K\(^{+}\)) and depolarizing recording solutions (10 mM K\(^{+}\)) for young and aged BF cholinergic neurons. \(C\): graphs show the percent inhibition of frequency by TTX in 3 and 10 mM K\(^{+}\) solutions. Note that TTX (0.5–1 \(\mu\)M) reduces the PSC frequency significantly more in aged BF cholinergic neurons. These data indicate that, in aged neurons, the newly recruited spontaneous events in 10 mM K\(^{+}\) are TTX-sensitive and action potential dependent. Bar graphs show means ± SE; numbers in parentheses indicate no. of cholinergic neurons. *P < 0.05, significant difference (1-way ANOVA).
Baclofen has been shown to be a potent inhibitor of presynaptic release in a number of preparations including the BF (Henderson and Jones 2005). Baclofen was equally effective at reducing sPSC frequency in young and aged rats (Fig. 9B), whereas amplitudes of PSCs were not significantly altered (Fig. 9C). The amplitude values for young neurons were 77.8 ± 21.5 pA in control and 68.1 ± 22.1 pA in baclofen. In aged neurons the values were 59.6 ± 5.8 pA in control and 43.5 ± 4.3 pA in baclofen. The frequency values in young neurons were 1.14 ± 0.25 Hz in control and 0.27 ± 0.05 in baclofen. In aged neurons, these values were 1.03 ± 0.32 Hz in control and 0.30 ± 0.09 Hz in baclofen. Similar results were observed in the hippocampus of aged rats, where GABA_B receptor function was maintained (McQuail et al. 2012).

Fig. 9A. Baclofen has been shown to be a potent inhibitor of presynaptic release in a number of preparations including the BF (Henderson and Jones 2005). Baclofen was equally effective at reducing sPSC frequency in young and aged rats (Fig. 9B), whereas amplitudes of PSCs were not significantly altered (Fig. 9C). The amplitude values for young neurons were 77.8 ± 21.5 pA in control and 68.1 ± 22.1 pA in baclofen. In aged neurons the values were 59.6 ± 5.8 pA in control and 43.5 ± 4.3 pA in baclofen. The frequency values in young neurons were 1.14 ± 0.25 Hz in control and 0.27 ± 0.05 in baclofen. In aged neurons, these values were 1.03 ± 0.32 Hz in control and 0.30 ± 0.09 Hz in baclofen. Similar results were observed in the hippocampus of aged rats, where GABA_B receptor function was maintained (McQuail et al. 2012).
DISCUSSION

Our results with cholinergic BF neurons show that inhibitory presynaptic function is compromised during aging and that this reduction is significantly linked to cognitive impairment. This deficit appears to be specific to presynaptic baseline excitability, because it can be overcome by increasing excitability (elevated [K⁺]) and can be modulated by mechanisms that decrease excitability (increased presynaptic buffering, TTX), and no decrement is observed in general presynaptic function (frequency modulation by baclofen) or in postsynaptic functions (sPSC amplitude). These findings extend our earlier work showing increased intracellular Ca²⁺ buffering in cognitively impaired aged subjects (Murchison et al. 2009). Collectively, a working hypothesis can be proposed such that inhibitory input to BF cholinergic neurons is reduced with age under conditions of low-level activity in the local GABAergic system. This deficit contributes to a state of cognitive impairment and could represent a mechanistic link between increased Ca²⁺ buffering in BF neurons and cognitive decline. However, buffering changes in cholinergic BF neurons do not prove that Ca²⁺ buffering is increased at GABAergic terminals, despite the fact that IPSC frequency can be reduced by added buffer. Other mechanisms that control presynaptic excitability could be involved also.

