

# Local cholinergic-GABAergic circuitry within the basal forebrain is modulated by galanin

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**Abstract** The basal forebrain (BF) is an important regulator of hippocampal and cortical activity. In Alzheimer's disease (AD), there is a significant loss and dysfunction of cholinergic neurons within the BF, and also a hypertrophy of fibers containing the neuropeptide galanin. Understanding how galanin interacts with BF circuitry is critical in determining what role galanin overexpression plays in the progression of AD. Here, we examined the location and function of galanin in the medial septum/diagonal band (MS/DBB) region of the BF. We show that galanin fibers are located throughout the MS/DBB and intermingled with both cholinergic and GABAergic neurons. Whole-cell patch clamp recordings from MS/DBB neurons in acute slices reveal that galanin decreases tetrodotoxin-sensitive spontaneous GABA release and dampens muscarinic receptor-mediated increases in GABA release in the MS/DBB. These effects are not blocked by pre-exposure to  $\beta$ -amyloid peptide ( $A\beta_{1-42}$ ). Optogenetic activation of cholinergic neurons in the MS/DBB increases GABA release back onto cholinergic neurons, forming a functional circuit within the MS/DBB. Galanin disrupts this cholinergic-GABAergic circuit by blocking the cholinergic-induced increase in GABA release. These data suggest that galanin works in the BF to reduce inhibitory input onto cholinergic neurons and to prevent cholinergic-induced increase in inhibitory tone. This disinhibition of cholinergic neurons could serve as a compensatory mechanism to

counteract the loss of cholinergic signaling that occurs during the progression of AD.

**Keywords** Acetylcholine · Medial septum · Galanin · Muscarinic ·  $\beta$ -Amyloid · Optogenetics

## Introduction

Cholinergic neurons in the basal forebrain (BF) send projections to the hippocampus and cortex (Mesulam et al. 1983; Dutar et al. 1995), where they regulate synaptic plasticity and cognition (Huerta and Lisman 1993; Ovsepiyan et al. 2004; Hasselmo 2006; Gu and Yakel 2011; Hasselmo and Sarter 2011). A decrease in cholinergic signaling in the brain due to the loss or dysfunction of cholinergic neurons in the BF is one of the hallmark features of Alzheimer's disease (AD) (Davies and Maloney 1976; Bartus et al. 1982; Whitehouse et al. 1982; Mufson et al. 2008; Schliebs and Arendt 2011). Changes in cholinergic transmission have been shown to coincide with the onset of  $\beta$ -amyloid ( $A\beta$ ) accumulation, and are detectable even during the preclinical stages of AD (Beach et al. 2000; Lim et al. 2015). Altered input onto cholinergic cells has the potential to alleviate or exacerbate this diminished cholinergic neurotransmission by disinhibiting or inhibiting surviving cholinergic cells. Cholinergic neurons in the BF receive input from local GABAergic and glutamatergic neurons, as well as afferent projections from other brain regions (Zaborszky et al. 1999; Colom 2006). The relative strength of these inputs has been shown to change with age (Jasek and Griffith 1998; Griffith et al. 2014), and may also be affected during AD, resulting in an altered ratio of excitatory and inhibitory transmission (Nava-Mesa et al. 2014). However, the extent to which

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synaptic transmission onto cholinergic neurons in the BF is altered in AD is still unknown. Determining how BF circuitry is altered during AD will be fundamental to understanding disease progression and developing novel therapeutic strategies to alleviate the symptoms of cholinergic dysfunction.

One known alteration that happens to BF circuitry during AD is the hyperinnervation of the BF by fibers containing galanin (Chan-Palay 1988; Mufson et al. 1993; Bowser et al. 1997), a 29–30 amino acid peptide that is expressed throughout the central nervous system (Tatemoto et al. 1983; Perez et al. 2001; Lang et al. 2015). Concurrent with the increase in galanin fiber density, there is an increase in galanin receptor expression (Mufson et al. 2000). The elevated levels of galanin in the BF of AD patients could indicate an important role for this peptide in the etiology of the disease, either as a compensatory mechanism or as an exacerbating factor during disease progression. Several studies have examined the potential role of galanin in AD, with some showing that galanin overexpression impairs learning and memory, and decreases choline acetyltransferase (ChAT) levels in the BF (Steiner et al. 2001; Crawley 2008). Conversely, other studies have shown that hyperinnervation of the BF can increase ChAT levels (Counts et al. 2008) and preserve the expression of neuroprotective genes (Counts et al. 2009) in AD brains, and that galanin can protect against A $\beta$ -induced toxicity and learning deficits (Ding et al. 2006; Elliott-Hunt et al. 2011; Li et al. 2013).

The key to discerning the role of galanin in disease progression may lie in examining the interaction between cholinergic and galaninergic signaling within the BF. Galanin infused into the medial septum can increase acetylcholine release in the ventral hippocampus and improve learning and memory (Elvander et al. 2004; Elvander and Ogren 2005). This enhancement may be partly due to a direct excitation of cholinergic neurons by galanin binding to the galanin receptor subtype 2 (GAL<sub>2</sub>) (Jhamandas et al. 2002). However, galanin receptors are found throughout the BF, including non-cholinergic cells (Miller et al. 1997; O'Donnell et al. 1999; Mennicken et al. 2002; He et al. 2005), indicating that galanin may have additional roles in regulating synaptic transmission within the BF. Here, we sought to ascertain the role of galaninergic signaling in the MS/DBB region of the BF, which provides the primary cholinergic input to the hippocampus (Dutar et al. 1995). To do this, we examined the location of galaninergic fibers within the MS/DBB and determined whether galanin can modulate synaptic transmission in the MS/DBB or alter the function of endogenous cholinergic signaling. Our results show that galanin is involved in regulating inhibitory input onto cholinergic neurons and

can modulate a local cholinergic-GABAergic circuit by blunting acetylcholine-induced increases in GABAergic signaling.

## Methods

### Animals

Wild-type C57BL/6J mice were obtained from Charles River and ChAT-IRES-cre (Rossi et al. 2011) (ChAT-Cre) mice were initially obtained from Jackson labs and subsequently bred in-house. Mice were maintained on a 12 h light/dark cycle under constant temperature control with free access to food and water. All procedures were approved and performed in compliance with the NIEHS/NIH Humane Care and Use of Animals in Research protocols.

### Immunohistochemistry

Eight-week-old C57BL/6J mice,  $n = 4–6$  of each sex, were anesthetized with phenobarbital and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4 % paraformaldehyde (PFA) in PBS. Brains were removed from the skull and postfixed overnight in 4 % PFA at 4 °C. Following rinse in PBS, brains were cryoprotected in 30 % sucrose in PBS and embedded in tissue-freezing medium (Triangle Biomedical Sciences). 40  $\mu$ m free-floating coronal cryosections were collected in PBS and every fourth section was processed for immunolabeling. Following a 3 min antigen retrieval in dH<sub>2</sub>O at 100 °C, sections were rinsed in PBS, PBS with 0.3 % Triton X-100 (PBST), and then blocked in either 5 % normal goat serum or 5 % normal donkey serum in PBST at room temperature for 1 h. Sections were then incubated overnight at 4 °C with antibodies against Galanin (rabbit anti-Galanin 1:2000; T-4334 Peninsula Labs) and the cholinergic marker choline acetyltransferase (Goat anti-ChAT 1:1000; AB144P Millipore), the GABAergic marker parvalbumin (goat anti-Parvalbumin 1:2000; ab32895 Abcam), or the GABAergic marker somatostatin (mouse anti-Somatostatin [SOM-018] 1:500; GTX71935 Gene-Tex). Following rinse in PBST, tissue was incubated with Alexa Fluor secondary antibodies at a 1:1,000 dilution (488 goat anti-rabbit; A11034, 488 donkey anti-rabbit; A21206, 568 goat anti-mouse; A11031, and 568 donkey anti-goat; A11057) for two hours at room temperature. Sections were rinsed with PBS, mounted onto slides, and coverslipped with vectashield hard set mounting medium (Vector Laboratories, Inc.; H-1400). Images were collected on Zeiss LSM 780 and 880 inverted confocal microscopes.

