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Presynaptic Muscarinic M₂ Receptors Modulate Glutamatergic Transmission in the Bed Nucleus of the Stria Terminalis

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Abstract

The anterolateral cell group of the bed nucleus of the stria terminalis (BNST_{ALG}) serves as an important relay station in stress circuitry. Limbic inputs to the BNST_{ALG} are primarily glutamatergic and activity-dependent changes in this input have been implicated in abnormal behaviors associated with chronic stress and addiction. Significantly, local infusion of acetylcholine (ACh) receptor agonists into the BNST trigger stress-like cardiovascular responses, however, little is known about the effects of these agents on glutamatergic transmission in the BNST_{ALG}. Here, we show that glutamate- and ACh-containing fibers are found in close association in the BNST_{ALG}. Moreover, in the presence of the acetylcholinesterase inhibitor, eserine, endogenous ACh release evoked a long-lasting reduction of the amplitude of stimulus-evoked EPSCs. This effect was mimicked by exogenous application of the ACh analogue, carbachol, which caused a reversible, dose-dependent, reduction of the evoked EPSC amplitude, and an increase in both the paired pulse ratio and coefficient of variation, suggesting a presynaptic site of action. Uncoupling of postsynaptic G-proteins with intracellular GDP-β-S, or application of the nicotinic receptor antagonist, tubocurarine, failed to block the carbachol effect. In contrast, the carbachol effect was blocked by prior application of atropine or M₂ receptor-preferring antagonists, and was absent in M₂/M₄ receptor knockout mice, suggesting that presynaptic M₂ receptors mediate the effect of ACh. Immuno-electron microscopy studies further revealed the presence of M₂ receptors on axon terminals that formed asymmetric synapses with BNST neurons. Our findings suggest that presynaptic M₂ receptors might be an important modulator of the stress circuit and hence a novel target for drug development.

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Keywords

Acetylcholine; Eserine; Carbachol; Muscarinic receptor knockout mice; EPSCs

1. Introduction

The anterolateral cell group of the bed nucleus of the stria terminalis (BNST_{ALG}) is thought to play a pivotal role in modulating affective behaviors by serving as a critical interface between cortical and subcortical components of the emotion circuitry (Choi et al., 2008; Davis and Shi, 1999; Hammack et al., 2004; Massi et al., 2008; Walker et al., 2003). Thus, the BNST receives substantial afferent inputs from the amygdala, the hippocampus, and the infralimbic prefrontal cortex (Dong et al., 2001; Dong and Swanson, 2004; Krettek and Price, 1978; Swanson and Cowan, 1979; Walker et al., 2003). In turn the BNST projects to multiple target regions that can modulate the behavioral response of an organism to environmental challenges, including the paraventricular nucleus (PVN), the ventral tegmental area (VTA), the dorsal Raphe nucleus (DRN), and the nucleus of the solitary tract (NTS) (Sawchenko and Swanson, 1983).

Although the BNST is comprised of multiple sub-nuclei, activation of neurons in the anterolateral cell group (BNST_{ALG}), which contains a high density of neurons expressing the stress hormone corticotropin releasing factor (CRF), has been implicated in initiating the acute behavioral response to stress stimuli (Casada & Dafny, 1991). Limbic afferents to the BNST_{ALG} are primarily excitatory in nature and release glutamate to elicit postsynaptic currents in BNST_{ALG} neurons that are mediated by activation of AMPA and NMDA receptors (Adamec, 1989; Egli and Winder, 2003; Guo and Rainnie, 2010; Massi et al., 2008). Significantly, blockade of AMPA receptors in the BNST_{ALG} can abolish light-enhanced startle (Walker and Davis, 1997), suggesting that modification of glutamatergic transmission in the BNST_{ALG} plays an important role in regulating affective behavior. Consistent with this premise, recent evidence suggests that glutamate transmission in the BNST_{ALG} can be modified by chronic restraint stress or prolonged drug exposure (Whitehurst et al., 2006).

Stress has also been shown to increase ACh release in limbic structures (Fadda et al., 2000; Mark et al., 1996; Nail-Boucherie et al., 2000; Tajima et al., 1996), where it is thought to play a critical role in modulating cognition, attention, and synaptic plasticity (Hasselmo and Barkai, 1995; Ovsepian et al., 2004). Several markers for cholinergic terminals are expressed in the BNST, namely choline acetyltransferase (ChAT), and acetylcholinesterase (AChE) (Ben-Ari et al., 1977; Gaspar et al., 1985; Prensa et al., 2003; Ruggiero et al., 1990; Woolf and Butcher, 1982), suggesting that local ACh release may play an important role in modulating the activity of neurons in this region. The response to ACh is mediated by activation of either muscarinic receptors (mAChR) or nicotinic receptors (nAChR), and high levels of muscarinic receptors have been detected in the BNST (Kobayashi et al., 1978; Kushida et al., 1995; Spencer et al., 1986; Wamsley et al., 1984). Muscarinic receptors are classified into five subtypes (M₁–M₅), which couple to their respective effector systems through either G_{αq} (M₁, M₃, M₅) or G_{αi} (M₂, M₄) proteins (Caulfield & Birdsall, 1998). Significantly, *in vivo* extracellular single unit recording studies have shown that muscarinic receptor activation can modulate the firing activity of BNST_{ALG} neurons (Casada and Dafny, 1993). Moreover, microinjection of the cholinergic agonist, carbachol (CCh), into the BNST *in vivo* induces cardiovascular responses that are blocked by the non-selective muscarinic receptor antagonist, atropine, or by a selective M₂ receptor antagonist, but not by a selective M₁ receptor antagonist (Alves et al., 2007), suggesting that activation of different

muscarinic receptor subtypes in the BNST may modulate specific behavioral outcome measures.