Alternatively to a causative role, reduced inhibition to the BF cholinergic system could represent a compensatory mechanism to alleviate an age-related decrease in cholinergic transmission. This sort of compensation has been proposed as a mechanism in aging cortical neurons, where reduced excitation leads to a downregulation of genes involved in inhibitory synaptic transmission (Gleichmann et al. 2012). Similar compensatory mechanisms may be at work also in age-related changes in excitatory transmission in the hippocampus (reviewed by Burke and Barnes 2010). Basal forebrain cholinergic neurons are critically involved in attention and some forms of learning and memory (Sarter and Bruno 2004) and are also targets of a number of age-related disorders including nonpathological cognitive impairment associated with normal aging (McKinney 2005). Reduced cholinergic synaptic transmission from BF has been implicated in age-related deficits observed in hippocampus and cortex (Casu et al. 2002; Potier et al. 2006; Turrini et al. 2001; Wu et al. 1988), and this decrement could be compensated by reduced inhibitory input to cholinergic neurons.

Synaptic function in the basal forebrain. BF neurons receive a complex mixture of both excitatory and inhibitory synaptic inputs (Colom et al. 2005; Henderson and Jones 2005; Manseau et al. 2005; Schneeguenberger et al. 1992; Sim and Griffith 1996; Wu et al. 2000) that include multimodal afferents from numerous brain regions (Alreja et al. 2000; Colom 2006; Dutar et al. 1995; Sarter et al. 2003). The network includes a combination of traditional cholinergic and GABAergic projecting neurons and local GABAergic and glutamatergic interneurons (Colom et al. 2005; Manseau et al. 2005) along with peptidergic neurons and fibers (Zaborszky and Duque 2000). The local BF circuitry is characterized by complex feedforward and feedback pathways (Manseau et al. 2005). The approach that we have taken in the present study eliminates the complex circuit interactions and allows us to focus on synaptic contacts on isolated cells. Anatomically, identified cholinergic BF neurons are surrounded by GABAergic varicosities (Chang et al. 1995; Khatib et al. 1998; Zaborszky et al. 1986). The predominance of inhibitory GABAergic currents (>95%) that we recorded in the reduced synaptic BF preparation is consistent with this anatomic substrate.

Reduced synaptic preparation. Synaptic function of central neurons acutely dissociated with (Drewe et al. 1988) and without enzyme treatment (Vorobjev 1991) has been studied in various brain regions, including amygdala (Koyama et al. 1999; Zhu and Lovinger 2005), hippocampus (Akaike et al. 2002; Inada et al. 2010; Sheinin et al. 2008), preoptic nucleus (Haage et al. 1998; Karlsson et al. 1997; Matsuo et al. 2003), ventral tegmental area (Ye et al. 2004; Manseau et al. 2005; Schneggenburger et al. 1992; Sim and Griffith 2005; Turrini et al. 2001; Wu et al. 1988), and this reduction is significantly linked to cognitive impairment. This deficit could be compensated by reduced inhibitory input to cholinergic neurons.
have examined the properties of sIPSCs in the nucleus basalis of young Wistar rats (Akaike and Moorhouse 2003). Similar to our findings, these investigators show a preponderance of GABAergic IPSCs and persistent function without rundown over time. Spontaneous frequencies and current amplitudes are very similar in young rats of the two strains and are sensitive to TTX and other neuromodulators. We have now extended this preparation to the study of synaptic function in BF neurons of aged rats.

Mechanisms of sPSC frequency deficit. The finding that elevated \([K^+]\) solution is able to raise the frequency of sPSCs in aged BF neurons so that there is no difference from young is an important result of this investigation. This supports the interpretation that there is an age-related deficit in sPSC release properties, rather than simply fewer synaptic terminals on the aged neurons. If there were fewer terminals, it is likely that terminal depolarization would reveal an even greater age-related deficit in PSC frequency. By similar reasoning, this result suggests that the deficit is not due to reductions in vesicular availability or exocytotic docking sites, which could become rate-limiting under sustained stimulation (Neher 2010). We believe that our data support a model in which decreased excitability at the aged presynaptic terminal reduces spontaneous transmitter release, but release can be enhanced by depolarization of the terminal. This depolarization is more dependent on TTX-sensitive function with age. We interpret this to support the idea that spontaneous synaptic activity in young BF neurons is less reliant on depolarization of the terminal by TTX-sensitive sodium channels.

