Cholinergic Neurons Excite Cortically Projecting Basal Forebrain GABAergic Neurons

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The basal forebrain (BF) plays an important role in the control of cortical activation and attention. Understanding the modulation of BF neuronal activity is a prerequisite to treat disorders of cortical activation involving BF dysfunction, such as Alzheimer's disease. Here we reveal the interaction between cholinergic neurons and cortically projecting BF GABAergic neurons using immunohistochemistry and whole-cell recordings *in vitro*. In *GAD67-GFP* knock-in mice, BF cholinergic (choline acetyltransferase-positive) neurons were intermingled with GABAergic (*GFP*⁺) neurons. Immunohistochemistry for the vesicular acetylcholine transporter showed that cholinergic fibers apposed putative cortically projecting GABAergic neurons containing parvalbumin (PV). In coronal BF slices from *GAD67-GFP* knock-in or *PV-tdTomato* mice, pharmacological activation of cholinergic receptors with bath application of carbachol increased the firing rate of large (>20 μm diameter) BF *GFP*⁺ and PV (*tdTomato*+) neurons, which exhibited the intrinsic membrane properties of cortically projecting neurons. The excitatory effect of carbachol was blocked by antagonists of M_1 and M_3 muscarinic receptors in two subpopulations of BF GABAergic neurons [large hyperpolarization-activated cation current (I_h) and small I_h , respectively]. Ion substitution experiments and reversal potential measurements suggested that the carbachol-induced inward current was mediated mainly by sodium-permeable cation channels. Carbachol also increased the frequency of spontaneous excitatory and inhibitory synaptic currents. Furthermore, optogenetic stimulation of cholinergic neurons/fibers caused a mecamylamine- and atropine-sensitive inward current in putative GABAergic neurons. Thus, cortically projecting, BF GABAergic/PV neurons are excited by neighboring BF and/or brainstem cholinergic neurons. Loss of cholinergic neurons in Alzheimer's disease may impair cortical activation, in part, through disfacilitation of BF cortically projecting GABAergic/PV neurons.

Key words: brain slices; optostimulation; patch-clamp; transgenic mice

Introduction

Impairments of cortical activation are observed in many neurological and psychiatric disorders (Herrmann and Demiralp, 2005; Brown et al., 2012). Improved treatment of these diseases requires a precise understanding of how ascending subcortical projections interact to mediate state-dependent changes in brain activity. One such subcortical projection arises in the basal forebrain (BF). Studies in animals have shown that pharmacological inhibition (Cape and Jones, 2000) or neurotoxic lesions (Buzsaki et al., 1988; Burk and Sarter, 2001; Kaur et al., 2008; Fuller et al., 2011) of this region cause dramatic impairments in cortical activation and attention. Furthermore, in humans with Alzheimer's disease, degeneration of regions of the BF containing cholinergic

neurons is one of the earliest signs of the disease (Teipel et al., 2011; Grothe et al., 2012).

Electrical stimulation of the caudal BF promotes high-frequency cortical electroencephalogram oscillations via release of acetylcholine in the cortex (Metherate et al., 1992; Metherate and Ashe, 1993). Accordingly, drugs targeting the cholinergic system have been proposed as treatments for disorders involving abnormal cortical activation such as Alzheimer's disease and schizophrenia (Conn et al., 2009; Jones et al., 2012). However, lesions of BF, which preferentially target noncholinergic neurons, have effects on cortical activation and attention, which are at least as potent as selective lesions of cholinergic neurons (Burk and Sarter, 2001; Kaur et al., 2008), suggesting that noncholinergic BF neurons are equally important.

In addition to cholinergic neurons, the BF cortical projection includes a substantial proportion of GABAergic neurons (Freund and Meskenaite, 1992; Gritti et al., 1993, 2003; Henny and Jones, 2008; McKenna et al., 2013), many of which contain the calcium-binding protein, parvalbumin (PV; Gritti et al., 2003). These PV-positive, BF GABAergic neurons are of particular interest since they are projection neurons that target GABAergic neocortical interneurons and pyramidal neurons (Freund and Meskenaite, 1992; Gritti et al., 2003; Henny and Jones, 2008). Juxtacellular labeling following single-unit recordings *in vivo* identified a subpopulation of BF GABAergic neurons (Manns et al., 2000; Has-

Received July 22, 2013; revised Jan. 7, 2014; accepted Jan. 10, 2014.

Author contributions: C.Y., J.T.M., J.C.Z., R.B., and R.E.B. designed research; C.Y., J.T.M., J.C.Z., S.W., R.B., and R.E.B. performed research; C.Y., J.T.M., and R.E.B. analyzed data; C.Y., J.T.M., and R.E.B. wrote the paper.

This work was supported by VA Merit Awards to R.W. McCarley and R.B., and by National Institute of Mental Health Grants R01 MH039683 and R21 MH094803; National Heart, Lung, and Blood Institute Grant HL095491; and National Institute of Neurological Disorders and Stroke Grant R21 NS079866.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.3235-13.2014

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sani et al., 2009) or PV neurons (Duque et al., 2000), which are fast firing and increase their firing rate further during cortical activation. Furthermore, we recently found that optogenetic stimulation of BF PV neurons preferentially entrains fast cortical oscillations (Kim et al., 2011). Together, these findings suggest that these neurons may play an important role in the control of cortical activation.

Cholinergic BF projection neurons exhibit local axon collaterals terminating onto other BF neurons, including GABAergic neurons (Záborszky et al., 1986; Záborszky and Duque, 2000). In addition, the BF receives a cholinergic input from the brainstem (Semba et al., 1988). However, the functional effect of acetylcholine release within the BF on the GABAergic/PV⁺ projection to the neocortex is unknown, due to difficulties in identification. Here, we use recently validated genetically modified mice expressing fluorescent markers in GABAergic and PV neurons (McKenna et al., 2013) to determine the relationship between these two important neurotransmitter systems involved in cortical activation and attention.

Materials and Methods

Animals. To identify BF GABAergic neurons, we used heterozygous *GAD67-GFP* knock-in mice (McKenna et al., 2013). Although heterozygous animals lack one copy of the *Gad1* gene encoding GAD67, the sleep–wake behavior and cortical rhythms of these animals are indistinguishable from those of wild-type animals (Chen et al., 2010; McNally et al., 2011). Male, heterozygous *GAD67-GFP* knock-in mice on a Swiss-Webster background were crossed in-house with wild-type Swiss-Webster mice (Charles River). To record from PV-containing neurons, we crossed *PV-Cre* mice (Strain 008069; Jackson Laboratories) with a Cre-reporter strain (Strain 007905; Jackson Laboratories), resulting in mice expressing red (tdTomato) fluorescence in neurons expressing PV (*PV-tdTomato* mice; McKenna et al., 2013). To investigate the endogenous effect of acetylcholine on BF GABAergic neurons, we used mice constitutively expressing channelrhodopsin 2 (ChR2) under the control of the ChAT promoter (Zhao et al., 2011). Male, hemizygous ChAT-ChR2-EYFP mice (Strain 014546; Jackson Laboratories) were crossed with female wild-type Swiss-Webster mice.

Since the extensive myelination in the BF after postnatal day 22 (P22) makes infrared visualization of the neurons extremely difficult, for *in vitro* electrophysiological recordings we used juvenile (12- to 22-d-old) animals. Adult animals were used for immunohistochemical staining and mapping. Mice were housed under constant temperature and a 12 h light/dark cycle (7:00 A.M./7:00 P.M.), with food and water available *ad libitum*. All experiments conformed to US Veterans Administration, Harvard University, and the US National Institutes of Health guidelines.

Target area within the BF. For both immunohistochemical staining and electrophysiological experiments, we focused on intermediate areas of the basal forebrain [substantia innominata (SI), horizontal limb of the diagonal band (HDB), magnocellular preoptic nucleus (MCPO) and ventral pallidum (VP)], where previous studies in the rat (Rye et al., 1984; Gritti et al., 2003; Henny and Jones, 2008) have found neurons projecting to the neocortex. Our analysis did not include the rostral aspect of BF (medial septum and vertical limb of the diagonal band), a portion of which contains neurons projecting to the hippocampus or the caudal magnocellular basal nucleus, although GFP⁺ neurons were also located in these areas. Therefore, our investigations were largely of intermediate levels of the basal forebrain.