Elsewhere in the brain, activation of presynaptic muscarinic receptors has been shown to potently modulate glutamate transmission (Qian and Saggau, 1997; Valentino and Dingledine, 1981). Recently, McElligott and colleagues have proposed that long-term depression of synaptic transmission in the BNST_{ALG} is dependent on activation of Gq-coupled receptors (McElligott et al., 2010). Hence, activation of M₁, M₃, and M₅ receptors, which selectively couple to Gq, would be predicted to reduce glutamate transmission in the BNST_{ALG}. However, M₂ and M₄ receptors have also been reported to modulate presynaptic neurotransmitter release (Trendelenburg et al., 2003; Fukudome et al., 2004), and electron microscopy studies have revealed the presence of M₂ receptors on the axon terminals of glutamatergic afferents suggesting that these receptors may also play a key role in regulating glutamate release (Aoki and Kabak, 1992; Mrzljak et al., 1993).

Despite convergent evidence suggesting that glutamate transmission in the BNST_{ALG} plays a central role in modulating anxiety-like behavior (ibid), and that muscarinic receptor activation modulates the firing activity of BNST neurons and alters cardiovascular tone, nothing is known about the effect of ACh on glutamate neurotransmission in this region. In this study, we have used a multidisciplinary approach to 1) examine the effects of ACh release on excitatory neurotransmission in the BNST_{ALG}, and 2) determine the identity and location of those cholinergic receptor subtype(s) mediating this effect.

2. Methods

2.1 Animals

The majority of experiments in this study were conducted on *ex vivo* brain slices taken from 5–7 week old, male, Sprague-Dawley rats (Charles River, Raleigh, NC). However, several experiments were conducted on tissue taken from 8–12 weeks old, male, transgenic mice. Hence, to examine the relative expression of presumed glutamatergic fibers and cholinergic fibers in the BNST, we conducted an immunohistofluorescence study on tissue sections taken from a Thy-1 eYFP-H mouse line (Thy-1 mouse), which were first described by Sanes and colleagues (Feng et al., 2000), and obtained from Jackson labs (Bar Harbor, ME, USA). To examine the involvement of select muscarinic receptors in the presynaptic modulation of glutamate transmission we conducted studies in tissue obtained from either M₁- or M₂/M₄-knockout mice. The generation and genetic background of the knockout mice have been described previously (Duttaroy et al., 2002; Zhang et al., 2002). For all studies, animals were group housed in cages with 4–5 per cage and had access to food and water *ad libitum*. The care of the animals and all procedures used in this study were performed in accordance with the National Institutes for Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Emory University.

2.2 Immunofluorescence

As previously described (Dabrowska and Rainnie, 2010), immunofluorescence experiments were performed on mouse brain sections derived from two 60 day old Thy-1 eYFP-H mice. Thy-1 eYFP-H mice have been shown to express enhanced yellow fluorescent protein (eYFP) at the high levels in glutamatergic neurons of the prefrontal and somatosensory cortex, as well as the basolateral amygdala (Sugino et al., 2006). To determine if ChAT labeled fibers were observed in close association with glutamatergic fibers in the anterolateral cell group of the bed nucleus of the stria terminalis (BNST_{ALG}), we incubated sections taken from Thy-1-eYFP mice with a goat polyclonal antibody raised against ChAT

(Chemicon-Millipore, AB144P, dilution 1:500). Here, animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg), and then transcardially perfused with ice-cold phosphate buffer saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were rapidly removed and post-fixed in 4% paraformaldehyde for 2 hrs before being cryoprotected in 30% sucrose in PBS overnight at 4°C. Coronal brain sections (40 µm) were cut on a microtome and stored at -20°C in a cryoprotective medium consisting of 25% glycerol and 30% ethylene glycol in 0.05 M phosphate buffer until needed. Representative sections from Bregma 0.26 mm to Bregma 0.10 mm were rinsed 3x for 10 mins in PBS, permeabilized with 0.5 % Triton-X 100 in PBS, and incubated for 48 hrs at 4°C with the primary antibody in 0.5% Triton-X/PBS solution. Sections were then rinsed 3x for 10 mins in PBS and incubated at room temperature for 2 hrs with Alexa-Fluor 568 donkey anti-goat IgG (1:500, Invitrogen, Carlsbad, CA, USA). Sections were then rinsed 3x for 10 mins in PBS, and 1x in phosphate buffer (PB), mounted on gelatin-coated glass slides and coverslipped using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Confocal laser scanning microscopy was used to analyze stained sections and to obtain high-resolution photomicrographs (10x and 63x magnification) using an Orca R2 cooled CCD camera (Hamamatsu, Bridgewater, NJ) mounted on a Leica DM5500B microscope (Leica Microsystems, Bannockburn, IL).

2.3 Patch clamp recording

2.3.1 Slice preparation—Slices containing the anterolateral BNST were obtained as previously described (Guo et al., 2009). Briefly, under deep isoflurane anesthesia (Henry Schein Inc, Melville, NY, USA) animals were decapitated, the brain removed, and immersed in an ice-cold (4°C) 95%–5% O₂/CO₂ oxygenated “cutting solution” with the following component (mM): NaCl (130), NaHCO₃ (30), KCl (3.50), KH₂PO₄ (1.10), MgCl₂ (6.0), CaCl₂ (1.0), glucose (10), supplemented with kynurenic acid (2.0). Coronal sections of 350 µm containing the BNST were cut using a Leica VTS-1000 vibratome (Leica Microsystems, Bannockburn, IL, USA). Slices were kept in oxygenated “cutting solution” at room temperature for 1 hr before transferring to regular artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (130), NaHCO₃ (30), KCl (3.50), KH₂PO₄ (1.10), MgCl₂ (1.30), CaCl₂ (2.50), and glucose (10). Slices were kept in the regular ACSF for at least 30 min before recording. Coronal slices of mouse BNST tissue were prepared in the same way as in the rat.

2.3.2 Patch clamp recording—Individual slices were transferred to a recording chamber mounted on the fixed stage of a Leica DMLFS microscope (Leica Microsystems). The slices were maintained fully submerged and continuously perfused with oxygenated 32°C ACSF, with a speed of ~2 ml/min. Individual BNST neurons were identified by using differential interference contrast (DIC) optics and infrared (IR) illumination with an IR sensitive CCD camera (Orca ER, Hamamatsu, Tokyo Japan). All recordings were confined to the anterolateral cell group as previously reported (Guo and Rainnie, 2010). Whole-cell recordings were obtained using recording pipettes pulled from borosilicate glass and having a resistance of 4–6 MΩ. The recording patch solution had the following composition (in mM): 130 K-gluconate, 2 KCl, 10 HEPES, 3 MgCl₂, 2 K-ATP, 0.2 NaGTP, and 5 phosphocreatine, which was titrated to pH 7.3 with KOH, and 290 mOsm. Data acquisition and analysis were performed using a Multiclamp 700B amplifier in conjunction with pClamp 10.0 software, and a DigiData 1322A AD/DA interface (Molecular Devices, Sunnyvale, CA, USA). The membrane potential was held at -60 mV for all neurons and only those BNST neurons had a stable membrane potential more negative than -55 mV were used. The access resistance were monitored throughout experiment and neurons had more than 15% change of access resistance were discarded.