## Electrophysiology

At 6–10 weeks of age, mice were decapitated under isoflurane anesthesia. Their brains were removed and immediately placed in an ice-cold cutting solution-containing (in mM) 230 sucrose, 3 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 28 NaHCO<sub>3</sub>, 5 glucose, 0.5 CaCl<sub>2</sub>, 7 MgCl<sub>2</sub>, 1 ascorbate, and 3 sodium pyruvate. Coronal slices (300  $\mu$ m) were taken through the MS/DBB using a Leica VT1200 s vibratome (Leica Biosystems Inc.). Slices were then transferred to an artificial cerebrospinal fluid (ACSF) solution-containing (in mM) 126 NaCl, 3.5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 11 glucose, 2 CaCl<sub>2</sub>, and 1.3 MgCl<sub>2</sub> that was continuously oxygenated with 95 % O<sub>2</sub>/5 % CO<sub>2</sub>. Slices were warmed to 35° C for 30 min, and then cooled to room temperature for 1 h prior to recording.

Following a 1 h recovery period, slices were transferred to a recording chamber that was continuously perfused with oxygenated ACSF at a rate of 2 ml/min. Whole-cell patch clamp recordings were taken from MS/DBB neurons in slices using patch pipettes that were pulled with a P-97 horizontal puller (Sutter Instrument Co.) to a resistance of 3–7 M $\Omega$ . For voltage clamp recordings, cells were held at -60 mV. Cells were only used for analysis if access resistance was below 40 M $\Omega$  and varied by less than 20 % throughout the experiment. Inhibitory postsynaptic current (IPSC) recordings were performed using an internal pipette solution that contained (in mM) 135 CsCl, 10 EGTA, 2 MgCl, 10 HEPES, 4 MgATP, and 0.3 NaGTP, with pH 7.2–7.3 and osmolarity 280–290 mOsm. IPSCs were isolated by bath applying 10  $\mu$ M DNQX and 40  $\mu$ M AP5 to block AMPA and NMDARs, respectively. Excitatory postsynaptic current (EPSC) and current clamp recordings were performed using an internal pipette solution that contained (in mM) 130 K<sup>+</sup>-gluconate, 10 KCl, 10 HEPES, 1 EGTA, 2 MgCl<sub>2</sub>, 3 MgATP, and 0.3 NaGTP with pH 7.2–7.3 and osmolarity 280–290 mOsm. EPSCs were isolated by bath applying 10  $\mu$ M gabazine to block GABA<sub>A</sub>Rs. In a subset of experiments, 0.5  $\mu$ M tetrodotoxin (TTX) was added to the bath solution to isolate action potential-independent postsynaptic currents. Signals were digitized using an Axon Digidata 1550 digitizer (Molecular Devices) and collected using pClamp 10 software (Molecular Devices) with an Axopatch 200B amplifier (Molecular Devices). Data were filtered at 2 kHz and sampled at 10 kHz.

Peptides were allowed to wash-on for at least 5 min. Recordings of spontaneous postsynaptic currents were analyzed for 3–4 min prior to and after drug application. Carbachol often resulted in transient changes in spontaneous activity. Therefore, responses to carbachol were recorded throughout a 6 min wash-on and the peak 30 s of activity was used for statistical analysis. In cells where there was a decrease or no change in frequency, data were

analyzed 90–120 s after the start of carbachol wash-on, at which point complete exchange of the bath solution would have occurred.

## Stereotaxic injections

Expression of mCherry and ChR2 into cholinergic neurons solely in the MS/DBB was achieved by stereotaxic injection of virus directly into the MS/DBB of ChAT-Cre mice. pAAV-EF1a-double-floxed-hChR2(H134R)-mCherry-WPRE-HGHpA (Addgene plasmid #20297 from Karl Deisseroth) and was packaged with AAV stereotypic 9 by the viral core facility at the NIEHS. For stereotaxic injections, 5–7-week-old ChAT-Cre mice were anesthetized with an intraperitoneal (IP) injection of 100 mg/kg ketamine and 7 mg/kg xylazine. Once fully anesthetized, Marcaine (50–75  $\mu$ l of 0.5 % solution of bupivacaine) was delivered subcutaneously to the scalp as a local analgesic. Mice were secured in a stereotaxic instrument (Kopf Instruments) using ear bars. A Hamilton syringe with a 30 gauge needle (Hamilton Co.) was slowly lowered into position and 1  $\mu$ l of virus was infused at a rate of 0.1  $\mu$ l/min for 10 min. Stereotaxic coordinates used were 0.05 mm ML, 0.8 mm AP, and -4.4 mm DV. Following surgery, the mouse was given 0.1 mg/kg buprenorphine subcutaneously (SC), 0.5 cc of sterile saline SC, and 0.1 cc of Antisedan (0.2 mg/ml) IP to reverse the effects of the ketamine/xylazine. The mouse was allowed to recover on a heating pad and monitored for any signs of distress. 2–3 weeks following surgery, mice were decapitated under isoflurane anesthesia and their brains were used for electrophysiology as described above.

## Optogenetic stimulation

Activation of ChR2 was achieved by delivering square pulses of a 470 nm blue LED light through a 400  $\mu$ m diameter, and 0.39 numerical aperture fiber optic cannula (ThorLabs, Inc.) positioned directly over the slice. Output power of the LED was 12.5 mW, as measured by an X-Cite XR2100 power meter (EXFO, Inc.).

## Drugs

All drugs were purchased from Tocris Bioscience unless otherwise noted. Working solutions of all drugs were made fresh from frozen stock solutions each morning. Human A $\beta$ <sub>1–42</sub> (Anaspec, Inc.) was prepared according to package instructions to prevent fibril formation; lyophilized powder was reconstituted in 1 % NH<sub>4</sub>OH and then diluted in ACSF to a stock concentration of 200  $\mu$ M. Stock solutions of A $\beta$ <sub>1–42</sub> were aliquoted and stored at -20 °C and used within 10 days.

## Data Analysis

Electrophysiology data were analyzed using MiniAnalysis 6.03 (Synaptosoft, Inc.). SigmaPlot 13 (Systat Software, Inc.) was used for statistical analysis and graphical illustrations. Data were tested for normality using a Shapiro–Wilk test and then analyzed using the appropriate statistical test: a paired *t* test or Wilcoxon signed-rank test for single-paired comparisons and one-way repeated measures ANOVA with Holm–Sidak post-hoc analysis or Friedman test with Tukey’s post-hoc analysis for multiple comparisons. Significance was defined as a *p* value of <0.05. Data are presented  $\pm$  SEM.

## Results

### Galanin-containing fibers are found throughout the medial septum and diagonal band of Broca

To examine what role galanin may play in regulating BF function, we first sought to determine where galanin fibers are located in relation to two main cell types in the MS/DBB: cholinergic and GABAergic neurons. Galanin expression in the MS/DBB has been studied previously, but there is still some debate as to the precise location of galanin in this region (Melander et al. 1985; Miller et al. 1998; Perez et al. 2001; Keimpema et al. 2014). We performed double immunofluorescence labeling to examine the expression of galanin-containing fibers relative to cholinergic and GABAergic neurons in the MS/DBB of adult C57BL/6J mice. We targeted choline acetyltransferase (ChAT) as a marker for cholinergic neurons, somatostatin (SST) as a marker for GABAergic interneurons, and parvalbumin (PV) as a marker for GABAergic projection neurons (Zaborszky and Duque 2000; Xu et al. 2015). We found that galaninergic fibers are located throughout the MS/DBB, and are intermingled with cholinergic neurons as well as both SST<sup>+</sup> and PV<sup>+</sup> GABAergic neurons (Fig. 1). We did not observe any co-expression of galanin with either cholinergic or GABAergic neurons. These data show that galanin is expressed in fibers throughout the MS/DBB, and may, therefore, be capable of altering synaptic transmission.

### Galanin reduces inhibitory input to basal forebrain neurons

We performed whole-cell patch clamp recordings from basal forebrain neurons of unknown neurochemical identity in acute slices taken from 6- to 10-week-old C57BL/6J mice to determine how galanin affects synaptic transmission within the basal forebrain. To examine the impact of

galanin on GABAergic transmission, we recorded spontaneous IPSCs (sIPSCs) from MS/DBB neurons. We found that 500 nM galanin (1–29 rat, mouse) significantly decreased the frequency of sIPSCs without altering their amplitude (Fig. 2a). This effect was consistently seen in recorded neurons, with all cells responding to galanin with at least a 5 % decrease in sIPSC frequency, and 10 of 11 cells showing at least a 15 % decrease in sIPSC frequency. The *in vivo* galanin receptor antagonist M40 (500 nM) also caused a significant decrease in sIPSC frequency (*n* = 5, data not shown). These results are consistent with the previous studies showing that *in vivo* galanin receptor antagonists often act as partial agonists *in vitro* (Papas and Bourque 1997; Kinney et al. 1998). To determine if the galanin-induced decrease in GABA release was action potential-dependent, we recorded miniature IPSCs (mIPSCs) in the presence of 0.5  $\mu$ M TTX to block voltage-gated Na<sup>+</sup> channels. Galanin had no effect on mIPSC frequency or amplitude (Fig. 2b), indicating that galanin decreases GABA release in a TTX-dependent manner.