Immunohistochemistry methods. Immunohistochemical staining and analysis were performed as in our previous study (McKenna et al., 2013). Mice were deeply anesthetized with sodium pentobarbital (50 mg/ml), exsanguinated with saline solution, and perfused transcardially with 10% buffered formalin. Brains were postfixed for 4 h and then transferred to a 30% sucrose solution for 48 h at 4°C. The 40 μ m sections were cut on a freezing microtome and collected into four wells of PBS. Tissue was mounted onto chrome-alum gelatin-coated slides, dried, and coverslipped using Vectashield HardSet Mounting Medium (H-1400; Vector

Laboratories). Staining and quantification was conducted in four animals. For each stain [ChAT, vesicular acetylcholine transferase (vAChT), and PV], we quantified three representative coronal section levels per animal (measured from bregma: rostral, 0.38 mm; medial, 0.014 mm; and caudal, −0.10 mm) using Neurolucida software (version 8; MicroBrightField) and an Olympus BX51 microscope.

Choline acetyltransferase immunohistochemistry. To determine the relative location of cholinergic and GABAergic neurons in *GAD67-GFP* knock-in mice, as well as to confirm that ChR2-enhanced yellow fluorescent protein (EYFP) was correctly localized to cholinergic neurons in ChAT-ChR2-EYFP mice, we performed ChAT staining. Coronal slices from one well were placed in a blocking solution, and then incubated in rabbit anti-choline acetyltransferase [ChAT (the synthetic enzyme for acetylcholine) 1:200; AB143; Millipore (<http://antibodyregistry.org>, identification (ID) number 2079760] for 2 nights at 4°C. After incubation, tissue was rinsed and treated for 3 h at room temperature (RT) in secondary antibody, donkey anti-rabbit IgG conjugated with Alexa Fluor 594 (red; 1:100; A21207; Invitrogen).

vAChT and PV immunohistochemistry. To determine whether cholinergic varicosities are located in the vicinity of GABAergic and PV⁺ neurons, we performed staining for PV and vAChT in *GAD67-GFP* knock-in mice. Coronal BF slices were treated as follows: (1) incubated in goat anti-vAChT [1:500; AB1578 (now listed as AG260); Millipore; <http://antibodyregistry.org>, ID:10000324] for 1 night at 4°C; (2) treated for 3 h at RT in secondary antibody, donkey anti-goat IgG conjugated with Alexa Fluor 594 (red; 1:100; A11058; Invitrogen); (3) incubated in rabbit anti-PV (1:200; PA1-933; Thermo Scientific; Antibody Registry, <http://antibodyregistry.org>, ID number 2173898) for 40 h at 4°C; (4) treated with donkey anti-rabbit (1:300; 711-005-152; Jackson ImmunoResearch) for 2 h at RT; (5) treated with streptavidin Alexa Fluor 350 (blue; 1:100; S-11249; Invitrogen) for 1 h at RT; and, finally, (6) to amplify the blue signal, sections were treated with primary antibody biotinylated goat anti-streptavidin (1:100; BA-0500; Vector Laboratories) for 45 min at RT. Sections were then restained with Streptavidin Alexa Fluor 350 (1:100) for 30 min at RT.

Cell mapping and photography of GABAergic (GFP⁺) neurons, cholinergic neurons, and PV⁺ neurons. The perimeter and landmarks of BF were first traced at a low magnification (10 $\times$). Neuronal location was then plotted at higher magnifications (20–40 $\times$) and imposed on the low-magnification Neurolucida sketches. The distribution of labeled neurons was determined using a mouse brain atlas (Franklin and Paxinos, 2008). We mapped the representative case that best matched the template sub-nuclei boundaries. Once tracing and cell plotting was completed in Neurolucida, this sketch was placed on top of the Adobe Illustrator templates (using the “Transparency” tool). Symbols were then replotted in Adobe Illustrator (CS5, version 15.1.0) superimposed on the Neurolucida cell mapping.

GFP⁺ neurons in *GAD67-GFP* knock-in mice and EYFP⁺ neurons in ChAT-ChR2-EYFP mice were identified by the presence of green fluorescence (excitation/emission ratio, 488:509 nm). ChAT⁺ and vAChT⁺ cells were identified by the presence of red fluorescence (excitation/emission ratio, 590:617 nm), and PV⁺ neurons were identified by blue fluorescence (excitation/emission ratio, 343:442 nm). Digital images of fluorescently labeled neurons were captured using a deconvolution Zeiss Axioplan 2 microscope and the Slidebook system (3i: Intelligent Imaging Innovations). Image clarity was enhanced by adjusting the contrast and brightness in Slidebook and/or Adobe Photoshop image-processing software. Individual low-power photomicrographs, as well as all figures that include multiple panels, were assembled using Adobe Photoshop (CS5, version 12.1, $\times 64$), except for cellular mapping (Fig. 1G,H), where they were assembled using Adobe Illustrator (CS5, version 15.1.0).

Preparation of BF slices for electrophysiological recordings. Mice were deeply anesthetized with isoflurane and then decapitated. Two 300- μ m-thick coronal BF slices were cut rostrocaudally between 0.26 and −0.22 mm from bregma (Franklin and Paxinos, 2008). After slicing, they were placed into ACSF containing the following (in mM): 124 NaCl, 1.8 KCl, 25.6 NaHCO₃, 1.2 KH₂PO₄, 2 CaCl₂, 1.3 MgSO₄, and 10 glucose (osmolarity, 300 mOsm), saturated with 95% O₂/5% CO₂

for >1 h at room temperature before being transferred to the recording chamber and superfused with warmed ACSF (32°C) at 2–3 ml/min.

Whole-cell recordings. Electrophysiological recordings focused on intermediate/caudal parts of the HDB and MCPPO, where our previous study (McKenna et al., 2013) found that large GABAergic and GABAergic/PV⁺ neurons are most highly concentrated. Putative cortically projecting neurons were identified *in vitro* based on their large size (Gritti et al., 2003) and typical intrinsic membrane properties (McKenna et al., 2013). GABAergic (GFP⁺) neurons in *GAD67-GFP* knock-in mice or PV⁺ neurons in *PV-tdTomato* mice were selected for recording based on their expression of the fluorescent marker. Neurons were photographed before recording using a Hamamatsu ORCA-AR CCD camera. Long-axis cell diameter was measured from these images and calibrated using a standard grid. For most experiments, patch pipettes (3–6 M Ω) were filled with intracellular solution containing the following (in mM): 130 potassium gluconate, 5 NaCl, 2 MgCl₂, 10 HEPES, 0.1 EGTA, 2 Na₂ATP, 0.5 NaGTP, 4 MgATP, 1 spermine, and 0.5% biocytin, pH 7.25 with KOH (280 mOsm). Membrane potential measurements were adjusted for a –15 mV liquid junction potential between pipette and bath solutions (calculated using pClamp 9.0 software). For recording of IPSCs, potassium gluconate was replaced with KCl. Recordings were made using a Multiclamp 700B amplifier and pClamp 9.0 software (Molecular Devices). Bridge balance was adjusted after gaining access to the whole cell and maintained throughout the experiment. Recordings were accepted if action potentials were overshooting and series resistance changed by <10% during the experiment. In voltage-clamp recordings, series resistance was 6–15 M Ω and was not compensated. Continuous recordings of membrane voltage and current were made using a MiniDigi 1A system and Axoscope 9.2 software (Axon Instruments) with a sampling frequency of 1 kHz.

Characterization of intrinsic membrane properties. As described previously (McKenna et al., 2013), a series of 1-s-long hyperpolarizing and depolarizing current pulses were applied in current-clamp from the resting membrane potential (RMP). The size of the first hyperpolarizing step was titrated according to the input resistance of the neuron so that the negative peak of the membrane potential during the step reached –100 mV. Progressively more depolarizing current steps (in increments one-fifth the size of the initial step) were applied until the firing rate did not increase further. The percentage of depolarizing sag was determined from the largest hyperpolarizing step as [(steady-state voltage at end of the step – RMP)/(peak voltage – RMP)] $\times$ 100.