However, it could be argued that the depolarization induced activity represents a different sort of release from true spontaneous release, possibly involving different vesicle pools. There is some debate regarding the existence of a distinct spontaneous vesicle pool (Denker and Rizzoli 2010), but we cannot rule out the possibility that such a pool could be reduced with age, without changes to other pools. Another interesting alternative explanation could involve an increase in the ratio of silent synapses in aged neurons. In this scenario, depolarization of the terminals activates more silent synapses in aged BF neurons, the total active and silent synapses being about equal in young and aged neurons. The ratio of silent synapses is known to change during development (Kerchner and Nicoll 2008) and might continue to change with age. In hippocampus, however, postsynaptically silent excitatory synapses are maintained during aging, and there appears to be no change in the ratio of active/silent synapses (Sametsky et al. 2010). Although silent

Fig. 9. Synaptic modulation by the GABA\(_\text{A}\) receptor agonist baclofen is not altered with age. In 7 BF cholinergic neurons each from young and aged rats, baclofen (10 \(\mu M\)) significantly reduced the frequency of sPSCs but did not change the amplitude. These experiments utilized the reduced synaptic preparation in 10 mM K\(^+\) recording solution as the control to normalize the starting frequencies. A: sequential voltage-clamp records (\(V_h = -60\) mV) of sPSCs are shown stacked in this example from an aged rat. Representative records from control, baclofen-exposed, and wash conditions are depicted. B: summary graph of young and aged control and baclofen-inhibited PSC frequency. C: summary graph of young and aged sPSC amplitude in control and in baclofen. *\(P < 0.05\), significant difference from control (1-way ANOVA).
GABAergic synapses have not been investigated thoroughly, silent inhibitory synapses that display functional plasticity are known in the central nervous system (Charpier et al. 1995; Wolszon and Faber 1989). Future studies using evoked PSCs will be required to address the issues of silent synapses and changes in plasticity in the BF with age.

Inhibitory synaptic transmission and aging. Reductions of synaptic inhibition during aging have been reported in a number of brain areas, including the lateral septum (Garcia and Jaffard 1993), hippocampal formation (Billard et al. 1995; Potier et al. 2006; Stanley et al. 2012), and auditory cortex (Caspar et al. 2008; Llano et al. 2012). However, diminished inhibition is not a universal attribute of brain aging because enhanced GABAergic tone also is associated with unsuccessful cognitive aging in the prefrontal cortex (Bories et al. 2013). Regardless of the direction of change in specific brain structures, there is much evidence to suggest that alterations in number or in function of inhibitory interneurons contribute to age-related behavioral impairment (Bories et al. 2013; Llano et al. 2012; Stanley et al. 2012). A recent anatomical study found no cell loss in the rostral BF during aging; however, the number of GAD67 immunopositive neurons was selectively elevated in aged rats that exhibited impaired spatial learning (Banuelos et al. 2013).

It is possible that the decreased GABAergic transmission that we observed in cognitively impaired rats is a homeostatic compensatory response intended to offset an ongoing imbalance between excitation and inhibition underlying cognitive dysfunction. For example, an increase in inhibitory tone in the prefrontal cortex is associated with age-related cognitive impairment (Bories et al. 2013). Homeostatic changes in presynaptic function are known to occur in response to altered postsynaptic excitation/inhibition (Paradis et al. 2001; Turrigiano and Nelson 2004). In support of this possibility, postsynaptic GABAA receptor function is increased with age in BF neurons (Griffith and Murchison 1995), which could trigger a compensatory reduction in presynaptic GABA input. Similarly, experimental silencing of excitatory synaptic input to cortical neurons triggers a compensatory downregulation of genes involved in GABAergic transmission, among others (Gleichmann et al. 2012), and many of these expression changes reflect patterns of gene expression seen in aging and cognitive impairment (Blalock et al. 2004; Lu et al. 2004; Rowe et al. 2007). Interestingly, reduced excitation to the BF from the hippocampus (Gant and Thibault 2009) could represent the sort of physiological network silencing that could trigger a compensatory reduction in BF inhibitory transmission.