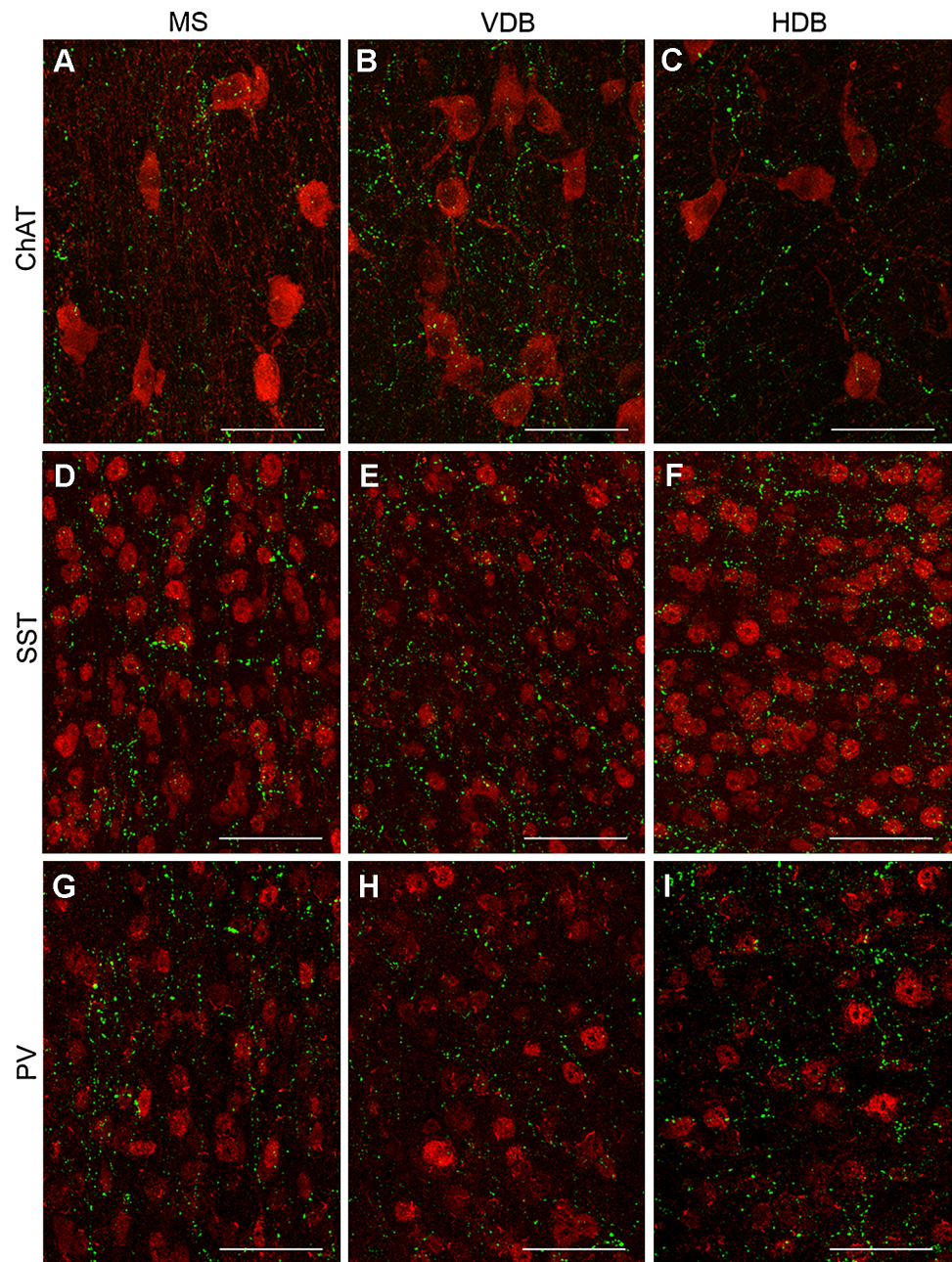
To determine if galanin could alter spontaneous glutamate release, we recorded sEPSCs from MS/DBB neurons. Galanin had no effect on sEPSC frequency or amplitude (Fig. 2c). There was a small, but significant, decrease in mEPSC frequency, but not amplitude, induced by galanin (Fig. 2d). These data suggest that galanin may work directly at glutamatergic presynaptic terminals to decrease TTX-independent glutamate release without altering TTX-dependent glutamate release.

The most pronounced effect of galanin on synaptic transmission that we observed was a decrease in TTX-dependent GABA release. Galanin binds to three known receptors, GAL<sub>1-3</sub> (Habert-Ortoli et al. 1994; Howard et al. 1997; Wang et al. 1997), so we next wanted to determine which galanin receptor subtypes were responsible for decreasing TTX-dependent GABA release in the MS/DBB. To test this, we recorded sIPSCs from MS/DBB neurons prior to and following bath application of the GAL<sub>1</sub>-specific agonist M617, and the GAL<sub>2/3</sub>-specific agonist galanin (2–11) (Gal 2–11, also known as AR-M 1896). M617 (500 nM) significantly reduced the frequency, but not amplitude, of sIPSCs (Fig. 3a), whereas Gal 2–11 (500 nM) had no effect on sIPSC frequency or amplitude (Fig. 3b). These results suggest that galanin reduces GABA release in the MS/DBB via activation of the GAL<sub>1</sub> receptor.

### Galanin blocks muscarinic receptor-induced GABA release

Previous studies have shown that the muscarinic agonist carbachol can excite GABAergic neurons and increase GABA release in the BF (Alreja et al. 2000; Wu et al.

**Fig. 1** Galanin<sup>+</sup> fibers are present throughout the MS/DBB. Fluorescent images show expression of galanin and ChAT in the MS/DBB. Galanin<sup>+</sup> fibers (green) were observed in the vicinity of ChAT<sup>+</sup> neurons (red) in the medial septum (MS) (a), vertical limb of the diagonal band (VDB) (b), and horizontal limb of the diagonal band (HDB) (c). Fluorescent images show expression of galanin and SST in the MS/DBB. Galanin<sup>+</sup> fibers (green) were observed in the vicinity of SST<sup>+</sup> neurons (red) in the MS (d), VDB (e) and HDB (f). Fluorescent images show expression of galanin and PV in the MS/DBB. Galanin<sup>+</sup> fibers (green) were observed in the vicinity of PV<sup>+</sup> neurons (red) in the MS (g), VDB (h), and HDB (i). Scale bars 50  $\mu$ m