Pharmacological experiments to determine the effect of carbachol on firing frequency and membrane potential. Carbachol (50 μ M) was bath applied for 2 min. Action potential frequency was determined on-line in 15 s bins by crossing a threshold set at 0 mV (Clampex 9.0 software; Molecular Devices). To determine the receptor mediating the effect of carbachol, the cholinergic antagonists atropine (muscarinic antagonist; 5 μ M), pirenzepine dihydrochloride (0.1, 1, 10, and 50 μ M; M₁ receptor antagonist), and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP; 0.3, 1, and 3 μ M; M₃ receptor antagonist) were bath applied at least 5 min before the application of carbachol. Small 200 ms hyperpolarizing current steps were applied every 15 s to monitor input resistance. Drugs were purchased from Sigma-Aldrich. Stock solutions were prepared

Figure 1. GABAergic and cholinergic neurons intermingle in the BF of *GAD67-GFP* knock-in mice. **A–C**, Photographic montage of GFP⁺ (green, **A**) and ChAT⁺ (red, **B**) neuronal perikarya in the intermediate region of the BF, which contains neurons projecting to the neocortex. ChAT⁺ neurons extended throughout BF, largely distributed more medially and dorsally (centered in HDB/MCPPO), compared with the main cluster of GFP neurons, which was predominantly centered more laterally in MCPPO (merged image, **C**). **D–F**, Higher-magnification image demonstrates the close proximity of GFP⁺ (green, **D**) and ChAT⁺ (red, **E**) neurons in BF. ChAT⁺ neurons were not labeled with GFP (Merge, **F**). **G, H**, Areas targeted for electrophysiological recordings are shaded in yellow (**G**); schematic representation of GFP⁺ (green) and ChAT⁺ (red) neuronal perikarya in BF (**H**). Scale bars: **A–C**, 1 mm; **D–F**, 50 μ m; **G, H**, 1 mm. aca, anterior commissure, anterior aspect; CPu, caudate putamen; CTX, cortex; LPO, lateral preoptic area; Tu, olfactory tubercle.

in distilled water and stored at –4°C. The concentrations used were determined by conducting a literature search for both known receptor binding specificity values and previous experimental usage of drugs in *in vitro* slice preparations (Shen and Johnson, 2000; Busch and Borda, 2003; Pressler et al., 2007). We note that binding affinities of antagonists measured in expression systems can be used only as a guide to effective concentrations in brain slice experiments since the steady-state concentration in the immediate vicinity of the receptors within the slice are likely to be much lower than the concentration in the perfusate. In addition, post-translational modifications or accessory protein coupling *in situ* can modify binding affinity. Nonetheless, differences in responsiveness and concentration dependence of antagonism are suggestive of differences in the receptor subtype involved.

Voltage-clamp experiments to investigate the ion channels involved in the carbachol response. Neurons were clamped at –60 mV. To determine whether the carbachol responses reversed at the K⁺ equilibrium potential, slow (4 s) voltage ramps from –120 to –60 mV were applied at 30 s intervals. To determine whether the carbachol response was mediated by mixed cation channels, slices were incubated in a modified Ca²⁺-free ACSF (in mM: 124 NaCl, 1.8 KCl, 25.6 NaHCO₃, 1.2 KH₂PO₄, 2 MgCl₂, 1.3 MgSO₄ and 10 glucose) containing 500 nM tetrodotoxin (TTX) and tested with a ramp from –100 to 40 mV (20 s duration) every 2 min. To determine the involvement of Na⁺ in the carbachol response, the same voltage ramps from –100 to 40 mV were applied in Ca²⁺-free solution where most of the extracellular sodium was replaced by the impermeant ion *N*-methyl-D-glucamine [NMDG; low extracellular Na⁺-Ca²⁺-free solution as follows (in mM): 127 NMDG, 1.8 KCl, 25.6 NaHCO₃, 1.2 KH₂PO₄, 2 MgCl₂, 1.3 MgSO₄, and 10 glucose, osmolality 300 mOsm].

Measurement and analysis of synaptic currents. Spontaneous postsynaptic currents were recorded without TTX, while miniature postsynaptic

currents were recorded in the presence of 500 nM TTX. EPSCs were recorded with 10 μ M GABAzine in the bath to block GABA_A receptor-mediated currents. IPSCs were recorded in the presence of 20 μ M DNQX and 50 μ M AP-5 to block glutamatergic AMPA and NMDA receptor-mediated currents, respectively. To enhance the resolution of GABA_A receptor-mediated events, we enhanced the driving force for chloride entry by using a KCl-based pipette solution. A 1 min period immediately before carbachol application and a 1 min period 2 min after the application of carbachol, when the peak effect was apparent, were used for statistical analysis (Igor software). Only well resolved events with amplitudes >10 pA were analyzed.

Optical stimulation of cholinergic neurons. Brain slices were prepared from 13- to 22-d-old ChAT-EYFP-ChR2 mice, as described above. Cholinergic neurons were selected for recording based on their expression of EYFP. Putative GABAergic neurons were determined by their fluorescent-negative property, morphology, and electrophysiological properties (McKenna et al., 2013). A BLS-series BioLED light source (Mightex) connected to the microscope via a Mightex multiwavelength beam combiner and controlled by a BioLED light source control module was used to deliver 470 nm wavelength blue light to the brain slices through the full field of a 40 $\times$ water-immersion objective attached to the microscope, illuminating the area surrounding the recorded neuron. The light power delivered to the tissue was 30 mW/mm², which was determined by dividing the power of light under the objective measured with a slim photodiode power sensor (Sensor S130C; Thorlabs) by the area of the objective field (0.26 mm² for the W N-Achroplan, 40 $\times$ Zeiss objective). The response of cholinergic neurons to light stimulation was recorded in regular ACSF, while the response of GABAergic-like neurons to light stimulation was recorded in the presence of 20 μ M DNQX plus 50 μ M AP-5 plus 10 μ M GABAzine to block indirect effects of light stimulation mediated through the stimulation of glutamatergic and GABAergic neurons. Since cholinergic neurons have prominent A-type potassium currents, which limit the rate of depolarization and long-lasting action potentials, we used a pulse width of 10 ms to ensure reliable elicitation of action potentials in response to light. Atropine and mecamylamine hydrochloride (nicotinic receptor antagonist; 100 μ M; Sigma) were bath applied to determine the receptor types in mediating the response.

Statistics. Data are presented as the mean $\pm$ SEM. Neuroanatomical percentages of overall counts of BF ChAT⁺ cells were analyzed using one-way ANOVA, followed by a pairwise independent *t* test comparison among the different BF subnuclei, using Bonferroni's correction. The effect of the drug or light stimulation on membrane potential/current or input resistance were assessed by comparing membrane potential/current or input resistance immediately before the application of the drug/light and at the peak of the effect. A paired/unpaired *t* test was used for statistical analysis of significance. Statistical analysis used SPSS software (release 11.5), and differences were considered significant at *p* < 0.05.

Results

GABAergic neurons are intermingled with cholinergic neurons in the mouse BF and are surrounded by cholinergic fibers

As depicted in Figure 1, cholinergic and GABAergic neurons are in close proximity to each other in the BF. Immunohistochemical labeling for the acetylcholine synthesizing enzyme ChAT, in the BF of mice where GABAergic neurons are labeled with green fluorescent protein (*GAD67-GFP* knock-in mice; Tamamaki et al., 2003; McKenna et al., 2013), revealed that the distribution of cholinergic and GABAergic neurons overlapped. Quantification revealed that the highest density of BF cholinergic neurons was in the HDB (97.6 $\pm$ 17.1 ChAT⁺ neurons/mm²/slice; *n* = 4). Lower densities were found in the MCPO (47.4 $\pm$ 3.5 ChAT⁺ neurons/mm²/slice) and dorsal BF nuclei (VP, 24.6 $\pm$ 3.1 ChAT⁺ neurons/mm²/slice; SI, 20.0 $\pm$ 1.3 ChAT⁺ neurons/mm²/slice). One-way ANOVA confirmed significant differences between the ChAT density of the four BF subnuclei ($F_{(3,12)} = 16.035$, *p* < 0.001), and follow-up pairwise comparisons (Bonferroni cor-

Horizontal Limb of the Diagonal Band (HDB)

Magnocellular Preoptic Nucleus (MCPO)

Figure 2. A–H, Cholinergic (vAChT⁺) varicosities are closely apposed to BF GABAergic/PV neurons in the HDB (A–D) and the MCPO (E–H) of *GAD67-GFP* mice. Fluorescent images show GFP⁺ (green, A and E) and PV⁺ (blue, B and F) neurons. vAChT varicosities (red, C and G) are apposed to GABA⁺/PV⁺ neurons in both BF regions (merged images, D and H, arrowheads). Punctate green fluorescence, perhaps suggestive of axon terminals, was also observed over the cell bodies of some GFP⁺ (E) and GFP[−] neurons (A). Scale bars, 25 μ m.

rected) revealed the following significant density differences: HDB > MCPO (*p* = 0.011); and HDB > VP, SI (*p* < 0.001). Although the main cluster of cholinergic neurons was located more medially than the main cluster of large-sized (>20 μ m) GABAergic neurons, which we previously found were clustered in the MCPO (McKenna et al., 2013), ChAT⁺ neurons were intermingled with large GABAergic (GFP⁺) neurons throughout the BF. In particular, cholinergic and large GABAergic neurons were closely intermingled in the lateral part of the HDB region and the medial part of the MCPO (Fig. 1). ChAT⁺ neurons were not labeled with GFP, indicating that the GFP label is not expressed ectopically in cholinergic neurons (Fig. 1D–F).