2.3.3 Recording of stimulus-evoked EPSCs—To record postsynaptic currents in BNST neurons, a concentric bipolar stimulation electrode (FHC, Bowdoinham, ME) was placed in the dorsal stria terminals, the major afferent input to the BNST. Evoked EPSCs were recorded in the presence of the selective GABA_A receptor antagonist SR 95531 (5 μ M) to block the GABA_A receptor-mediated IPSCs. One train of five single square wave pulses (150 μ s, 0.2 Hz) was delivered every 2 min throughout the experiment to induce EPSCs. For analysis the peak EPSC amplitude was calculated as the mean response to each train of 5 stimulations. The mean of three stable EPSC trains obtained before drug treatment was considered as baseline EPSCs. All EPSCs values were normalized to the baseline level and expressed as the percentage of baseline EPSCs.

2.3.4 Paired-pulse stimulation paradigm—To examine the potential site of action of any ACh response, a paired pulse paradigm was employed in which two electrical stimuli were delivered with an inter-stimulus-interval of 50 ms. Five traces with an interval of 5 s between traces were averaged to measure the peak amplitude of both EPSCs. The paired pulse ratio (PPR) was then calculated as the peak amplitude of the second EPSC (EPSC2) divided by the first, EPSC1 (PPR = EPSC2/EPSC1). Alterations in the PPR are thought to represent changes in release probability in the presynaptic terminal (Hess and Ludin, 1987; Manabe et al., 1993).

2.3.5 Coefficient of variation of synaptic transmission—The coefficient of variation (CV) of the postsynaptic response is defined as the ratio of the standard deviation (δ) to the mean (μ), and was used as an alternative measure to determine the site of drug action. A change of CV is associated with either a change of release probability (Pr) or the number of release sites (n) (Choi and Lovinger, 1997). CV is calculated as δ/μ , where δ is the standard deviation of the peak eEPSC amplitude and μ is the mean eEPSC amplitude. Here, we used five eEPSCs immediately before CCh application, and five eEPSCs during the maximal CCh effect to calculate CV in the baseline and CCh respectively.

2.3.6 Miniature EPSCs (mEPSCs) recording—To further determine the site of action of any ACh response, spontaneous mEPSCs were recorded in the presence of TTX (1 μ M) and SR 95531 (5 μ M). A drug-induced change of mEPSCs frequency suggests a presynaptic action whereas a change of mEPSCs amplitude would suggest a postsynaptic action site. Synaptic events were captured continuously for 2 min before and during drug application. All events were detected offline and their amplitude and frequency calculated using MiniAnalysis 6.0 (Synaptosoft Inc., Decatur, GA).

2.3.7 Postsynaptic activation of AMPA receptors—To determine if any drug manipulation had a direct effect on postsynaptic AMPA currents, AMPA (1 mM) was transiently applied (20–100 ms, 5–20 psi) to recorded neurons through a microelectrode connected to a picospritzer II (Parker Hannifin Instrumentation, Cleveland, OH) as previously reported (Guo & Rainnie, 2010). Here, an AMPA containing microelectrode (tip resistance $\sim$ 3 M Ω) was placed near the soma of the neuron to be recorded and AMPA applied every 2 min by transiently increasing the pressure in the electrode. After three stable AMPA currents were recorded, drug was applied by gravity perfusion and the AMPA current evoked in the presence of drug was recorded.

2.3.8 Drug application—Drugs were obtained from 1) Sigma-Aldrich (St. Louis, MO): Carbachol (CCh), tetrodotoxin (TTX), methocarbamol, tubocurarine, muscarine, pirenzepine, 6,7-dinitroquinoxaline-2,3-dione (DNQX), Guanosine 5-(β -thio)diphosphate (GDP- β -s), or 2) Tocris Bioscience (Ellisville, MO):, telencephal, dimethindene, and 3,6a,11,14-Tetrahydro-9-methoxy-2-methyl-(12H)-isoquinolo[1,2-b]pyrrolo[3,2-f][1,3]benzoxazine-1-

carboxylic acid, ethyl ester (PD 102807). Drugs were made as concentrated stock solutions in distilled water, except DNQX, which were made in 100% DMSO. The final concentration of DMSO on application was no more than 0.1%. All drugs were applied in the ACSF using a continuous gravity fed bath application unless specifically stated.

2.4 Whole tissue RT-PCR

Whole tissue BNST RNA was prepared from 500 μ m coronal sections of the mouse brain from which the BNST was micro-dissected out. BNST tissue was homogenized in Trizol and the RNA isolated. The isolated RNA was then reverse transcribed to cDNA as previously described (Guo et al, 2009). The cDNA was amplified using 10X PCR buffer (Qiagen, Germantown, MD), 3 mM MgCl₂ (Qiagen), 10 mM dNTPs, 2.5U of Taq DNA Polymerase (Qiagen) and 100 nM primers. PCR primers used for each of the muscarinic receptors were developed from GenBank sequences with commercially available Oligo software (IDT Tools, Coralville, IA, USA). The housekeeping gene glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used in all experiments as a positive control. The primers used for M₁ receptor were 5'-AGC AGC TCA GAG AGG TCA CAG CCA-3' and 5'-GGG CCT CTT GAC TGT ATT TGG GGA -3' (273 bp); for M₂ receptors were 5'-CAA GAC CCA GTA TCT CCA AGT CTG -3' and 5'-CGA CGA CCC AAC TAG TTC TAC AGT-3' (369 bp); for M₃ receptor 5'-ACA GAA GCG GAG GCA GAA AAC TTT -3' and 5'-CTT GAA GGA CAG AGG TAG AGT AGC-3' (561 bp); for M₄ receptor were 5'-AAG GAG AAG AAG GCC AAG ACT CTG-3' and 5'-GCG AGC AAT GCT GGC AAA CTT TCG -3' (444bp); and for M₅ receptor were 5'-TGT AGC AGC TAC CCC TCT TCA GAG -3' and 5'-AGC AGC AGC TGG AGA CAG AAA GTA-3' (198). Standard PCR was performed on a PTC-200 Peltier thermal cycler (MJ Research) using the following program: 94°C for 40 sec, 56°C for 40 sec and 72°C for 1 min for 40 cycles. PCR products were visualized by staining with ethidium bromide and separated by electrophoresis in a 1% agarose gel.