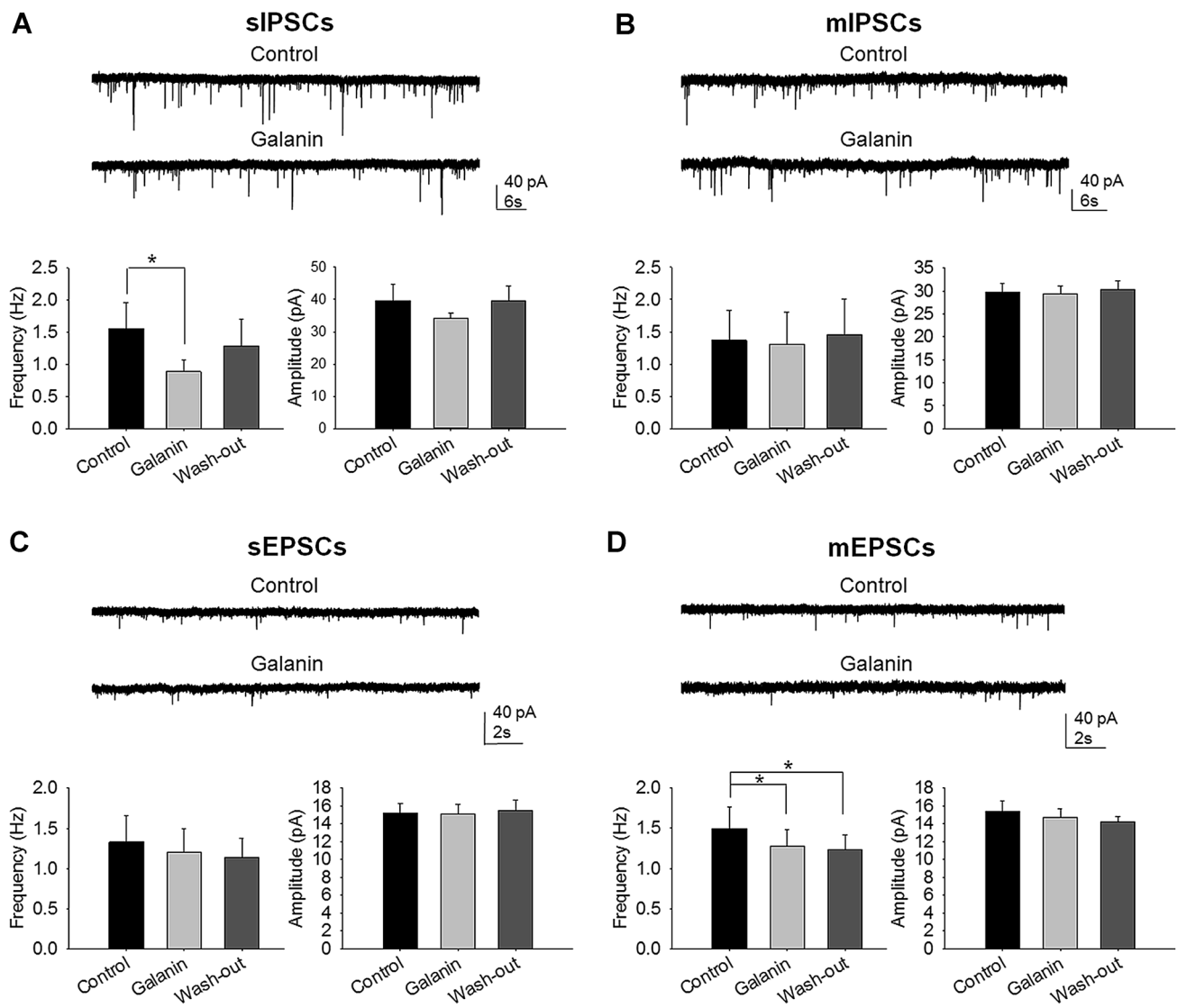


2000; Yang et al. 2014). Because these effects are opposed to those of galanin, we wanted to determine if galanin was capable of interfering with the actions of carbachol. We found that bath application of 50  $\mu$ M carbachol significantly increased sIPSC frequency, but not amplitude, in MS/DBB neurons (Fig. 4a–c). The effects of carbachol were blocked by the muscarinic acetylcholine receptor (mAChR) antagonist atropine (5  $\mu$ M) (Fig. 4d). To determine if galanin could alter carbachol-induced GABA release, we bath applied galanin prior to and during the wash-on of carbachol. In the presence of galanin, carbachol had no significant effect on sIPSC frequency or amplitude

(Fig. 4e–g). Atropine did not block the inhibitory effect of galanin on GABA release, indicating that galanin's blockade of carbachol-induced GABA release is not due to a direct antagonism of muscarinic antagonists (Fig. 4h).

#### **$\beta$ -amyloid peptide decreases glutamate release and does not block the disinhibition caused by galanin**

Galanin expression is increased in the basal forebrain of AD patients (Chan-Palay 1988; Mufson et al. 1993; Bowser et al. 1997), at the same time in which A $\beta$  levels in the



**Fig. 2** Galanin decreases TTX-dependent GABA release in the MS/DBB. **(a)** Galanin (500 nM) significantly decreased the frequency, but not amplitude, of spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from MS/DBB neurons in slices. This effect was reversible following a 10 min wash-out,  $n = 11$ . **(b)** Galanin had no effect on frequency or amplitude of miniature (m)IPSCs,  $n = 10$ .

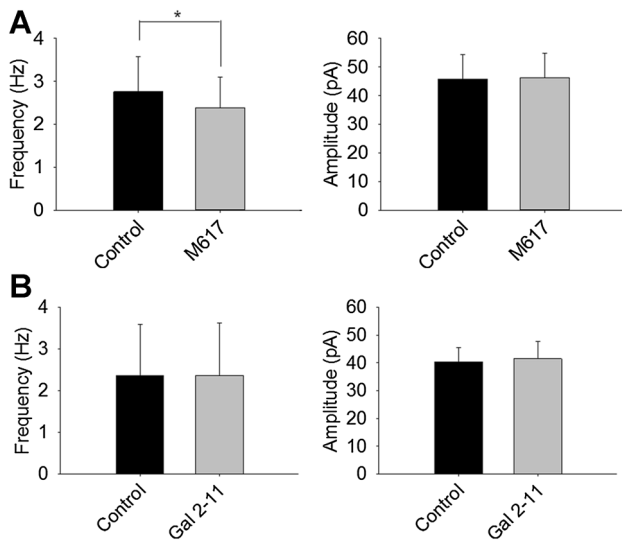
**(c)** Galanin had no effect on the frequency or amplitude of spontaneous excitatory postsynaptic currents (sEPSCs),  $n = 10$ . **(d)** Galanin significantly reduced the frequency, but not amplitude, of mEPSCs,  $n = 10$ . Data presented  $\pm$  SEM. Asterisk indicates  $p < 0.05$

brain will also be high (McLean et al. 1999; Wang et al. 1999). In addition, there is some evidence that galanin and  $A\beta$  can be co-released from dense core vesicles (Toneff et al. 2013). Because these peptides will be highly expressed at the same time, we sought to classify the effects of  $A\beta$  on synaptic transmission within the BF, and determine if there is any interaction between the actions of galanin and  $A\beta$  on synaptic transmission. We found that bath application of 100 nM  $A\beta_{1-42}$  significantly decreased sEPSC frequency, with no change in amplitude (Fig. 5a).  $A\beta_{1-42}$  did not significantly alter sIPSC frequency or amplitude, and did not inhibit the effects of galanin or

carbachol on sIPSC frequency (Fig. 5b, c). Thus, galanin and  $A\beta_{1-42}$  have distinct and opposing actions on synaptic transmission in the MS/DBB; i.e., galanin decreases inhibitory synaptic transmission, while  $A\beta_{1-42}$  decreases excitatory synaptic transmission.

#### Galvanin reduces GABA release onto cholinergic neurons

The galanin-induced decrease in sIPSC frequency shown above was recorded from neurons that likely represented a mixture of cell types. We next sought to confirm that



**Fig. 3** Activation of GAL<sub>1</sub> decreases GABA release in the MS/DBB. **a** The GAL<sub>1</sub> agonist M617 (500 nM) significantly decreased the frequency, but not amplitude, of sIPSCs recorded from MS/DBB neurons,  $n = 8$ . **b** The GAL<sub>2/3</sub> agonist Gal 2–11 (500 nM) had no effect on the frequency or amplitude of sIPSCs,  $n = 5$ . Data presented  $\pm$  SEM. Asterisk indicates  $p < 0.05$

galanin could selectively decrease GABA release specifically onto cholinergic neurons in the MS/DBB by utilizing knock-in mice that expressed Cre recombinase under the control of the ChAT promoter (Rossi et al. 2011). At 5–7 weeks of age, ChAT-Cre mice were stereotaxically injected with a Cre-inducible AAV-containing double-flxed ChR2 (Tsai et al. 2009; Witten et al. 2010) fused with mCherry into the MS/DBB, resulting in selective expression of mCherry and ChR2 in cholinergic neurons (Gu and Yakel 2011). Two to three weeks following injection, whole-cell patch clamp recordings were made from mCherry-expressing neurons in MS/DBB slices (Fig. 6a), which had the electrophysiological characteristics of BF cholinergic neurons, including a low spontaneous firing rate ( $1.17 \pm 0.28$  Hz,  $n = 7$ ), inward rectification, and lack of depolarizing sag (Fig. 6b) (Griffith and Matthews 1986; Unal et al. 2012; McKenna et al. 2013). Bath application of galanin significantly reduced the frequency, but not amplitude, of sIPSCs onto identified cholinergic neurons (Fig. 6c). These results confirm that galanin is reducing GABAergic input directly onto cholinergic neurons in the MS/DBB.