To determine whether there are cholinergic synaptic varicosities in the vicinity of GABAergic BF neurons, and, in particular, cortically projecting (PV⁺) BF GABA neurons, we performed immunohistochemical staining for the vAChT and PV in brain tissue from *GAD67-GFP* mice. Multiple, punctate, vAChT⁺ varicosities were observed surrounding and apposed to GFP⁺/PV⁺ and GFP⁺/PV[−] neurons in both the HDB and MCPO (Fig. 2), as well as in other regions of the BF, suggesting innervation by cholinergic fibers.

Pharmacological activation of cholinergic receptors excites BF GABAergic and PV⁺ neurons

To identify BF GABAergic neurons for electrophysiological recordings, we used *GAD67-GFP* knock-in mice (Tamamaki et al., 2003; McKenna et al., 2013). We focused our electrophysiological recordings on large (>20 μ m) GFP⁺ neurons, which fired spontaneously at high rates and had a prominent depolarizing sag

Figure 3. *A–L*, The cholinergic agonist carbachol excites two types of large BF GABAergic neurons, large I_h (*A–F*) and small I_h (*G–L*). *A*, A 1 s hyperpolarizing current injection (-1.05 nA) reveals a large depolarizing sag (49.4%) with a biexponential decay ($\tau_{\text{fast}} = 39.6$ ms, $\tau_{\text{slow}} = 402.7$ ms), defining this type of neuron. *B*, A $50 \mu\text{M}$ dose of carbachol strongly increased the spontaneous firing frequency ($n = 9$). *C, D*, A representative recording from a large I_h neuron showing the carbachol-induced depolarization and firing. *E*, Dose dependence of carbachol response. $\text{EC}_{50} = 23 \mu\text{M}$. The number of neurons for each concentration of carbachol is given above each data point ($n = 4–9$). *F*, Mean data for the effect of $50 \mu\text{M}$ carbachol on the firing frequency of large I_h neurons. Control data were taken from the time point 2 min before application, whereas the carbachol response data were taken from the 5 min time point. Significance was assessed with a paired t test. *G*, A 1 s hyperpolarizing current injection (-0.30 nA) reveals a small depolarizing sag (27.6%) with a monoexponential decay ($\tau = 273.0$ ms), defining this type of neuron. *H*, Time course of carbachol effect on action potential firing ($n = 9$). *I, J*, A representative recording from a small I_h neuron showing the carbachol-induced depolarization and firing. *K*, Dose dependence of carbachol response. $\text{EC}_{50} = 33 \mu\text{M}$. The number of neurons for each concentration of carbachol is given above each data point ($n = 4–9$). *L*, Mean data for the effect of $50 \mu\text{M}$ carbachol on the firing frequency of small I_h neurons.

during hyperpolarizing current pulses. These neurons had the same size (Gritti et al., 2003) and intrinsic membrane properties as those identified in cortically projecting GABAergic neurons (McKenna et al., 2013). As in our previous study (McKenna et al., 2013), we subdivided these neurons into two groups based on the amplitude and kinetics of the depolarizing sag during hyperpolarizing current pulses. Since this sag was blocked by pharmacological blockers of the H-current (McKenna et al., 2013), we termed these subgroups large hyperpolarization-activated cation current (I_h) and small I_h .

A brief (2 min) bath application of the broad-spectrum cholinergic agonist carbachol ($50 \mu\text{M}$) increased the firing rate of both large I_h and small I_h GABAergic neurons (Fig. 3; large I_h neurons: 11.8 ± 2.9 to 22.1 ± 3.9 Hz, $p = 0.015$, $n = 9$; small I_h neurons: 3.5 ± 0.9 to 15.1 ± 2.8 Hz, $p = 0.0016$, $n = 9$). This response was dose dependent with an EC_{50} value of $23 \mu\text{M}$ in large I_h neurons and $33 \mu\text{M}$ in small I_h neurons (Fig. 3). Carbachol also reduced spike amplitude, likely due to increased inactivation of voltage-gated sodium channels at depolarized membrane potentials (Fig. 3D).

An important subset of the cortically projecting BF GABAergic projection contains PV (Gritti et al., 2003), comprising $\sim 25\%$ of the large GABAergic neurons in the ventral BF (McKenna et

al., 2013). To confirm that the cortically projecting PV⁺ subpopulation is also excited, we recorded from PV⁺ neurons in slices prepared from mice selectively expressing a red fluorescent marker in PV neurons (McKenna et al., 2013). Indeed, six of eight PV⁺ neurons recorded in slices prepared from PV-tdTomato mice were excited by carbachol ($50 \mu\text{M}$).

Carbachol increases the firing frequency of BF GABAergic neurons via activation of M_1/M_3 receptors

The broad-spectrum muscarinic receptor antagonist atropine ($5 \mu\text{M}$) completely blocked the carbachol-induced increase in firing frequency in both large and small I_h GABAergic neurons, without having an effect on its own (large I_h : control, 9.1 ± 2.1 Hz; atropine, 8.8 ± 1.7 Hz; atropine with carbachol, 8.2 ± 1.6 Hz; no significant differences, repeated-measures ANOVA with Bonferroni *post hoc* test, $n = 6$; small I_h : control, 9.5 ± 5.1 Hz; atropine, 9.9 ± 4.8 Hz; atropine with carbachol, 7.6 ± 3.4 Hz; no significant differences, $n = 6$). Thus, excitatory carbachol responses are mediated through muscarinic receptors, but there is no basal cholinergic tone in the slice.

We next tested more selective muscarinic antagonists. Excitatory muscarinic effects in other brain regions are mediated via M_1 -type

Figure 4. The excitatory effect of carbachol on BF GABAergic neurons is mediated by M_1/M_3 receptors. **A–J**, The effect of carbachol is mainly mediated by the M_1 receptor in large I_h GABAergic neurons (**A–D**) and by the M_3 receptor in small I_h GABAergic neurons (**E–J**). **A**, Mean time course of carbachol response in the presence of an M_1 receptor antagonist,

receptors (M_1, M_3, M_5), whereas M_2 -like receptors (M_2, M_4) mediate inhibitory responses (Nicoll et al., 1990). Therefore, we focused on M_1 -type receptors, and in particular M_1 and M_3 receptors, since M_5 receptors appear to be at low levels or absent from BF (Allen Mouse Brain Atlas, <http://mouse.brain-map.org>). Although the effect of carbachol was blocked by atropine in both large I_h and small I_h neurons, there were differences between the two groups in their response to more specific muscarinic antagonists. In large I_h neurons, in the presence of the M_1 receptor antagonist pirenzepine dihydrochloride (10 μ M), the neuronal firing frequency (9.6 ± 2.6 Hz) was similar to that before drug application (10.5 ± 3.2 Hz, $p = 0.45$, $n = 10$). Most of the neurons ($n = 7/10$) exposed to 10 μ M pirenzepine did not show any significant change in firing frequency when carbachol was applied (Fig. 4A; baseline, 10.3 ± 2.9 Hz; in carbachol, 12.7 ± 3.8 Hz; $t_{(20)} = 1.83$; $p > 0.05$, repeated-measures ANOVA with Bonferroni *post hoc* test, $n = 7$). This inhibition was dose dependent. When the firing rate was normalized to that before carbachol application, the firing rate was increased by 2.87 ± 0.68 -fold ($n = 9$), 3.03 ± 0.63 -fold ($n = 9$), 1.72 ± 0.26 -fold ($n = 5$), and 1.23 ± 0.15 -fold ($n = 7$) in the presence of 0, 0.1, 1, and 10 μ M pirenzepine, respectively. Three neurons still showed a significant increase in firing frequency from 8.2 ± 6.6 to 15.7 ± 7.5 Hz in 10 μ M pirenzepine in response to carbachol stimulation (Fig. 4C; $p < 0.05$, Friedman's test with Dunn's

← pirenzepine dihydrochloride (10 μ M). The carbachol response was inhibited in seven of nine neurons. **B**, Summary of the pirenzepine effect on carbachol response of the seven neurons shown in **A**. The firing frequency at three time points (2 min for control, 7 min for pirenzepine alone, and 10 min for pirenzepine with carbachol) were compared by one-way ANOVA with Bonferroni's multiple-comparison test. **C**, Mean time course of carbachol response in the presence of pirenzepine for the remaining three neurons. The carbachol response was not blocked by pirenzepine in these neurons but was subsequently inhibited by incubation with an M_3 receptor antagonist, 4-DAMP (3 μ M), as shown in **D** summary data. **E**, Mean time course of carbachol response in the presence of pirenzepine for small I_h GABAergic neurons. The carbachol response was not blocked in six of eight neurons. **F**, Summary of the pirenzepine effect on carbachol response for the six cells in **D**. **G**, Mean time course of carbachol response in the presence of pirenzepine for the remaining two neurons. The carbachol response was completely blocked by pirenzepine in these neurons, as shown in **H** summary data. **I**, Mean time course of carbachol response in the presence of 3 μ M 4-DAMP for five neurons with small I_h . The carbachol response was inhibited in all five cells. **J**, Mean data of the neurons recorded in **I**. The carbachol response in small I_h neurons was significantly inhibited by the M_3 receptor antagonist.