2.5 Electron Microscopy

For immunoelectron microscopy, rats were sacrificed and perfused with 4% paraformaldehyde, 0.2% glutaraldehyde and 0.2% picric acid in PBS. The brains were blocked and postfixed in 4% paraformaldehyde for 4 hrs. Coronal, 50 μ m thick vibratome sections of the medial temporal lobe were cut and stored frozen at -80 °C in 15% sucrose until immunohistochemical experiments were performed. Single-label immunoperoxidase labeling was performed using rabbit anti-M₂ AChR at a 1:1000 dilution (Millipore). The single-label immunoperoxidase labeling was performed as described previously (Muly et al., 2003). Briefly, sections were thawed, incubated in blocking serum (3% normal goat serum, 1% bovine serum albumin, 0.1% glycine, 0.1% lysine in 0.01 M phosphate buffered saline, pH 7.4) for 1 hr and then placed in primary antiserum diluted in blocking serum. After 36 hrs at 4°C, the sections were rinsed and placed in a 1:200 dilution of biotinylated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA) for 1 hr at room temperature. The sections were then rinsed, placed in avidin-biotinylated peroxidase complex (ABC Elite, Vector, Burlingame, CA) for 1 hr at room temperature, and then processed to reveal peroxidase using 3, 3'-diaminobenzidine (DAB) as the chromagen. Sections were then post-fixed in osmium tetroxide, stained *en bloc* with uranyl acetate, dehydrated, and embedded in Durcupan resin (Electron Microscopy Sciences, Fort Washington, PA). Selected regions of the BNST were mounted on blocks, and ultrathin sections were collected onto pioloform-coated slot grids and counterstained with lead citrate. Control sections processed as above except for the omission of the primary immunoreagent, did not contain DAB label upon electron microscopic examination.

Ultrathin sections were examined with a Zeiss EM10C electron microscope and immunoreactive elements were imaged using a Dualvision cooled CCD camera (1300 × 1030 pixels) and Digital Micrograph software (version 3.7.4, Gatan, Inc., Pleasanton, CA). Images selected for publication were saved in TIFF format and imported into an image processing program (Canvas 8; Deneba Software, Miami, FL). The contrast was adjusted, and the images were cropped to meet size requirements.

2.6 Statistical analysis

Statistical analysis was performed using Graphpad Prism 4.0 (Graphpad software Inc, La Jolla, CA). Data are presented as means ± S.E.M, and were analyzed using either a t-test for paired or unpaired observations, a one-way ANOVA followed by a post-hoc Tukey test, or a two-way ANOVA with Bonferroni post-test. For all comparisons, a *p* value < 0.05 was considered as statistically significant.

3. Results

3.1 Putative glutamate fibers and ChAT-positive fibers are in close association in the BNST_{ALG}

In coronal sections obtained from Thy-1-H eYFP mice, many eYFP containing (eYFP+) cell bodies were observed in the prefrontal cortex, hippocampus, and the BLA (Fig. 1A), regions that have been previously reported to send glutamate projections to the BNST. Consistent with this observation, high levels of eYFP+ fibers were seen to exit the BLA in a medio-dorsal direction through the stria terminalis (st) towards the BNST, and then enter the BNST_{ALG} in its dorsal aspect (Fig 1B). Somatodendritic expression of eYFP+ neurons was rarely observed in the BNST_{ALG}, suggesting that the eYFP+ fibers were predominantly afferent inputs. Using the Thy-1 mice to identify putative glutamatergic afferents to the BNST_{ALG} (Fig 1C), we next used an antibody directed against ChAT to examine the relative association of the eYFP+ fibers with cholinergic compartments in the BNST_{ALG}. No ChAT+ cell somas were observed in the BNST_{ALG}, and only sparse (1–2 cells/per region) ChAT+ neurons were observed in the medial posterior-lateral division of the BNST. However, high to moderate somatodendritic ChAT-immunoreactivity was observed in areas immediately adjacent to the BNST, such as the medial septal nucleus, the triangular septal nucleus, as well as caudate putamen. ChAT+ fibers were observed at moderate levels in the BNST_{ALG}, and were seen to course dorso-ventrally towards the anterior commissure in the same fiber tract as the eYFP+ fibers of the stria terminalis (Fig 1C'). Significantly, eYFP+ fibers and ChAT-immunoreactive fibers with multiple beaded varicosities were seen to run parallel to each other in the stria terminalis (Fig. 1C'') suggesting a potential role for cholinergic afferents in modulating glutamate release in the BNST_{ALG}.

3.2 Endogenous acetylcholine modulates glutamate transmission in the BNST

Having demonstrated the close proximity of possible release sites of cholinergic and glutamatergic fibers, we next examined whether endogenous ACh release from cholinergic terminals could modulate the response of BNST_{ALG} neurons to glutamatergic input. Here, monosynaptic EPSCs (eEPSCs) were evoked by electrical stimulation of the stria terminalis in the presence of the GABA_A receptor antagonist, SR 95531 (5 μM), before, during, and after application of the acetylcholinesterase (AChE) inhibitor, eserine. As illustrated in Figure 2A (open squares), bath application of eserine (20 μM) reproducibly reduced the amplitude of eEPSCs. The eserine effect started after 2 minutes, reached a peak response at 6 minutes, reducing the amplitude of eEPSCs to 66 ± 4% of baseline, and persisted for more than 20 minutes of washout (data not shown). One way ANOVA revealed a significant effect of eserine on eEPSCs amplitude ($F_{1,6}=13.6$, $p<0.01$) and post-hoc Tukey test indicated a significant difference between baseline and post-eserine treatment (Fig 2A, open squares).