### Galanin disrupts a local cholinergic-GABAergic circuit in the MS/DBB

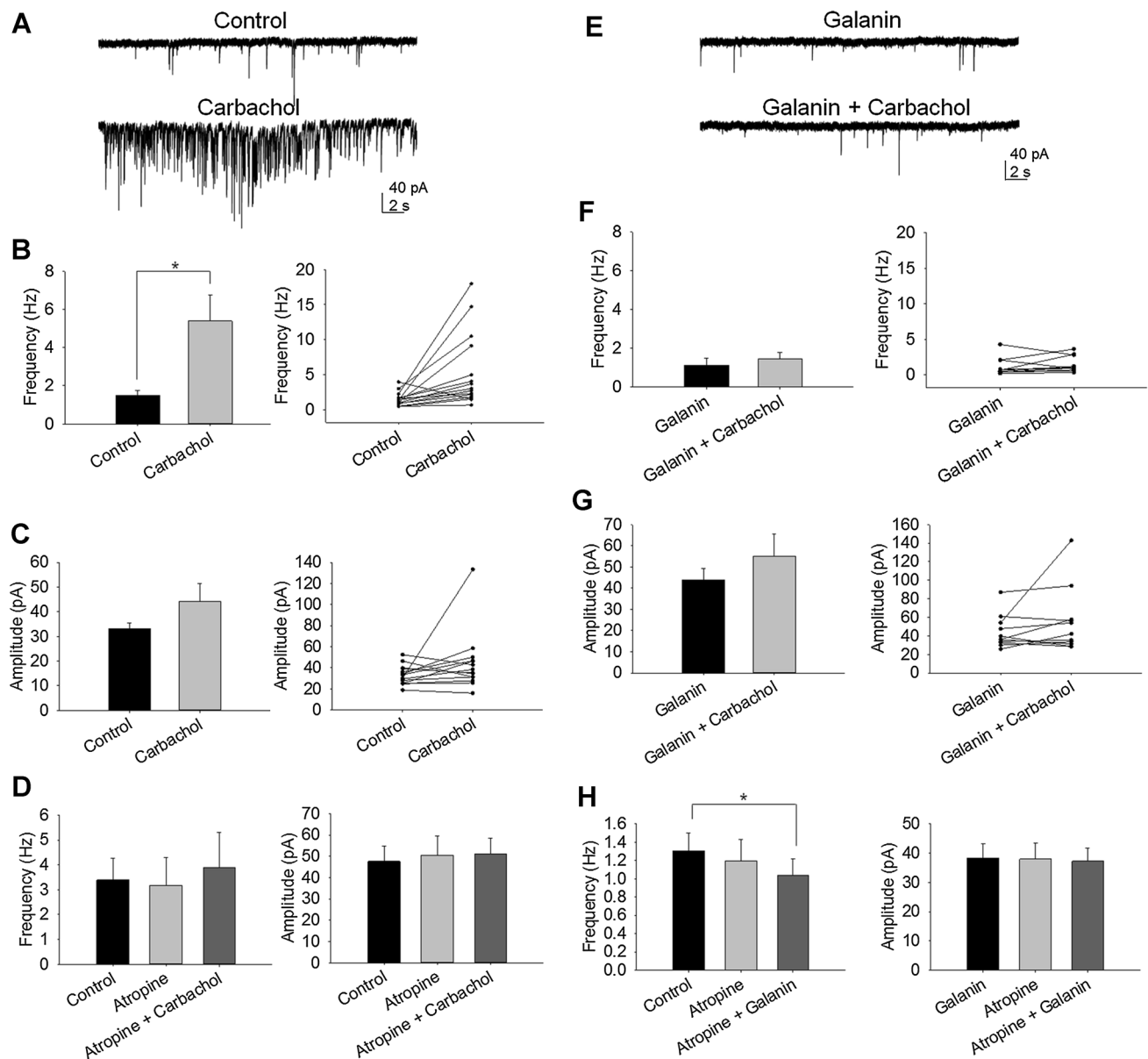
Data presented here and elsewhere (Alreja et al. 2000; Wu et al. 2000; Yang et al. 2014) show that cholinergic activation of muscarinic receptors can activate GABAergic neurons in the BF. However, it has not been shown that activation of

cholinergic neurons within the MS/DBB can increase GABA release back onto cholinergic neurons. To examine the potential existence of a cholinergic-GABAergic circuit within the MS/DBB, we utilized optogenetics to selectively stimulate MS/DBB cholinergic neurons. In ChAT-Cre mice that had undergone stereotaxic injections as described above, cholinergic neurons-expressing mCherry responded to 10 Hz 470 nm blue light stimulation by firing action potentials, confirming the presence and activation of ChR2 in these neurons (Fig. 7a). To test the impact of cholinergic activation on GABA release onto cholinergic neurons, we recorded sIPSCs from identified cholinergic neurons for 45 s prior to and 30 s after a light stimulation protocol that consisted of 4 bursts of 25 ms pulses at 10 Hz that lasted for 1 s and were separated by 3 s inter-burst intervals (Fig. 7b). We found that this light stimulation protocol resulted in a significant increase in sIPSC frequency, but not amplitude, in cholinergic neurons (Fig. 7c). Individual data revealed that 8/17 cells (47 %) had at least a 20 % increase in frequency, and 5/17 cells (29 %) had at least a 50 % increase in frequency (Fig. 7d). In the presence of gabazine (10  $\mu$ M), light stimulation still resulted in large direct inward ChR2-mediated currents, but no sIPSCs were present before or after the light stimulation, confirming that all events are GABA<sub>A</sub>R-mediated ( $n = 5$ , data not shown). Bath application of atropine (5  $\mu$ M) completely abolished the optically induced increase in sIPSC frequency, confirming that cholinergic-induced increases in GABA release in the MS/DBB are predominately mediated by muscarinic receptors (Fig. 7e).

To determine if galanin could disrupt the endogenous cholinergic-induced increases in GABA release in the same way that it blocked carbachol-induced GABA release, we performed our optogenetic stimulation protocol in the presence of galanin (Fig. 8a). In slices that were exposed to galanin prior to and during our light stimulation protocol, light stimulation did not significantly alter the frequency or amplitude of sIPSCs recorded from cholinergic neurons (Fig. 8b). Compared to what was observed under control conditions, there were very few cells that responded to the stimulation with an increase in frequency; 3/15 cells (20 %) had at least a 20 % increase in frequency, and only 2/15 cells (13 %) had at least a 50 % increase in frequency (Fig. 8c). These results indicate that galanin is dampening the ability of endogenous cholinergic activation to increase GABA release onto cholinergic neurons.

### Discussion

Understanding circuitry within the BF is critical to determining how the BF regulates activity in the hippocampus and cortex, and the overall impact of the cholinergic dysfunction that occurs during AD. The overexpression of



**Fig. 4** Galanin blunts carbachol-induced GABA release in the MS/DBB. **a** Sample traces from a MS/DBB neuron showing sIPSCs before and after bath application of carbachol (50  $\mu$ M). **b** Mean data and individual cell data showing the effects of carbachol on sIPSC frequency,  $n = 15$ . **c** Mean data and individual cell data showing the effects of carbachol on sIPSC amplitude,  $n = 15$ . **d** Atropine (5  $\mu$ M) blocked the effects of carbachol,  $n = 8$ . **e** Sample traces showing sIPSCs recorded in the presence of galanin (500 nM) before and after