multiple-comparison test, $n = 3$). After washing out carbachol and pirenzepine from the bath, these three neurons were incubated with an M_3 receptor antagonist, 4-DAMP ($3 \mu\text{M}$), for 5–8 min, and then the effect of carbachol was retested. Their firing frequency was similar in 4-DAMP, and there was no significant difference from that before carbachol application (Fig. 4D; control, 8.6 ± 6.9 Hz; 4-DAMP, 11.4 ± 7.3 Hz; $p > 0.05$, Friedman's test with Dunn's multiple-comparison test, $n = 3$). In control experiments without any antagonist, carbachol induced a consistent and repeatable increase in firing during the second application (control, 6.2 ± 2.1 Hz; first carbachol application, 22.3 ± 6.7 Hz; washout, 5.1 ± 1.6 Hz; second carbachol application, 17.0 ± 6.1 Hz; there were no significant differences in the firing frequencies at baseline or in the presence of carbachol; control vs washout, $p = 0.6289$; first vs second carbachol application, $p = 0.205$, paired t test). These data suggest that the response to carbachol in the majority of large I_h GABAergic neurons is mediated via M_1 receptors, and in a smaller population via M_3 receptors.

In small I_h neurons, the effects of M_1 and M_3 receptor-selective antagonists were reversed. The carbachol effect on firing frequency persisted in most small I_h neurons (six of eight neurons) with a 5 min incubation in $10 \mu\text{M}$ of the M_1 receptor antagonist pirenzepine (Fig. 4E,F). Their firing frequencies in regular ACSF, pirenzepine, and carbachol with pirenzepine were respectively 0.37 ± 0.12 , 0.96 ± 0.37 , and 9.13 ± 3.48 Hz (ACSF vs pirenzepine, $p > 0.05$; pirenzepine with carbachol vs ACSF/pirenzepine; $p < 0.05$, repeated-measures ANOVA with Bonferroni post-test, $n = 6/8$). The remaining two neurons did not respond to carbachol in the presence of $10 \mu\text{M}$ pirenzepine (control, 10.5 ± 8.2 Hz; pirenzepine, 8.9 ± 7.1 Hz; pirenzepine plus carbachol, 7.4 ± 5.8 Hz; Fig. 4G). In contrast to the effect of pirenzepine in large I_h neurons, increases in firing rate in response to carbachol were observed even in the presence of pirenzepine, even at concentrations as high as $50 \mu\text{M}$, although at the highest dose ($50 \mu\text{M}$) there was a trend-level ($p = 0.12$) reduction in the increase in firing produced by carbachol. When the firing rate was normalized to that before carbachol application, the firing rate was significantly increased by 5.67 ± 1.12 -fold ($n = 9$), 7.72 ± 3.04 -fold ($n = 5$), 5.84 ± 3.62 -fold ($n = 8$), and 3.2 ± 0.86 -fold ($n = 6$) in the presence of 0, 0.1, 10, and $50 \mu\text{M}$ pirenzepine, respectively. In contrast, in all small I_h neurons tested ($n = 5$), 4-DAMP completely blocked the carbachol response (Fig. 4I). The firing frequencies in ACSF, $3 \mu\text{M}$ 4-DAMP, and 4-DAMP with carbachol were, respectively, 3.68 ± 2.20 , 2.95 ± 1.90 , and 3.31 ± 2.05 Hz ($p > 0.05$, repeated-measures ANOVA with Bonferroni post-test, $n = 5$). The normalized increase in firing induced by carbachol was inhibited by 4-DAMP with a steep dose dependence, as follows: 5.67 ± 1.12 -fold increase without 4-DAMP ($n = 9$); 7.1 ± 2.8 with $0.3 \mu\text{M}$ 4-DAMP; 5.5 ± 2.7 with $1 \mu\text{M}$ 4-DAMP ($n = 4$); and 1.3 ± 0.18 with $3 \mu\text{M}$ 4-DAMP ($n = 5$); that is, doses of 0.3 and $1 \mu\text{M}$ did not block the effect of carbachol, but a dose of $3 \mu\text{M}$ caused a complete block. These results suggest that the M_3 receptor mediates the carbachol response in most small I_h GABAergic neurons.

Bath-applied carbachol-induced inward currents in GABAergic neurons via muscarinic receptors and opening sodium-permeable cation channels

Carbachol depolarized both large and small I_h neurons (large I_h , 8.1 ± 1.1 mV; small I_h , 11.3 ± 1.6 mV) in the presence of TTX ($0.5 \mu\text{M}$), suggesting a direct postsynaptic effect. Under voltage-clamp at a holding potential of -60 mV, carbachol-induced inward currents of -171 ± 44 pA (baseline current changed from

-190 ± 55.8 to -363.6 ± 68.0 pA; $p = 0.0036$, paired t test) and -52 ± 14 pA (baseline current changed from -113.2 ± 27.1 to -164.8 ± 39.8 pA; $p = 0.0088$, paired t test) in large ($n = 11$) and small I_h ($n = 8$) neurons, respectively (Fig. 5). In both types of neurons, the inward current was accompanied by an increase in membrane current noise [large I_h : root mean square (RMS) of baseline, 4.8 ± 0.7 pA; vs carbachol, 13 ± 3.0 pA; $p = 0.0257$, paired t test, $n = 11$; small I_h : RMS baseline, 4.0 ± 0.7 pA; vs carbachol, 6.2 ± 1.2 pA; $p = 0.0130$, paired t test, $n = 8$], suggesting that more channels are opening and closing in the presence of carbachol. In the presence of atropine, carbachol did not induce any significant change in baseline current for either large I_h neurons ($n = 5$) or small I_h neurons ($n = 6$; large I_h holding current in atropine vs atropine plus carbachol: -166.5 ± 56.3 vs -166.0 ± 56.9 pA, $p = 0.7686$; small I_h holding current in atropine vs atropine plus carbachol: -69.2 ± 21.2 vs -76.5 ± 17.4 pA, $p = 0.2730$; paired t test), suggesting that the postsynaptic current response was mediated via muscarinic receptors.

To test whether the carbachol-induced inward current reverses at the equilibrium potential for potassium ions (-100 mV under our conditions), we applied slow (4 s) voltage ramps from -120 to -60 mV every 30 s and compared the response in baseline from that in carbachol. However, these responses did not cross in any of the neurons tested. In most large I_h neurons (7 of 10) and small I_h neurons (6 of 8), the current difference between carbachol application and control converged as the voltage became less negative, suggesting a reversal at more positive potentials. In the remaining neurons, the curves remained parallel in this voltage range. Thus, a decrease in leak potassium conductance is not the major mediator of the carbachol excitation.

To test whether the carbachol-induced inward current is due to the opening of mixed cation channels, which normally have a reversal potential ~ 0 mV, we applied ramps from -100 to 40 mV. To remove the influence of calcium spikes and voltage-gated Na^+ channels, we used a calcium-free extracellular solution containing TTX ($0.5 \mu\text{M}$). The amplitude of the carbachol-induced inward current under these conditions (large I_h : 82.3 ± 15.2 pA, $n = 10$; small I_h : 99.6 ± 16.1 pA, $n = 14$) was not significantly different from that in regular ACSF ($p > 0.05$, unpaired t test). In 9 of 13 large I_h neurons and 7 of 14 small I_h neurons tested with this protocol, the carbachol-induced inward current reversed close to 0 mV (Fig. 5; large I_h : 0.6 ± 5.4 mV, $n = 9$; small I_h : 4.4 ± 8.5 mV, $n = 7$). In three large I_h neurons and five small I_h neurons, there was no reversal in the range from -100 to 40 mV. One large I_h neuron and two small I_h neurons showed outward currents from -100 to -75 mV, but inward currents from -75 to 40 mV. These data indicated that in most neurons, carbachol opened mixed cation channels permeable to both Na^+ and K^+ . However, closure of leak potassium channels may also be involved in some neurons.