Importantly, the inhibitory effect of eserine was completely blocked by pre-application of the non-selective muscarinic receptor antagonist, atropine (5 μ M, Fig 2A, filled diamonds). Two way ANOVA indicated a significant effect of atropine treatment versus eserine application alone ($F_{(1, 164)}=172$, $p<0.001$). Together these results suggest that endogenously released ACh can activate muscarinic receptors in the BNST_{ALG} and thereby suppress glutamate transmission.

3.3 Exogenous CCh mimics the effects of ACh on eEPSCs

We next tested the effect of exogenous application of the non-hydrolysable ACh analogue, carbachol (CCh), on the amplitude of eEPSCs. In all neurons tested, bath application of CCh (10 μ M) caused a significant reduction in the amplitude of the eEPSCs. No significant difference was found in the response to CCh application between Type I – III BNST_{ALG} neurons ((Hammack et al., 2007), data not shown). After 6 minutes of application, the amplitude of the eEPSC was reduced to $30.6\pm2.9\%$ of baseline (Fig 2B, $n=21$, $p<0.001$). Unlike the eserine-induced response, the CCh response was fully reversible with the eEPSC amplitude returning to basal levels after 10 minutes of washout with ACSF. A typical response to CCh application and the group response are shown in Figure 2B. We then examined the dose-dependency of the CCh response. Low concentrations of CCh (0.1 μ M) caused a slight decrease of eEPSCs amplitude ($93.0\pm0.5\%$ of baseline, $n=3$, $p>0.05$), whereas high concentrations (100 μ M) markedly reduced the eEPSC amplitude to $11.4\pm1.5\%$ of baseline ($n=5$, $p<0.01$), and a plot of the full dose-response relationship for CCh revealed an $EC_{50} = 4.0 \mu$ M (Fig. 2C).

Because the effect of CCh was reversible and more potent than eserine, in all subsequent studies we used exogenous CCh application to mimic endogenous ACh, to determine its site action, and to identify the ACh receptor subtype/s mediating the response.

3.4 Exogenous CCh decreases the eEPSCs amplitude by acting at a presynaptic locus

The CCh-induced reduction of eEPSCs amplitude could be due to either pre- or postsynaptic mechanisms, or both. Hence, we next performed a series of experiments to identify the locus of CCh action.

First, we used a paired-pulse paradigm to examine the effects of CCh application on the paired-pulse ratio (PPR) of two eEPSCs elicited in short succession (see Methods). A change in the PPR is considered as indicative of a presynaptic site of action. In the majority of BNST_{ALG} neurons examined, the peak amplitude of the second eEPSC was greater than that of the first eEPSC, i.e., showed paired-pulse facilitation. A typical response is shown in Figure 3A (left trace). The mean PPR of the eEPSCs at baseline in control ACSF was 1.39 ± 0.07 ($n=15$). In the presence of CCh (10 μ M), although the amplitude of both eEPSCs was reduced, the PPR of the eEPSCs was increased (Fig. 3A right trace), and the mean PPR increased significantly to 2.03 ± 0.17 ($n=15$, $p<0.01$, paired t-test, Fig. 3B). The decreased amplitude of the eEPSCs accompanied by an increase in PPR suggested that CCh may act at a presynaptic site to reduce the probability of release from glutamate terminals.

To confirm a possible presynaptic locus for CCh action, we next examined the coefficient of variation (CV) of the eEPSCs before and during drug application. Consistent with the observed increase in PPR, CV was significantly increased in the presence of CCh ($302\pm45\%$ of baseline, $p<0.01$, $n=16$), further confirming a presynaptic site of action for CCh.

To test this hypothesis, we next examined the effect of CCh application on the frequency and amplitude of miniature spontaneous EPSCs (mEPSCs) recorded in BNST_{ALG} neurons. Here, mEPSCs were recorded in the presence of TTX (1 μ M) and SR 95331 (5 μ M) to block action potential dependent synaptic transmission and GABA_A receptor-mediated inhibitory

currents, respectively (Fig. 4A, left traces). In the presence of CCh (10 μ M), the mean frequency of mEPSCs decreased from 4.4 ± 0.7 Hz in control ACSF to 3.2 ± 0.4 Hz ($n=15$, $p<0.01$; Fig 4B). In contrast, the amplitude of the mEPSCs was unaffected by CCh application (ACSF = 9.0 ± 0.9 pA; CCh = 8.6 ± 0.7 pA, $n=15$, $p=0.5$; Fig 4C). Quantitative analysis showed that CCh caused a rightward shift of the cumulative probability curve for the mEPSCs, suggesting that CCh increased the inter-event interval but not the amplitudes for these spontaneous events (Fig. 4D, E). The CCh-induced decrease of mEPSCs frequency together with its lack of effect on mEPSCs amplitude further suggested that CCh acts at a presynaptic locus to decrease glutamate release.

To further confirm that CCh had no direct postsynaptic effect on AMPA currents, we next examined the response of BNST_{ALG} neurons before and during CCh application, to pressure ejection of AMPA. Pressure application of AMPA (1 mM) to the cell soma induced a transient inward current that ranged between 100 – 300 pA, which was completely blocked by the selective AMPA receptor antagonist, DNQX (20 μ M). A typical postsynaptic AMPA response and its block by DNQX are illustrated in Figure 5A. Consistent with our hypothesis, bath application of CCh failed to change the amplitude of the AMPA current (6 min CCh application: $102 \pm 2.8\%$ of baseline, $p>0.05$ Fig. 5B). Moreover, in 3 neurons we simultaneously recorded eEPSCs as well as the postsynaptic AMPA current. Whereas CCh application reduced the amplitude of the eEPSCs to $28.7 \pm 3.1\%$ of baseline ($n=3$, $p<0.01$), it had no effect on the amplitude of the postsynaptic AMPA current. Moreover, the inhibitory effect of CCh on the eEPSCs was fully reversible after 10 min of washout ($93.7 \pm 9.6\%$ of baseline, $n=3$, $p<0.05$ vs CCh, $p>0.5$ vs baseline). To exclude the possibility that pressure ejected AMPA might be acting on adjacent neurons to exert an indirect buffering effect on the amplitude of the postsynaptic AMPA current, a second set of experiments were conducted in the presence of TTX (1 μ M). Consistently, CCh had no effect on the AMPA-induced inward current in all 8 neurons examined in this condition (one way ANOVA, $F_{69}=0.57$, $p=0.81$, Fig 5B).