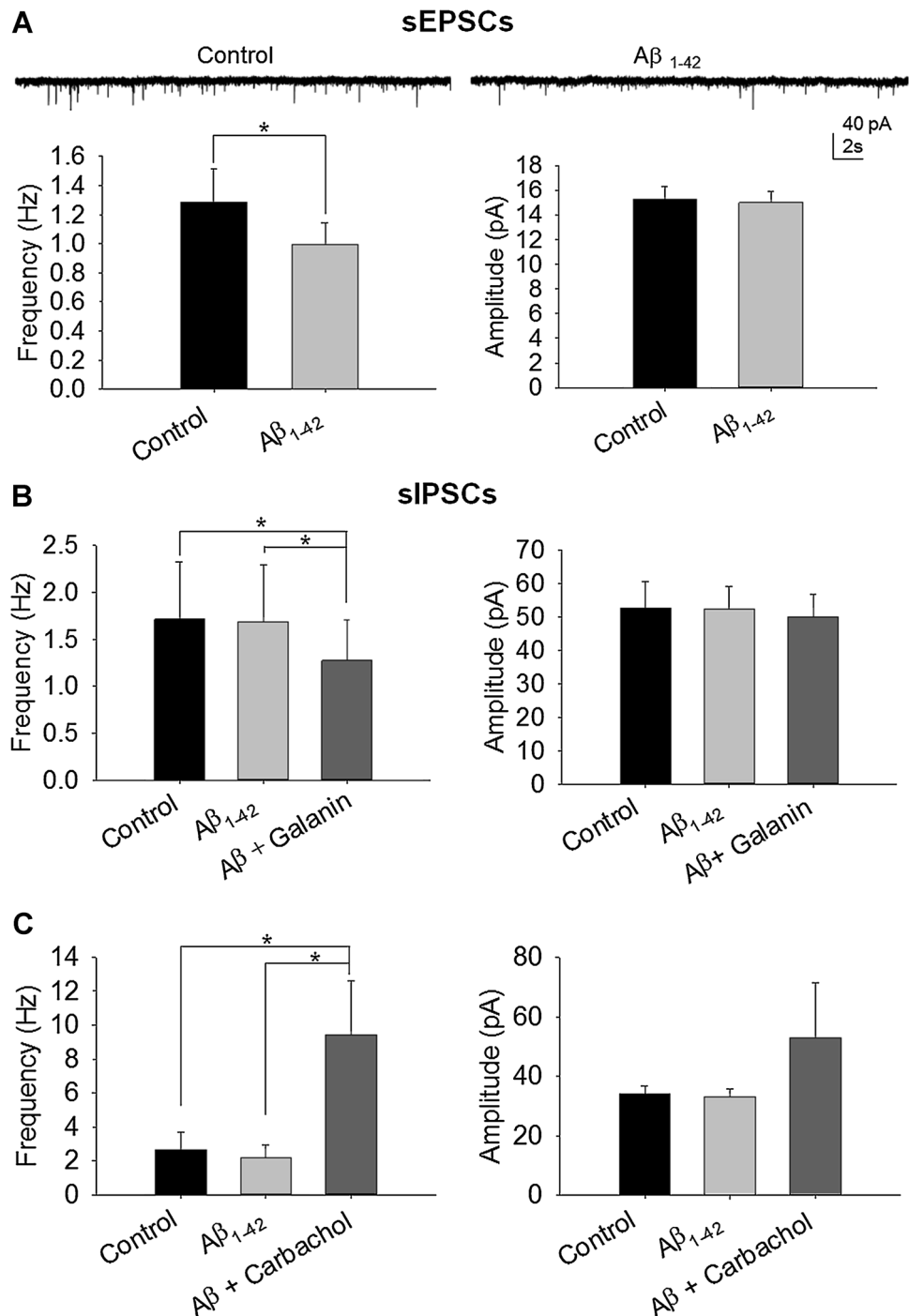
bath application of carbachol. **f** Mean data and individual cell data showing the effects of carbachol on sIPSC frequency in the presence of galanin,  $n = 11$ . **g** Mean data and individual cell data showing the effects of carbachol on sIPSC amplitude in the presence of galanin,  $n = 11$ . **h** Atropine did not block the inhibitory effect of galanin on sIPSC frequency,  $n = 9$ . Data presented  $\pm$  SEM. Asterisk indicates  $p < 0.05$

galanin in the BF of patients with late-stage AD (Chan-Palay 1988; Mufson et al. 1993; Bowser et al. 1997) points to a potentially critical role for this peptide in regulating BF function during the progression of the disease. However, little is known about the function of galanin in the BF, and whether it acts in a neuroprotective or detrimental manner in AD. Here, we show that galanin significantly decreases TTX-dependent GABA release via activation of

the GAL<sub>1</sub> receptor and disrupts a local cholinergic-GABAergic circuit in the MS/DBB. Conversely, A $\beta$ <sub>1–42</sub> significantly decreases glutamate release in the MS/DBB, and does not interfere with the galanin-mediated decrease in GABA release. Together, these data suggest that galanin works in the BF to disinhibit cholinergic neurons by reducing inhibitory input and preventing cholinergic-induced increases in GABA release.



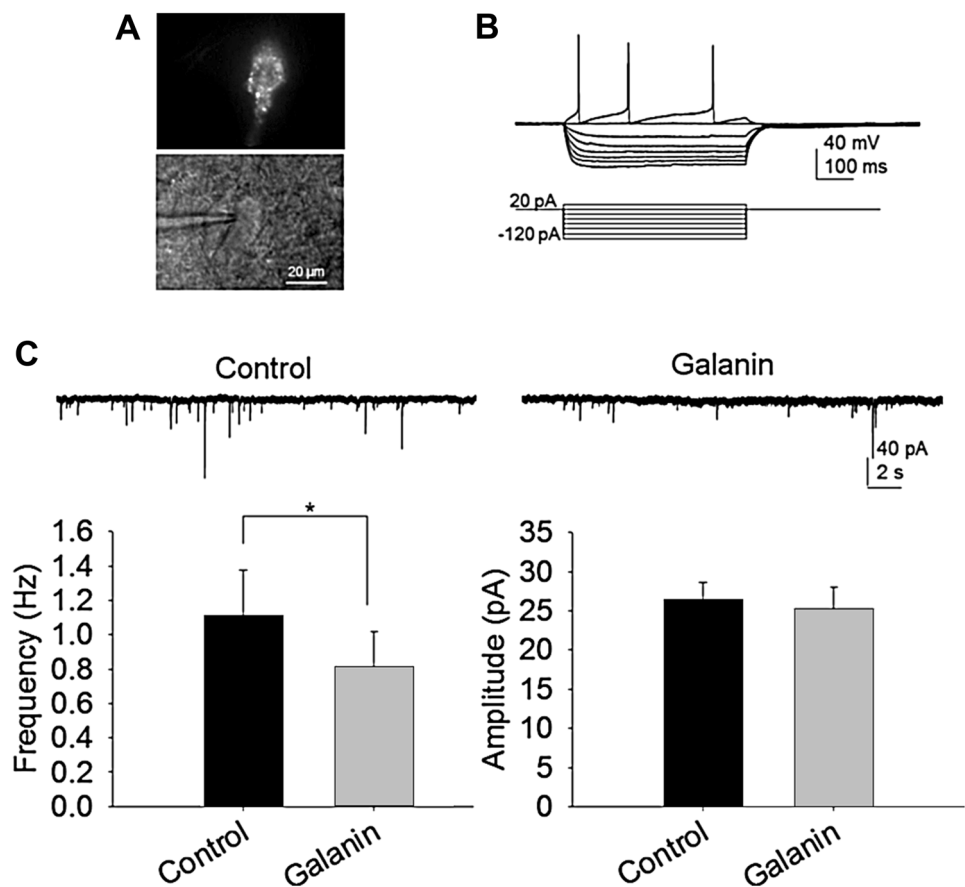
**Fig. 5**  $\beta$ -Amyloid 1–42 peptide ( $A\beta_{1-42}$ ) decreases glutamate, but not GABA, release in the MS/DBB. **a**  $A\beta_{1-42}$  (100 nM) significantly decreased the frequency, but not amplitude, of sEPSCs recorded from MS/DBB neurons in slices,  $n = 13$ . **b**  $A\beta_{1-42}$  had no effect of sIPSC frequency or amplitude, and did not block the inhibitory effects of galanin on sIPSC frequency,  $n = 13$ . **c**  $A\beta_{1-42}$  did not block the excitatory effects of carbachol on sIPSC frequency,  $n = 4$ . Data presented  $\pm$  SEM. Asterisk indicates  $p < 0.05$



Anatomical studies have shown that projecting cholinergic neurons in the BF possess local axon collaterals (Zaborszky and Duque 2000; Duque et al. 2007), but the functional connectivity of these cholinergic fibers has only recently been explored. Recent studies using optogenetic techniques have shown that selective activation of cholinergic neurons can elicit responses in BF GABAergic and glutamatergic neurons (Dannenberg et al. 2015; Xu et al. 2015). Here, we show that not only do MS/DBB

cholinergic neurons synapse onto local GABAergic neurons, they also increase GABA release back onto cholinergic neurons, forming a functional circuit within the BF. This circuit will allow cholinergic neurons to effectively inhibit themselves, thereby preventing prolonged and excessive periods of elevated excitation. This balance may be critical for maintaining normal firing levels and for regulating activity during periods of elevated or rhythmic firing, such as gamma and theta rhythms, which are critical

**Fig. 6** Galanin decreases GABA release directly onto cholinergic neurons in the MS/DBB. **a** Example of an mCherry-expressing cholinergic neuron in the MS/DBB 2–3 weeks following stereotaxic injection of Cre-inducible AAV-containing double-floxed ChR2(H134R) and mCherry into the MS/DBB. **b** mCherry-expressing neurons had electrophysiological properties characteristic of cholinergic neurons in the basal forebrain. **c** Galanin significantly decreased the frequency of sIPSCs onto mCherry-expressing cholinergic neurons,  $n = 11$ . Data presented  $\pm$  SEM. Asterisk indicates  $p < 0.05$