If Na^+ is the main ion carrying the carbachol-induced inward current in most neurons, removing extracellular Na^+ should strongly reduce the carbachol-induced inward current. To test this hypothesis, we reduced the extracellular Na^+ concentration from 152.6 to 25.6 mM by replacing NaCl by equimolar NMDG chloride. Switching the bath solution from ACSF to the low- Na^+ /calcium-free/TTX-containing solution induced outward currents in both large I_h (149.4 ± 35.1 pA, $n = 9$) and small I_h (63.0 ± 7.9 pA, $n = 15$) neurons, likely due to a reduction in hyperpolarization-activated cation currents active at the holding potential (-60 mV; McKenna et al., 2013). In both large and small I_h neurons, the carbachol-

Figure 5. Carbachol activates a noisy, sodium-permeable cation current in BF GABAergic neurons. Large I_h neurons are shown in **A–E**. Small I_h neurons are shown in **F–J**. **A**, Carbachol induces an inward current at -60 mV in large I_h neurons. Downward deflections are voltage ramps from -120 to -60 mV. No reversal was seen in this voltage range. **B**, Carbachol significantly increases membrane current noise. **C**, The carbachol-induced current reverses at ~ 0 mV in regular extracellular $[\text{Na}^+]$ (152.6 mM). **D**, The reversal potential of the carbachol-induced current shifts in the negative direction when extracellular Na^+ is decreased. Insets in **C** and **D** show current–voltage relationships obtained by subtracting the control response from the response in the presence of carbachol. **E**, Decreasing extracellular sodium significantly reduces the amplitude of the carbachol-induced current measured at -60 mV. **F**, Carbachol induces an inward current at -60 mV in small I_h neurons. Downward deflections are voltage ramps from -120 to -60 mV. No reversal was seen in this voltage range. **G**, Carbachol significantly increases membrane current noise in small I_h GABAergic neurons. **H**, The carbachol-induced current reverses at ~ 0 mV in regular extracellular $[\text{Na}^+]$ (152.6 mM). **I**, The reversal potential of the carbachol-induced current shifts in the negative direction when extracellular Na^+ is decreased. Insets in **H** and **I** show current–voltage relationships obtained by subtracting the control response from the response in the presence of carbachol. **J**, Decreasing extracellular sodium significantly reduces the amplitude of the carbachol-induced current measured at -60 mV.

induced inward current in low- Na^+ /calcium-free ACSF was significantly smaller than that in regular Na^+ /calcium-free solution (Fig. 5; large I_h : -11.1 ± 9.2 pA; $n = 8$; $p = 0.0017$, unpaired t test; small I_h : -17.4 ± 6.9 pA; $n = 9$; $p = 0.0008$, unpaired t test). Furthermore, the reversal potentials were significantly more negative (large I_h : -56.6 ± 6.0 mV; $n = 8$;

$p < 0.01$, unpaired t test; small I_h : -71.4 ± 2.0 mV; $n = 5/7$; $p < 0.01$, unpaired t test) than in normal Na ACSF. Thus, both reversal potential measurements and sodium-substitution experiments suggested that the inward current is mediated by sodium-permeable cation channels.

Carbachol increases the frequency of synaptic inputs to GABAergic neurons

Finally, we investigated the effect of carbachol on the synaptic inputs to BF GABAergic neurons. Spontaneous EPSCs (sEPSCs) were recorded at a holding potential of -60 mV in the presence of $10 \mu\text{M}$ GABA_A receptor-mediated events. In both large I_h and small I_h neurons, carbachol caused a large increase in the sEPSC frequency (Fig. 6; large I_h : control, 5.7 ± 1.9 Hz; vs carbachol, 11.6 ± 3.2 Hz; $p = 0.028$, paired t test; $n = 6$; small I_h : control, 1.9 ± 0.8 Hz; vs carbachol, 4.7 ± 1.1 Hz; $p = 0.0267$; paired t test; $n = 10$) and a smaller increase in the sEPSC amplitude (large I_h : control, -18.0 ± 0.9 pA; vs carbachol, -25.2 ± 3.3 pA; $p = 0.0469$, paired t test; $n = 6$; small I_h : control, -16.6 ± 1.7 pA; vs carbachol, -21.0 ± 3.3 pA; $p = 0.0406$, paired t test; $n = 10$), suggesting that carbachol causes both presynaptic and postsynaptic changes. Carbachol did not cause a significant change in the frequency or amplitude of miniature EPSCs (mEPSCs) recorded in the presence of TTX ($0.5 \mu\text{M}$) in large I_h GABAergic neurons. The frequency and amplitude of mEPSCs for large I_h GABAergic neurons were 2.90 ± 0.95 Hz and -18.2 ± 1.5 pA in control, and 1.91 ± 0.72 Hz and -19.9 ± 1.3 pA in carbachol ($p = 0.1788$ for frequency; $p = 0.1290$ for amplitude, paired t test; $n = 4$). Thus, carbachol likely increased the frequency of sEPSCs by increasing the excitability of local glutamatergic neurons synapsing on the recorded neuron. For small I_h GABAergic neurons, there was a small but significant decrease in the frequency of mEPSCs with carbachol application (mEPSC frequency: control, 2.87 ± 0.51 Hz; vs carbachol, 1.33 ± 0.34 Hz; $p = 0.0106$; mEPSC amplitude: control, -17.6 ± 0.9 pA; vs carbachol, -20.5 ± 1.9 pA; $p > 0.05$; $n = 6$). Thus, as with large I_h neurons, increases in the frequency of sEPSCs by carbachol were due to increased excitability of local glutamatergic neurons.

Carbachol had similar effects on inhibitory synaptic inputs. Spontaneous IPSCs (sIPSCs) were recorded at a holding potential of -90 mV using a KCl-based patch solution to increase the driving force for chloride entry in the presence of glutamate receptor antagonists. In both large and small I_h neurons, carbachol significantly increased the frequency and am-

plitude of sIPSCs (Fig. 7; large I_h neurons: sIPSC frequency: control, 13.3 ± 5.9 Hz; vs carbachol, 28.5 ± 7.1 Hz; $p = 0.0028$; sIPSC amplitude: control, -31.6 ± 7.5 pA; vs carbachol, -43.5 ± 10.1 pA; $p = 0.0484$; $n = 7$; small I_h neurons: sIPSC frequency: control, 6.2 ± 2.0 Hz; vs carbachol, 27.6 ± 7.9 Hz; $p = 0.0201$; sIPSC amplitude: control, -33.1 ± 8.0 pA; vs carbachol, -57.9 ± 16.0 pA; $p = 0.0410$; $n = 6$). There was no significant change in the frequency or amplitude of miniature IPSCs (mIPSCs) in large or small I_h GABAergic neurons (large I_h neurons: mIPSC frequency: control, 4.3 ± 2.6 Hz; vs carbachol, 3.3 ± 0.7 Hz; $p = 0.6616$; mIPSC amplitude: control, -23.3 ± 3.1 pA; vs carbachol, -25.6 ± 3.6 pA; $p = 0.5772$; $n = 5$; small I_h neurons: mIPSC frequency: control, 2.6 ± 1.0 Hz; vs carbachol, 1.5 ± 0.5 Hz; $p = 0.0786$; mIPSC amplitude: control, -26.0 ± 3.2 pA; vs carbachol, -31.8 ± 5.9 pA; $p = 0.2405$; $n = 6$). Thus, in both large and small I_h neurons, increases in sIPSC frequency were likely to be due to increased excitability of local GABAergic neurons.

Optical stimulation of cholinergic neurons in ChAT-ChR2-EYFP mice causes an inward current in putative GABAergic/PV neurons

Our results presented above reveal strong pharmacological evidence for cholinergic effects on BF GABAergic neurons. Next, we investigated whether BF GABAergic neurons are excited by acetylcholine released from cholinergic neurons. We used mice in which a ChR2 and EYFP fusion protein is constitutively expressed under the control of the ChAT promoter (Zhao et al., 2011). Immunohistochemical staining for ChAT in ChAT-ChR2-EYFP mice confirmed that $87.3 \pm 2.2\%$ of EYFP⁺ neurons stained positively for ChAT, while 90.2% of ChAT⁺ neurons were EYFP⁺ ($n = 4$; Fig. 8A–C). Thus, ChR2-EYFP was expressed selectively in cholinergic neurons, and almost all cholinergic neurons expressed ChR2-EYFP.