Finally, McElligott and colleagues have reported that activation postsynaptic $G_{\alpha q}$ -coupled receptors can act to decrease the amplitude of eEPSCs in the BNST (McElligott et al 2010). Three muscarinic receptor subtypes (M_1 , M_3 , and M_5) are $G_{\alpha q}$ -coupled receptors. To determine if activation of postsynaptic $G_{\alpha q}$ -coupled muscarinic receptors may contribute to the CCh response, we included a non-hydrolyzable GDP analogue, GDP- β -S, in the patch recording solution to uncouple G-protein coupled receptors from their effector system. Inclusion of GDP- β -S (1 mM) in the patch solution failed to block the CCh-induced attenuation of the eEPSC amplitude ($29.3 \pm 2.6\%$ of baseline, $n=10$, $p<0.001$, Fig. 6A, B), which closely resembled that recorded with regular patch solution ($30.6 \pm 2.9\%$ of baseline; $p>0.05$). A similar increase of PPR was also observed (baseline = 1.28 ± 0.07 ; CCh = 1.67 ± 0.18 ; $n=10$, $p<0.01$; Fig. 6C, D). Together these results demonstrate that uncoupling postsynaptic G proteins had no effect on CCh-evoked response, and provided further evidence that presynaptic ACh receptors mediate the inhibition of CCh on eEPSCs.

3.5 Presynaptic muscarinic receptors mediate the effect of CCh on eEPSCs

Although we have shown that CCh mimicked the eserine effect, which itself was blocked by the non-selective muscarinic receptor antagonist, atropine, CCh is a broad spectrum ACh agonist, and hence could act at either muscarinic or nicotinic ACh receptors. To exclude an action at nicotinic receptors we compared the CCh response in the presence of atropine with the response in the presence of the nicotinic receptor antagonist tubocurarine. As expected from its blockade of eserine's effects, atropine (5 μ M) fully blocked the CCh-induced attenuation of the eEPSC amplitude ($100.5 \pm 2.7\%$ of baseline, $n=6$, $p>0.05$ vs baseline; Fig 7A), as well as the associated change in PPR (1.30 ± 0.13 baseline, 1.31 ± 0.11 CCh, $n=5$, $p>0.05$; Fig 7B). In contrast, tubocurarine (10 μ M) failed to block the CCh effect on either

the eEPSCs amplitude ($31.6 \pm 8.7\%$ of baseline $n=6$, $p<0.01$), or the associated increase in PPR (baseline = 1.54 ± 0.03 ; CCh = 2.01 ± 0.11 , $n=6$, $p<0.05$; Fig 7A, B). These data strongly suggest that presynaptic muscarinic, but not nicotinic, receptors contribute to the inhibitory effect of CCh on glutamate release.

To confirm this hypothesis, we examined whether the selective muscarinic receptor agonist, muscarine, could mimic the attenuation of eEPSC amplitude induced by eserine and CCh. As expected, bath application of muscarine ($10 \mu\text{M}$), reduced the eEPSCs amplitude to $20.2 \pm 4.7\%$ of baseline ($n=7$, paired t-test, $p<0.01$), an effect that was accompanied by an increase of PPR (baseline 1.38 ± 0.16 , muscarine 1.87 ± 0.22 , $n=7$, $p<0.05$) (see Fig 7C,D).

3.6 Identification of the muscarinic receptor subtype(s) mediating the CCh response

Five muscarinic receptor subtypes have been identified (M_1 – M_5) that are encoded by five different genes (Caulfield and Birdsall, 1998; Wess, 2004). Among these receptors, M_1 , M_2 and M_4 receptors have all been shown to act as presynaptic heteroreceptors and modulate synaptic release in the CNS (Bellingham and Berger, 1996; Kremin et al., 2006; Kubista et al., 2009; Sim and Griffith, 1996; Zhang and Warren, 2002). Hence, we next tested the ability of different M_1 , M_2 and M_4 receptor preferring antagonists to block the CCh-induced decrease of glutamate release in the BNST_{ALG}. As summarized in Figure 7E, the CCh response was dose-dependently attenuated following application of the M_1 receptor-preferring antagonists, telenzepine (TZP, 1 – $10 \mu\text{M}$), and pirenzepine (PZP, 1 – $10 \mu\text{M}$) or the M_2 receptor-preferring antagonists, dimethindene (DMTD, 1 – $10 \mu\text{M}$) and methoctramine (1 – $10 \mu\text{M}$). Telenzepine appeared to have the highest inhibitory potency, followed by pirenzepine, methoctramine and dimethindene. In contrast, in the presence of the M_4 receptor-preferring antagonist, PD102807 ($10 \mu\text{M}$), CCh caused a significant attenuation of the eEPSCs amplitude ($37.0 \pm 5.4\%$ of baseline, $p<0.01$, $n=8$), and an increase of PPR (baseline = 1.28 ± 0.04 ; CCh = 1.71 ± 0.17 , $n=7$, $p<0.05$), effects that were not different from that of CCh application in ACSF ($p>0.05$, Fig 7E), suggesting that M_4 receptors may not play a significant role in the presynaptic effect of CCh.

However, the identity of the presynaptic receptor/s mediating the CCh response remained uncertain as the concentration of antagonists required to attenuate the CCh response were in ranges where multiple muscarinic receptor subtypes are occupied (Caulfield and Birdsall, 1998). To more directly address this issue, we used two lines of transgenic muscarinic receptor knockout mice ($M_1^{-/-}$ and $M_{2/4}^{-/-}$) (Gomez et al., 1999; Hamilton et al., 1997; Wess, 2004) to further examine the contribution of these receptors to the CCh response.