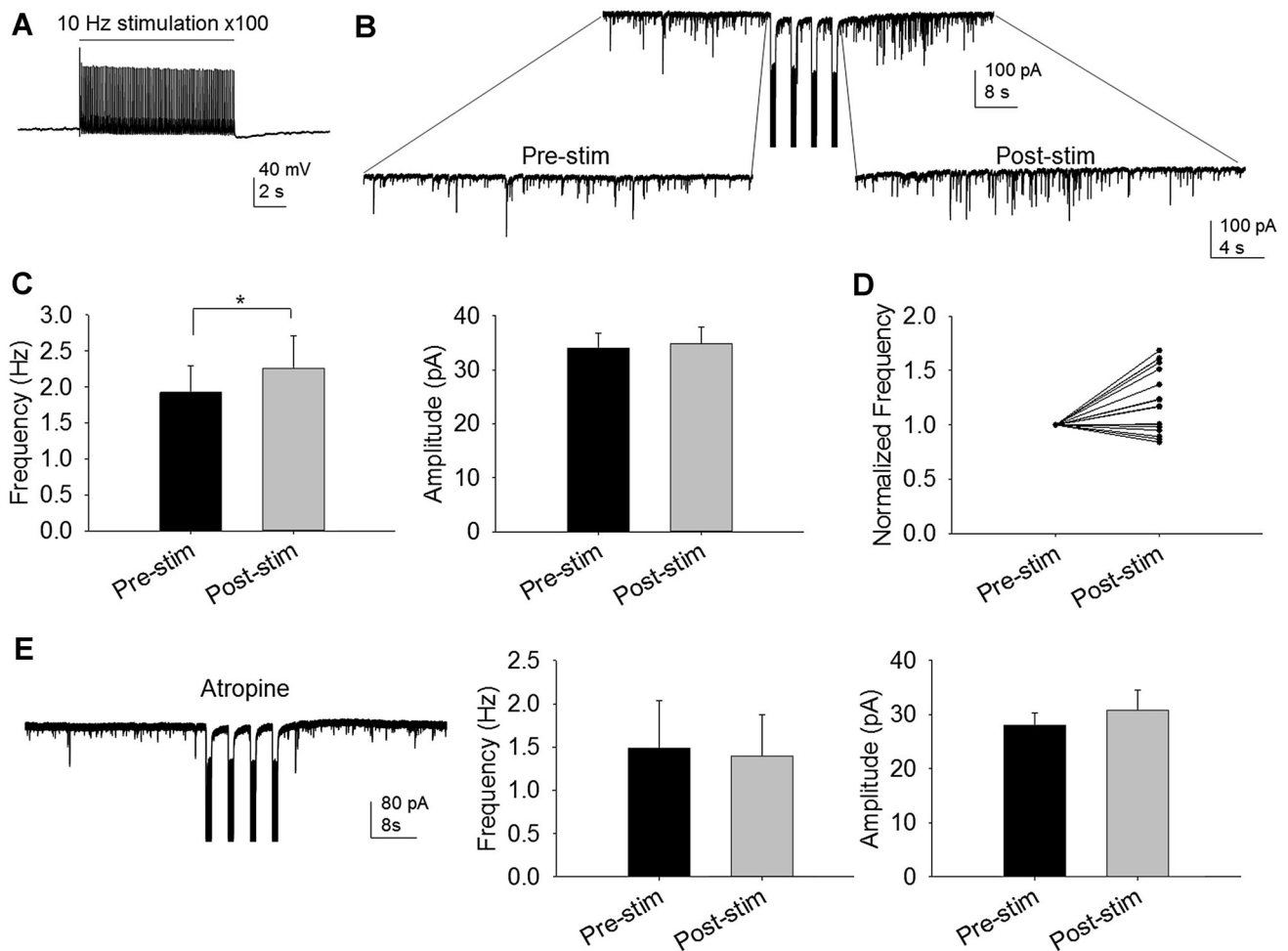


for memory encoding and retrieval (Hasselmo et al. 2002; Lisman and Buzsaki 2008; Colgin et al. 2009) and are altered in AD (Jelic et al. 2000; van Deursen et al. 2008). In the MS/DBB, projecting PV<sup>+</sup> GABAergic neurons can rhythmically fire at theta frequency, while cholinergic neurons fire at slow, non-rhythmic rates (Griffith and Matthews 1986; Sotty et al. 2003; Varga et al. 2008). A recent study reported that MS/DBB neurons regulate theta rhythmicity via an intra-septal relay that is dependent on mAChR activation and leads to the recruitment of PV<sup>+</sup> neurons (Dannenberg et al. 2015). The cholinergic-GABAergic interactions described here may be part of this intra-septal relay and aid in the generation of theta by maintaining the low firing rate of cholinergic neurons while allowing for increased activation of PV<sup>+</sup> GABAergic neurons.

An interplay between cholinergic and galaninergic signaling was first proposed when it was suggested that galanin may be co-expressed in cholinergic neurons of the BF (Melander et al. 1985). However, the extent to which this co-expression occurs has subsequently come into question (Miller et al. 1998; Perez et al. 2001; Keimpema et al. 2014). Here, we show that galanin fibers are found throughout the MS/DBB of the adult mouse. These fibers are intermingled with both cholinergic and GABAergic

neurons, putting them in ideal position to modulate BF function. We did not note co-localization of galanin in cholinergic or GABAergic cell bodies in the BF, suggesting that if galanin is expressed in GABAergic terminals in the BF, as previously shown (Keimpema et al. 2014), these processes may originate from neurons outside the BF. It is also possible that blocking axonal transport of galanin with colchicine treatment may have revealed some co-localization of galanin in cell bodies of the MS/DBB (Melander et al. 1985). However, colchicine has been shown to elevate galanin levels in the brain while reducing ChAT levels (Cortes et al. 1990), which could potentially confound data acquired using this method. Further studies will need to be conducted to definitively say from where galanin fibers in the MS/DBB originate. The diffuse expression of galanin fibers throughout the MS/DBB shown here is similar to what has been observed in higher apes and humans (Benzing et al. 1993; Mufson et al. 1993). These results indicate that the role that galanin plays in regulating BF function in mice may closely reflect the role that galanin plays in the human BF.

Several studies have shown that galanin can regulate acetylcholine release in the hippocampus in a manner that is highly dependent on the region of the brain in which galanin is expressed. For instance, galanin given



**Fig. 7** Activation of cholinergic neurons in the MS/DBB increases GABA release onto cholinergic neurons. **a** Cholinergic cells in the MS/DBB of ChAT-Cre mice-expressing Chr2 responded to 470 nm light stimulation by firing action potentials that followed light pulses at 10 Hz. **b** A sample trace from a MS/DBB cholinergic cell showing that 4 bursts of 10 Hz/1 s light stimulation resulted in an increase in

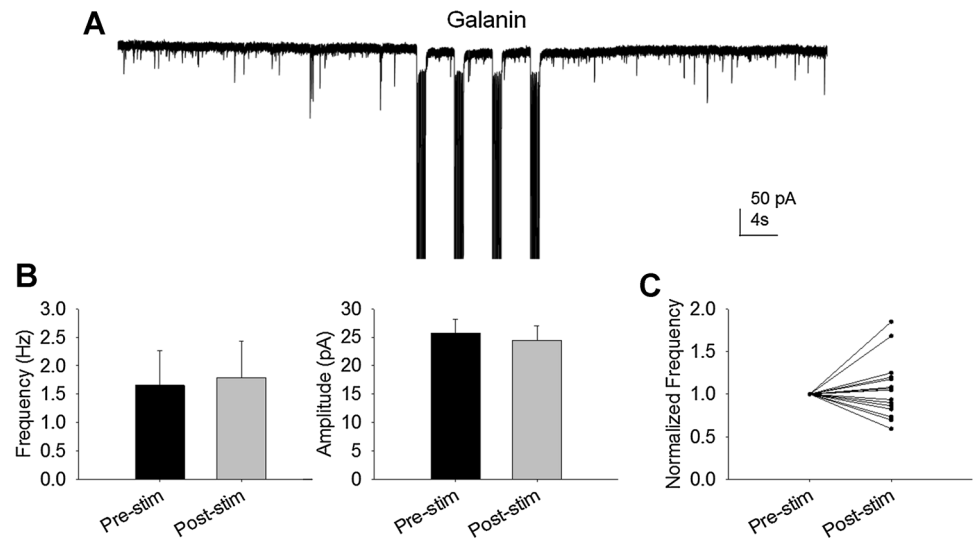
sIPSC frequency. **c** Mean data showing sIPSC frequency and amplitude before and after light stimulation,  $n = 17$ . **d** Individual cell data showing light-induced changes in sIPSC frequency. **e** In the presence of atropine (5  $\mu$ M), light stimulation had no significant effect on sIPSC frequency or amplitude,  $n = 9$ . Data presented  $\pm$  S.E.M. Asterisk indicates  $p < 0.05$