To verify that light could stimulate action potentials in cholinergic neurons in BF slices prepared from ChAT-ChR2-EYFP mice, we first recorded the response of EYFP⁺ neurons to blue (470 nm) light stimulation. As expected, EYFP⁺ neurons were large neurons (long-axis diameter, 21.6 ± 1.0 μ m; $n = 9$), which had the typical intrinsic membrane properties of ChAT⁺ cholinergic neurons (McKenna et al., 2013; Fig. 8D), including a low maximal firing frequency (15.3 ± 3.2 Hz; $n = 4$) and large afterhyperpolarizations (-22.6 ± 3.4 mV; $n = 9$). All EYFP⁺ neurons tested responded to 470 nm light with action potentials confirming the excitatory function of these channels ($n = 8$; Fig. 8D–G). When stimulated at 10 Hz for 10 s (10 ms pulse width), $78 \pm 21\%$ of light pulses elicited action potentials. Thus, optical

Figure 6. A–F, Carbachol increases the frequency and amplitude of sEPSCs in both large I_h (A–C) and small I_h (B–F) BF GABAergic neurons. The holding potential was -60 mV. Experiments were conducted in the presence of the GABA receptor antagonist GABA_zine to isolate excitatory events. A, D, Representative raw 10 s data traces in control (top) and in the presence of carbachol (50 μ M; middle) show that carbachol caused a large increase in the frequency of sEPSCs and a smaller increase in their amplitude. sEPSCs were completely blocked (bottom trace) by a mixture of glutamatergic receptor antagonists (AMPA/kainate receptor antagonist DNQX plus NMDA receptor antagonist AP-5), confirming that they are mediated by activation of ionotropic glutamate receptors. B, E, The cumulative probability histograms for the amplitudes of sEPSCs show a significant increase ($p < 0.001$, Kolmogorov–Smirnov two-sample test). C, F, The cumulative probability histograms for interevent intervals of sEPSCs show a significant increase ($p < 0.001$, Kolmogorov–Smirnov test). The inserts in B, C, E, and F show the data for all recorded neurons (large I_h , $n = 6$; small I_h , $n = 10$). In both groups, there was a statistically significant increase in the frequency and amplitude of sEPSCs ($p < 0.05$, paired t test).

stimulation is very effective in eliciting action potentials in cholinergic neurons.

To test whether the stimulation of cholinergic neurons/fibers causes postsynaptic responses in GABAergic neurons, we recorded light-induced responses from large-sized (21.5 ± 0.6 μ m long-axis diameter), EYFP-negative neurons ($n = 32$), which showed intrinsic membrane properties similar to those of identified cortical projecting GABAergic neurons (McKenna et al., 2013; Fig. 8H; large I_h , $n = 13$: depolarizing sag, $56.1 \pm 1.9\%$; RMP, -63.9 ± 1.8 mV; input resistance, 111.5 ± 17.2 M Ω ; spontaneous firing frequency, 13.2 ± 2.9 Hz; small I_h , $n = 19$: depolarizing sag, $29.4 \pm 2.6\%$; RMP, -62.6 ± 1.2 mV; input resistance, 197.6 ± 29.6 M Ω ; spontaneous firing frequency, $8.9 \pm$

Figure 7. *A–F*, Carbachol increases the frequency and amplitude of sIPSCs in both large I_h (*A–C*) and small I_h (*D–F*) BF GABAergic neurons. The holding potential was -90 mV. Experiments were conducted in the presence of a mixture of glutamatergic receptor antagonists (AMPA/kainate receptor antagonist DNQX plus NMDA receptor antagonist AP-5) to isolate inhibitory synaptic events. A high chloride intracellular solution was used to enhance resolution of sIPSCs. This causes a shift in the equilibrium potential of chloride to more positive values; thus, the inhibitory currents are inward going. *A, D*, Representative raw 10 s data traces in control (top) and in the presence of carbachol ($50 \mu\text{M}$, middle) show that carbachol caused a large increase in the frequency and amplitude of sIPSCs. sIPSCs were completely blocked (lower trace) by the GABA receptor antagonist GABAzine, confirming that they are mediated by the activation of GABA_A receptors. *B, E*, The cumulative probability histograms for the amplitudes of sIPSCs show a significant increase ($p < 0.001$, Kolmogorov–Smirnov two-sample test). *C, F*, The cumulative probability histograms for interevent intervals of sIPSCs show a significant increase ($p < 0.001$, Kolmogorov–Smirnov two-sample test). The inserts in *B, C, E*, and *F* show the data for all recorded neurons (large I_h , $n = 7$; small I_h , $n = 6$). In both groups, there was a statistically significant increase in the frequency and amplitude of sIPSCs ($p < 0.05$, paired t test).

2.0 Hz). In the presence of $20 \mu\text{M}$ DNQX, $50 \mu\text{M}$ AP-5 and $10 \mu\text{M}$ GABAzine to block ionotropic glutamatergic and GABAergic receptors, these neurons responded to a 10 s train stimulation of blue light at 10 Hz with an inward current [Fig. 8*I*; -21.3 ± 2.4 pA, $n = 32$ (13 large I_h neurons, 19 small I_h neurons)]. The current appeared after 2.6 ± 0.4 pulses (i.e., 260 ms) reached a maximum after 29.8 ± 4.5 pulses (~ 300 s) and decayed following the end of the light train with a time constant of 4.6 ± 1.7 s. The current response during the train exhibited some desensitization (Fig. 8*I*). The inward current at the end of the light train was statistically significantly different from the peak response ($73.6 \pm 6.8\%$ of peak; $p = 0.0194$, paired t test). In a control group, two current responses to 10 s light train stimulation (10 Hz, 10 ms width pulse) separated by 5 min were similar in peak amplitude

(first response, -21.2 ± 3.3 pA; second response, -22.5 ± 3.7 pA; $p = 0.643$, paired t test; $n = 9$) and charge within 10 s stimulation (first response, -170.2 ± 32.0 pC; second response, -192.8 ± 32.3 pC; $p = 0.3785$, paired t test). To confirm that the release of acetylcholine was action potential dependent, we examined the response in the presence of 500 nM TTX. With TTX, the response to light train was completely blocked [peak amplitude: without TTX, -16.3 ± 3.6 pA; with TTX, -0.12 ± 0.46 pA; $p = 0.015$; charge within 10 s stimulation: without TTX, -99.6 ± 23.8 pC; with TTX, 2.6 ± 1.2 pC; $p = 0.012$, paired t test; $n = 5$ (1 large I_h neuron; 4 small I_h neurons)]. To examine whether this current response was due to the activation of muscarinic receptors by acetylcholine, we applied atropine ($5 \mu\text{M}$) in the bath and tested its effect on the amplitude of the light-induced response. In the experimental group, putative GABAergic neurons were incubated with atropine ($5 \mu\text{M}$) for 5 min after the first current response. With atropine, the response to the light train was significantly decreased [peak amplitude: without atropine, -26.5 ± 8.6 pA; with atropine, -13.4 ± 9.1 pA; $p = 0.027$; charge within 10 s stimulation: without atropine, -172.5 ± 39.2 pC; with atropine, -67.7 ± 35.4 pC; $p = 0.018$, paired t test, $n = 7$ (3 large I_h neurons; 5 small I_h neurons)]. As atropine did not completely block the response, we added the nicotinic receptor antagonist mecamylamine ($100 \mu\text{M}$) to determine whether the atropine-insensitive component was due to activation of nicotinic receptors. In the presence of both atropine and mecamylamine, the response to the light train was completely blocked (peak amplitude: without atropine and mecamylamine, -20.7 ± 5.5 pA; with atropine and mecamylamine, -0.77 ± 0.57 pA; $p = 0.020$; charge within 10 s stimulation: without atropine and mecamylamine, 149.7 ± 20.0 pC; with atropine and mecamylamine, 2.2 ± 2.5 pC; $p = 0.0052$, paired t test; $n = 4$ (2 large I_h neurons, 2 small I_h neurons), supporting the idea that the inward current was mediated by the release of acetylcholine, and activation of both muscarinic and nicotinic receptors on BF GABAergic neurons.

Discussion

The BF represents the final node of the ventral arm of the brainstem ascending reticular activating system, and as such plays a critical role in activating the cortex during wakefulness and rapid eye movement (REM) sleep (Brown et al., 2012). Within the BF, both cholinergic and noncholinergic BF neurons play important roles in cortical activation and attention (Semba, 2000). Lesion studies in animals report pronounced deficits in cortical activation and attention following lesions of the BF, which are more

profound when both cholinergic and noncholinergic neurons are lesioned, suggesting a synergistic role of cholinergic and noncholinergic BF neurons (Buzsaki et al., 1988; Kaur et al., 2008; Fuller et al., 2011). Among the noncholinergic neurons, neuroanatomical (Freund and Meskenaite, 1992; Gritti et al., 2003; Henny and Jones, 2008), electrophysiological recording (Duque et al., 2000; Hassani et al., 2009), and optogenetic studies (Kim et al., 2011) suggest that the BF GABAergic/PV⁺ neurons may be particularly important in controlling neocortical fast activity, particularly in the gamma frequency (30–80 Hz) range. However, until now, the interaction between BF cholinergic and GABAergic/PV⁺ neurons had not been investigated. Here we show that (1) cholinergic neurons and fibers were located in close proximity to identified BF GABAergic and PV neurons; (2) activation of cholinergic receptors increased synaptic transmission onto BF GABAergic/PV⁺ neurons and strongly increased the firing rates; and (3) optogenetic release of acetylcholine from cholinergic neurons *in situ* excited putative GABAergic/PV⁺ neurons.