Ca2+ buffering and synaptic transmission. We have hypothesized that increased neuronal Ca2+ buffering in BF neurons contributes to cognitive impairment by a mechanism that alters synaptic function (Murchison et al. 2009). The idea that age-related changes in Ca2+ homeostatic mechanisms have a critical impact on synaptic transmission is supported by extensive evidence (Burke and Barnes 2006; Foster 2007). Seemingly small or subtle perturbations in Ca2+ handling can profoundly alter synaptic function (Catterall and Few 2008; Kochubey et al. 2011), and a variety of such perturbations are known to occur in different cell types with age (Kumar et al. 2009). Inhibitory synaptic transmission in the BF is regulated by both Ca2+ influx from voltage-gated channels and release from intracellular stores (Akaike et al. 1992). In this investigation, we have shown that inhibitory synaptic transmission to BF cholinergic neurons is reduced in cognitively impaired aged rats. We were able to mimic this deficit in BF neurons from young rats by exposing the cells to the exogenous Ca2+ buffer BAPTA-AM. This effect is likely to be due to presynaptic loading of the terminals with exogenous buffer, rather than a postsynaptic effect, because the exogenous buffer is expected to washout of the postsynaptic neuron into the patch pipette. These results suggest that increased Ca2+ buffering at synaptic terminals could mediate a deficit in BF synaptic transmission with age that contributes to cognitive impairment, although other mechanism that reduce presynaptic excitability have not been ruled out.

This possibility is further supported by the finding of considerable Ca2+ cooperativity in the relationship between Ca2+ concentration and sPSC frequency (Fig. 7B). A Hill coefficient of 1 indicates no Ca2+ cooperativity and a linear relationship between Ca2+ binding and transmitter release. Larger values indicate an exponential increase in release with additional Ca2+ binding. The cooperativity we report for these BF synapses is lower than that at the frog neuromuscular junction (Dodge and Rahamimoff 1967) or at the ribbon synapse in the goldfish retina (Heidelberger et al. 1994) where power values of 4 were found, but it is similar to that of squid giant synapses (Smith et al. 1985), which are considered highly cooperative. Functionally, high cooperativity implies that small changes in Ca2+ availability will have a large effect on transmitter release.

Altering the presynaptic buffer in nerve terminals can profoundly affect synaptic function (Rozov et al. 2001). BAPTA-AM has been shown to inhibit evoked IPSCs (Niesen et al. 1991) and field EPSPs (Ouanounou et al. 1996) in the hippocampus, presumably by a presynaptic mechanism. In addition to the capacity for exogenous BAPTA to reduce free Ca2+ available to the transmitter release machinery, it has been shown to alter Ca2+-dependent K+ channel function and thus influence neuronal firing properties in a cell type-specific manner (Ovespian et al. 2012; Roussel et al. 2006; Velumian and Carlen 1999). These latter effects on firing properties perhaps serve to explain the results of Carlen and colleagues (Ouanounou et al. 1999; Tonkikh et al. 2006; Tonkikh and Carlen 2009) in which exogenous BAPTA reverses age-related deficits.

Conclusion. We have shown that increased Ca2+ buffering with age in BF cholinergic neurons is related to cognitive impairment in a spatial learning task (Murchison et al. 2009) and can be prevented by a calorically restricted diet (Murchison and Griffith 2007). However, age-related changes in the membrane properties of BF neurons were subtle and were not correlated with cognitive status. This finding led us to propose that BF synaptic transmission might be the key component affected by increased buffering (Murchison et al. 2009). In this investigation we provide the first evidence that inhibitory transmission within the BF is reduced with age in cognitively impaired rats and that the mechanism involves decreased presynaptic excitability, possibly due to increased Ca2+ buffering. It is unknown whether this deficit is a causal mechanism or if it represents a sort of homeostatic compensation to maintain cholinergic function. Further experiments are required to determine whether these alterations in synaptic func-
tion extend to excitatory transmission or affect synaptic plasticity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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