First, we used reverse transcriptase – polymerase chain reaction (RT-PCR) to confirm the selective deletion of the respective ACh receptor subtypes in the M_1 or $M_{2/4}$ knockout mice. As illustrated in Figure 8A, mRNA transcripts for all five muscarinic receptors were detected in BNST tissue from wild-type mice. As expected, M_1 receptor mRNA transcripts were not observed in the $M_1^{-/-}$ mice, and no M_2 or M_4 receptor mRNA transcripts were detected in tissue from the $M_{2/4}^{-/-}$ mice.

We next confirmed that the CCh response in wild type mice was similar to that seen in the rat BNST_{ALG}. Here, CCh ($10 \mu\text{M}$) caused a similar attenuation of the amplitude of the eEPSCs in BNST_{ALG} neurons from wild type mice as was seen in rat tissue ($32.4 \pm 7.1\%$ of baseline, $n=8$, $p<0.01$), and the PPR also showed a similar increase (baseline = 1.12 ± 0.07 ; CCh = 1.51 ± 0.13 , $n=8$, $p<0.05$; Fig 8B left traces). Moreover, the CCh effect was significantly attenuated by pretreatment with either telenzepine ($1 \mu\text{M}$, $92.7 \pm 2.8\%$ of baseline, $n=6$, $p<0.05$), or methoctramine ($1 \mu\text{M}$, $60.1 \pm 4.9\%$ of baseline, $n=5$, $p<0.01$).

In $M_1^{-/-}$ mice, the mean amplitude of the eEPSC (231 ± 27 pA; $n=10$) was not significantly different from the mean amplitude of the eEPSC in wild type mice (257 ± 26 pA, $n=8$, $p>0.5$). Moreover, application of CCh ($10 \mu\text{M}$) attenuated the eEPSC amplitude to $32.6 \pm 3.4\%$ of baseline ($n=10$, $p<0.01$; Fig 8B, C), which was not significantly different from that observed in the wild-type mice ($32.4 \pm 7.1\%$ of baseline), and the PPR during CCh application increased (baseline = 1.31 ± 0.07 , CCh = 1.69 ± 0.14 ; $p<0.05$, $n=9$) as seen in wild-type mice. Significantly, the CCh effect in the $M_1^{-/-}$ mice was blocked by telenzepine ($1 \mu\text{M}$, $94.8 \pm 2.7\%$ of baseline, $p>0.05$), suggesting that presynaptic M_1 receptor were unlikely to modulate glutamate release in the BNST_{ALG}, and that the telenzepine effect in wild-type mice was most likely due to a non-specific action at other muscarinic receptor subtypes.

Deletion of the genes for the M_2 and M_4 receptor also had no direct effect on the amplitude of the eEPSC ($M_2/M_4^{-/-}$ = 242 ± 24 pA; $n=10$; wild-type = 257 ± 26 pA; $n=8$; $p=0.66$). However, in $M_2/M_4^{-/-}$ mice the mean eEPSC amplitude was only reduced to $70.2 \pm 4.9\%$ of baseline ($n=10$, $p<0.01$; Fig 8B, C) in the presence of CCh ($10 \mu\text{M}$), which was significantly different from that observed in wild-type mice ($32.4 \pm 7.1\%$ of baseline, $p<0.01$). Moreover, CCh application failed to induce a significant change of the PPR in $M_2/M_4^{-/-}$ mice (baseline = 1.19 ± 0.07 ; $M_2/M_4^{-/-}$ = 1.35 ± 0.15 , $n=10$, $p=0.23$; Fig 8B, D). Given that the M_4 antagonist PD102807 had minimal effect on the CCh response, these data suggest that presynaptic M_2 receptors play a major role in mediating the CCh effect on glutamate release in the BNST_{ALG}. The residual CCh effect in the $M_2/M_4^{-/-}$ mice further suggests that other muscarinic receptors may also contribute to the CCh effect. Consistent with this hypothesis, prior application of telenzepine ($1 \mu\text{M}$) blocked the residual CCh effect in the $M_2/M_4^{-/-}$ mice ($92.7 \pm 2.8\%$ of baseline, $n=6$, $p<0.05$).

3.7 Evidence for M_2 receptor expression on presynaptic terminals in the BNST_{ALG}

In order to provide a structural substrate for our physiological findings we used immunoelectron microscopy to directly examine the distribution of M_2 receptors in the neuropil of the BNST_{ALG}. Immunoreactivity for the M_2 receptor could be identified in a variety of different compartments of the BNST neuropil, including dendritic shafts and spines, axons and axon terminals, as well as occasional glial processes. Consistent with our physiological data, labeled axon terminals were observed to make asymmetric synaptic contacts with both dendritic spines and shafts (Fig 9A), suggesting a presynaptic locus for M_2 receptors on excitatory inputs. Interestingly, labeled axon terminals sometimes contain dense core vesicles (Fig. 9B and C), suggest presynaptic M_2 receptor might modulate the release of other neurotransmitters such as neuropeptides, which are stored in the dense core vesicles in the release site.

4 Discussion

In this study, we have shown that glutamatergic- and cholinergic fibers are found in close association in the primary input stream to the BNST_{ALG}, namely the stria terminalis. Moreover, endogenous ACh significantly decreased the amplitude of evoked EPSCs, an effect that was mimicked in a reversible, dose-dependent manner by exogenous CCh administration ($\text{EC}_{50} = 4 \mu\text{M}$). Furthermore, pharmacological studies in rats and knockout mice suggest that the ACh effect on glutamate transmission is mediated by presynaptic M_2 receptors. Consistent with these data, we found that a sub-population of M_2 receptors are situated on axon terminals that make asymmetric synaptic contacts onto dendritic spines and shafts of BNST_{ALG} neurons. Together these data strongly suggest that ACh acting at presynaptic M_2 receptors play a critical role in modulating the input-output function of the BNST_{ALG} by regulating the strength of the excitatory afferent drive onto neurons in this region.