Cholinergic and GABAergic cell bodies were found in close proximity in the medial portion of the mouse BF, similar to previous findings in the rat (Gritti et al., 1993, 1997, 2003). In addition, many GABAergic neurons were located in lateral portions of the BF (McKenna et al., 2013), where much of the brainstem cholinergic input terminates (Woolf and Butcher, 1986; Semba et al., 1988). Cholinergic terminals, arising from local axon collaterals of BF cholinergic projection neurons or from brainstem inputs have been shown to terminate on noncholinergic neurons, including GABAergic neurons, in the rat (Záborszky et al., 1986; Martinez-Murillo et al., 1990; Záborszky and Duque, 2000). Heretofore, the subtype of GABAergic neuron targeted by cholinergic neurons had not been identified, however. Here, using dual fluorescent immunohistochemistry for vAChT and PV in *GAD67-GFP* knock-in mice, we observed that cholinergic terminals closely apposed GABAergic/PV neurons. These results suggest that acetylcholine, released from local axon collaterals of BF cholinergic neurons (Záborszky and Duque, 2000) or from brainstem cholinergic afferent inputs (Woolf and Butcher, 1986; Semba et al., 1988) is anatomically positioned to modulate the activity of cortically projecting BF GABAergic/PV neurons.

Consistent with a modulatory role for acetylcholine in the control of BF GABAergic/PV neuron activity were our pharma-

Figure 8. Optogenetic excitation of cholinergic neurons in ChAT-ChR2-EYFP mice causes an inward current in putative GABAergic neurons. **A–C**, Immunohistochemical staining for ChAT confirms that ChR2-EYFP is selectively localized to cholinergic neurons in ChAT-ChR2-EYFP mice. Scale bar, 50 μ m. **D**, Intrinsic membrane properties of an EYFP⁺ neuron. Scale bar, 25 μ m. **E–G**, Cholinergic neurons are excited by light stimulation. EYFP⁺ cholinergic neurons reliably follow a 5 s train of 10 Hz optical stimuli. **H**, Intrinsic membrane properties of a putative GABAergic (EYFP[−]) neuron. **I**, Optical stimulation induces an inward current in putative GABAergic neurons in the presence of AMPA, NMDA, and GABA_A receptor antagonists (20 μ M DNQX plus 50 μ M AP-5 plus 10 μ M GABAzine), with a muscarinic receptor antagonist (atropine) and with a mixture of muscarinic plus nicotinic receptor antagonists (atropine plus mecamylamine). The optically induced inward current is reproducible when repeated at a 5 min interval, is partially blocked by 5 μ M atropine, and is completely blocked by a combination of 5 μ M atropine and 100 μ M mecamylamine. The *p* value and the number of neurons tested (one neuron/animal) under each condition are given above each data bar. Paired *t* test. IR-DIC, Infrared-differential interference contrast.

cological experiments, which revealed for the first time that the cholinergic agonist carbachol strongly increased the firing rate of identified GABAergic and PV neurons with the size and intrinsic membrane properties corresponding to cortically projecting neurons (McKenna et al., 2013). Experiments with subtype-selective antagonists suggested that the excitatory effect of carba-

chol on BF GABAergic neurons was due to activation of muscarinic M_1 and M_3 receptors. Interestingly, recent advances in pharmacology have identified selective M_1 -type receptor allosteric agonists, which show promise in preclinical models of Alzheimer's disease and schizophrenia (Conn et al., 2009; Digby et al., 2012). Our results suggest that one possible target of these agents is the BF GABAergic/PV projection to the cortex.

Previously, we showed that cortically projecting BF GABAergic/PV neurons can be subdivided into two subpopulations based on the amplitude and kinetics of their H-current (McKenna et al., 2013). Further evidence that these two subgroups likely represent functionally different subgroups was provided by our experiments in this study using selective muscarinic antagonists, which suggested that M_1 receptors mediated the effect of carbachol in most large I_h neurons, whereas M_3 receptors mediated the effect on small I_h neurons. However, it should be noted that currently available muscarinic antagonists, such as the ones we used (pirenzepine and 4-DAMP) have relatively limited selectivity between M_1 and M_3 receptors and concentrations of antagonists required to block effects in brain slices are often much higher than the binding affinities of these drugs tested in expression systems would suggest. Thus, other approaches, such as testing the responses in $M1/M3$ knock-out animals, will be required in the future to definitely establish this difference. The functional significance of such a difference is unclear at present but it is of note that M_1 and M_3 receptors differentially couple to calcium and cAMP signaling (Burford et al., 1995), which may impact the prominent H-currents of these neurons by altering their activation/deactivation kinetics (Biel et al., 2009) and thereby alter rhythmic firing by changing the depolarizing drive during the interspike/interburst interval. Thus, in addition to increasing the firing rate, acetylcholine may modulate the burst or cluster firing of identified GABAergic and PV neurons observed *in vivo* (Duque et al., 2000; Hassani et al., 2009).

Several lines of evidence suggested that the carbachol-induced postsynaptic excitation of BF GABAergic neurons was mediated mainly via activation of mixed cation currents, as follows: (1) in the majority of neurons, the reversal potential of the inward current was intermediate between the sodium and potassium reversal potentials; (2) the inward current was associated with an increase in membrane noise, suggesting increased channel opening; and (3) a reduction of extracellular sodium strongly reduced the amplitude of the current and shifted the reversal potential closer to the potassium equilibrium potential. However, in some neurons there was no clear reversal potential or a reversal close to the potassium equilibrium potential, suggesting an additional minor contribution of leak potassium currents. In addition to blocking the increase in firing rate by bath-applied carbachol, atropine also blocked the inward current. Together with the large size of the carbachol-induced inward current, these data suggest that the muscarinic receptor-mediated inward current is the main driver of the increase in firing rate caused by carbachol.

In addition to direct postsynaptic actions, recordings of spontaneous synaptic currents revealed that carbachol increased the frequency of both excitatory and inhibitory events. Thus, in addition to exciting GABAergic projection neurons, carbachol also increased the activity of local glutamatergic and GABAergic interneurons. However, miniature, action potential-independent, synaptic currents were largely unaffected by carbachol, indicating that this effect is due to depolarization of local neurons rather than being due to a direct effect on the axon terminals themselves.

Direct support for an endogenous action of acetylcholine on BF GABAergic neurons was obtained through optical stimulation of cholinergic neurons in BF slice prepared from ChAT-ChR2-EYFP mice. Light stimulation reliably elicited action potentials in cholinergic neurons and elicited an inward current in noncholinergic neurons with the same intrinsic membrane properties as cortically projecting GABAergic/PV neurons (McKenna et al., 2013). One caveat to these findings is that recent experiments have shown that the ChAT-ChR2-EYFP mice that we used here have enhanced acetylcholine release due to overexpression of the vesicular acetylcholine transporter (Kolisnyk et al., 2013). Thus, the magnitude of the inward current we recorded may have been increased due to increased release of acetylcholine in this strain. However, it is at present unclear whether this constitutive up-regulation of acetylcholine release leads to compensatory down-regulation of postsynaptic receptors. In fact, the magnitude of the inward current we recorded was less than that caused by bath application of carbachol. Thus, although we cannot make any conclusions about the magnitude of the cholinergic current in BF GABAergic neurons, our optogenetic results support our pharmacological and anatomical experiments, and suggest that release of acetylcholine *in situ* from cholinergic neurons can also excite BF GABAergic neurons. The light-induced current was partially blocked by atropine and completely blocked by a combination of atropine and mecamylamine, indicating that, in contrast to the inward current induced by bath-applied carbachol, nicotinic receptors are also involved. Presumably slow bath application of carbachol caused desensitization of the nicotinic receptors, whereas fast synaptic release of acetylcholine did not.

Our results here provide the first anatomical and physiological evidence for excitatory effects of cholinergic neurons on cortically projecting BF GABAergic neurons. Thus, the increased firing rate of these neurons during waking and REM sleep (Hassani et al., 2009) may be mediated by input from neighboring basal forebrain and/or brainstem cholinergic neurons. Furthermore, cortical activation deficits associated with neuronal loss in the BF observed in Alzheimer's disease (Grothe et al., 2012; Mesulam, 2012) may impair cortical activation not only via a loss of the cholinergic cortical projection but also via reduced activity of BF cortically projecting GABAergic neurons, due to either direct neuronal degeneration of these neurons or withdrawal of the cholinergic input. Similarly, the inhibition of BF cholinergic neurons by adenosine during sleep deprivation (Porkka-Heiskanen et al., 1997; Yang et al., 2013) may act to reduce the firing of cortically projecting BF GABAergic/PV neurons through a withdrawal of excitatory cholinergic input. Conversely, cholinergic agonists acting on nicotinic or M_1/M_3 receptors should be effective procognitive agents (Conn et al., 2009), not only due to their direct action on the cortex but also due to their excitation of the BF GABAergic/PV cortical projection.

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