intracerebroventricularly or infused directly into the hippocampus can lead to a decrease in acetylcholine release in the ventral hippocampus (Fisone et al. 1987; Dutar et al. 1989; Yoshitake et al. 2011). Conversely, galanin infused directly into the MS increases acetylcholine release in the hippocampus (Elvander et al. 2004; Elvander and Ogren 2005). The mechanisms for this increase in acetylcholine release are not known, but may be due to a direct activation of cholinergic neurons (Jhamandas et al. 2002) or a disinhibition of cholinergic neurons. In support of the latter possibility, we show that galanin significantly decreases action potential-dependent GABA release in the MS/DBB. This could be due, in part, to decreased firing of BF GABAergic neurons, which have relatively high intrinsic firing rates (Ovsepian et al. 2012; McKenna et al. 2013), and provide inhibitory synaptic input to cholinergic and non-cholinergic neurons in the BF (Colom 2006; Xu et al.

2015). The dichotomy of effects of galanin on the cholinergic system would make it difficult to interpret results from galanin overexpressing or knockout mice. Consequently, both galanin overexpressing and knockout mice display deficits in cognitive tasks (Crawley 2008). To tease apart the localized effects of galanin, there is a need for conditional knockout or overexpressing mice that have galanin altered only in a subset of cells, or within a certain brain region.

The ability of galanin to block mAChR-induced GABA release in the MS/DBB described here suggests an additional level of control over the cholinergic-GABAergic circuit within the BF. These results are in line with the previous studies showing that blocking mAChRs in conjunction with infusing galanin into the MS causes a dramatic increase in acetylcholine release in the hippocampus (Elvander et al. 2004). In the hippocampus, galanin has

**Fig. 8** Galanin blunts acetylcholine-induced increases in GABA release onto cholinergic neurons in the MS/DBB. **a** A sample trace recorded from a MS/DBB cholinergic neuron showing sIPSCs before and after 470 nm light stimulation in the presence of galanin (500 nM). **b** Mean data showing sIPSC frequency and amplitude before and after light stimulation in the presence of galanin,  $n = 16$ . **c** Individual cell data showing light-induced changes in sIPSC frequency in the presence of galanin. Data presented  $\pm$  SEM. Asterisk indicates  $p < 0.05$



also been shown to inhibit the actions of mAChR activation (Fisone et al. 1991; Palazzi et al. 1991). Similar to these studies, we found that this inhibition of mAChR function does not appear to be a result of a direct antagonism of the mAChRs, as indicated by the lack of effect of atropine on galanin-induced decreases in GABA release. It is possible that this inhibition of mAChR function is due to an effect of galanin on voltage-gated  $Ca^{2+}$  entry or an opening of G protein-coupled inwardly rectifying potassium channels (Palazzi et al. 1991; Smith et al. 1998; Endoh et al. 2008; Anselmi et al. 2009). Interestingly, long-term exposure to galanin can upregulate mAChR expression in primary cortical neuron cultures (Cheng and Yu 2015) and can preserve mAChR expression in the hippocampus following increases in ventricular pressure (Barreda-Gomez et al. 2015). Thus, there could be a dual level of control of mAChRs by galanin; an initial and acute inhibition of mAChR-mediated responses followed by a chronic upregulation of mAChRs. It remains to be determined if these mAChRs are functional in nature, or what the long-term consequences of this upregulation may be.

The overexpression of galanin during AD (Chan-Palay 1988; Mufson et al. 1993; Bowser et al. 1997) points to a potentially critical role for this peptide during the progression of the disease. While there have been several studies suggesting that galanin may exacerbate AD symptoms by impairing learning and memory (Steiner et al. 2001; Crawley 2008), the majority of recent evidence suggests a neuroprotective role for galanin (Ding et al. 2006; Counts et al. 2008, 2009; Elliott-Hunt et al. 2011; Li et al. 2013). The results presented here show that galanin can decrease GABAergic transmission in the BF and prevent cholinergic-induced increases in GABA release onto cholinergic neurons. Although further studies would need to be conducted to see what, if any, impact this has on BF

function during AD, it is possible that galanin may work to allow surviving cholinergic neurons to fire and release ACh in regions of the brain, such as the hippocampus and cortex, without the negative feedback in the BF.

Because galanin and  $A\beta$  are both found in high levels during AD, it is important to take into consideration any potential interactions with  $A\beta$  that may alter galanin's actions. Recent studies have shown that  $A\beta$  may significantly alter the function of glutamatergic neurons in the BF, leading to changes in synaptic transmission (Chin et al. 2007; Santos-Torres et al. 2007), and firing rate (Leao et al. 2012). Confirming a role for  $A\beta$  in regulating excitatory transmission in the BF, we found that  $A\beta_{1-42}$  by itself can significantly decrease glutamatergic transmission without altering GABAergic transmission. These data show that the effects of  $A\beta_{1-42}$  on synaptic transmission in the BF are distinct from the actions of galanin. Furthermore, because  $A\beta_{1-42}$  decreases excitation and galanin decreases inhibition in the BF, it is possible that the galanin-induced decreases in GABA release described here could, to some extent, compensate for the  $A\beta$ -induced decrease in glutamate release and partially restore the balance of inputs onto cholinergic neurons.

Interestingly, we found that  $A\beta_{1-42}$  did not interfere with carbachol-induced GABA release. This is in contrast to the previous reports demonstrating an antagonism of mAChR function by  $A\beta$  (Santos-Torres et al. 2007; Preda et al. 2008). Conversely, other studies have shown that  $A\beta$  interactions with mAChRs are more pronounced following prolonged, rather than acute, exposure (Kelly et al. 1996; Janickova et al. 2013). It is possible that longer periods of  $A\beta$  exposure could disrupt mAChR function, and impair acetylcholine-stimulated GABA release in the BF. If this is the case, it would be interesting to determine how this would affect the acute actions of galanin on mAChR-mediated GABA release

described here, or the enhancement of mAChR expression induced by chronic galanin described previously (Barreda-Gomez et al. 2015; Cheng and Yu 2015).

Because of the proposed impact of galanin on cholinergic transmission in the brain, the primary focus of this study was on the effects of galanin on synaptic transmission onto cholinergic neurons in the BF. Accordingly, our optogenetic data clearly show an impact of galanin on GABAergic input onto cholinergic neurons. However, our initial recordings were done on neurons that likely represented a mixture of neuronal cell types. In these experiments, galanin reduced sIPSC frequency by at least 15 % onto 10 of 11 recorded neurons, suggesting that in addition to decreasing GABA release onto cholinergic neurons, galanin is likely also reducing GABAergic input onto other neuronal cell types found in the MD/DBB (e.g., GABAergic and glutamatergic neurons). This could have a profound effect on synaptic transmission within the BF, as well as on the hippocampus and cortex, which receive GABAergic, glutamatergic and cholinergic input from the BF (Gritti et al. 1997; Manns et al. 2001; Sotty et al. 2003). Further examination of galanin-induced changes in synaptic transmission onto non-cholinergic neurons within the BF is warranted to understand the full extent of galanin's effects on BF function.

In conclusion, we show that inhibitory input onto cholinergic neurons in the MS/DBB is, in part, mediated by local cholinergic input onto GABAergic neurons. Exploring this circuit may be key to understanding the intricate circuitry within the BF, and how intra-BF synaptic transmission may affect output to regions of the brain, such as the cortex and hippocampus. We further show that this BF circuit is disrupted by galanin, resulting in a decrease in GABA release onto cholinergic neurons and a blunting of mAChR-mediated increases in GABA release. The resulting disinhibition of cholinergic neurons is likely to have an effect on intra-BF activity as well as on BF output. Continuing to examine galanin's role in BF function will be critical for understanding how galanin affects learning and memory, and determining the functional significance of galanin overexpression in the BF during AD.

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