Significantly, we have shown that the AChE inhibitor, eserine, induced a long lasting reduction of the eEPSC amplitude in BNST_{ALG} neurons, an effect that was fully blocked by prior application of the non-preferring muscarinic receptor antagonist, atropine. These data suggest that the response to locally released ACh is highly regulated by the activity of AChE, but when ACh metabolism was prevented by eserine, increased extracellular ACh levels could now activate presynaptic muscarinic receptors and modulate glutamate release in the BNST_{ALG}. Consistent with these results, our immunohistochemical study showed a close association of cholinergic and glutamatergic fibers in the stria terminalis, and previous studies have reported a similar, atropine-sensitive, eserine-induced suppression of excitatory neurotransmission in the entorhinal cortex (Hamam et al., 2007), hippocampus (Colgin et al., 2003; Goto et al., 2008), olfactory tubercle (Owen and Halliwell, 2001), and hypoglossal motoneurons (Singer et al., 1996).

Converging evidence from the current study suggest that the effect of ACh on glutamate transmission is mediated by presynaptic muscarinic receptors. These results are consistent with previous studies showing that presynaptic muscarinic receptor activation suppresses glutamate release elsewhere in the brain (Fernandez de Sevilla et al., 2002; Sim and Griffith, 1996; Williams and Johnston, 1990; Zhang and Warren, 2002). Nicotinic receptors can also modulate glutamate transmission in the CNS. However, in contrast to muscarinic receptor activation, nicotinic receptor activation appears to facilitate glutamate transmission in the CNS (for a review see (Albuquerque et al., 2009)). For example, in the nucleus accumbens, ACh or CCh attenuated the EPSC amplitude in control ACSF, but ACh increased the EPSCs amplitude in the presence of atropine, an effect that was mimicked by nicotinic receptor agonists and blocked by nicotinic receptor antagonists. Together these data suggest that ACh can bi-directionally modulate glutamate transmission via muscarinic and nicotinic receptor activation, respectively (Zhang & Warren 2002). However, no effect of nicotinic receptor activation was observed in our study, suggesting that ACh modulation of glutamate release is region specific.

We used a combination of muscarinic receptors-preferring antagonists and muscarinic receptor knockout mice in an attempt to delineate the receptor subtype(s) mediating the inhibitory effect of ACh on glutamate transmission. However, our data raised an apparent paradox regarding the role of M₁ receptors in the ACh response. Here, the CCh effect was effectively blocked by M₁ receptor-preferring antagonists, but remained intact in the M₁^{-/-} mice. The most parsimonious explanation for this observation is that the concentration of M₁-preferring antagonists used in this study might block other muscarinic receptor subtypes, including M₂ receptor. This explanation is supported by the observation that the CCh effect was insensitive to deletion of the M₁ receptor, and yet 1 μM telenzepine blocked the CCh effect in M₁^{-/-} mouse, suggesting that telenzepine was acting to non-selectively block other muscarinic receptor subtypes. Consistent with this premise, the CCh effect was significantly attenuated in the M₂/M₄^{-/-} mice, and the residual response was once again blocked by telenzepine. These data strongly suggest that presynaptic M₂ receptor play a significant role in modulating glutamate release in the BNST_{ALG}, but cannot exclude a role for other muscarinic receptor subtypes.

In agreement with our physiological observations, our electron microscopy study showed expression of M₂ receptors in presynaptic compartments. Significantly, presynaptic M₂ receptors were located on axon terminals that were seen to make asymmetric synaptic contacts with the spines of BNST_{ALG} neurons, which are presumably excitatory synapses, and hence consistent with the role of presynaptic M₂ activation in reducing glutamate release. A similar presynaptic location of M₂ receptor has been reported in several brain regions including the hippocampus, striatum and cerebral cortex (Aoki and Kabak, 1992; Decossas et al., 2005; Hajos et al., 1998; Hersch and Levey, 1995; Mrzljak et al., 1993;

Plummer et al., 1999; Rouse et al., 1998). Interestingly, we also found M₂ expressions in postsynaptic compartments, including dendritic shafts and spines. This finding is consistent with our whole tissue RT-PCR study showing that transcripts for the M₂ receptor (in addition to the other four muscarinic subtypes) are expressed in the BNST_{ALG}. Studies are in progress to determine the role of postsynaptic M₂ receptor, as well as the other muscarinic receptor subtypes in BNST_{ALG} neurons.

Computational models of associative memory formation have suggested that ACh-induced suppression of excitatory transmission may play a critical role in preventing previously encoded associations from interfering with the encoding of new associations (for reviews see (Hasselmo, 2006; Hasselmo and Giocomo, 2006)). Given the proposed role of the BNST in modulating anxiety-like behavior in response to unpredictable stressors (Walker et al. 2003), it is possible that release of ACh in the BNST_{ALG} may act to enhance the signal-to-noise ratio in afferent pathways such that only salient sensory information would be transmitted into efferent pathways. For example, the BNST is thought to act as a critical station that relays input from the infralimbic cortex to subcortical structures such as the ventral tegmental area (Massi et al, 2008), and excitatory neurotransmission in this pathway is dynamically modulated following chronic drug administration, or stress (Francesconi et al., 2009; McElligott et al., 2010; Weitlauf et al., 2004). Acute stress can induce ACh release in limbic regions, including the PFC (Laplanche et al., 2004) and the hippocampus (Mitsushima et al., 2003; Stillman et al., 1997), and cholinergic neurons of the lateral septum and diagonal band of Broca, which send projections to the hippocampus and PFC, also project to the BNST (Shin et al., 2008). Consequently, ACh release in the BNST may also increase in response to an acute stressor and act to selectively filter the response of BNST_{ALG} neurons only to strong, salient, sensory input by activating presynaptic M₂ receptors. Hence, stress-induced ACh release in the BNST_{ALG} may act to gate the flow of sensory information in this critical component of the stress circuitry. Furthermore, the BNST_{ALG} contains a heterogeneous population of neurons that differ, not only in their electrophysiological properties (Hammack et al, 2007), but also in their transcriptome expression (Hazra et al., 2011). Importantly, the stress hormone CRF is preferentially expressed by Type III BNST neurons (Dabrowska et al., 2011). Hence, M₂ receptor activation on glutamatergic terminals targeting CRF neurons may act to decrease the firing activity of CRF neurons and reduce anxiety-like behavior. Indeed, M₂ receptors have been implicated in the pathophysiology of emotional disorders. Among the 11 single nucleotide polymorphisms (SNPs) spanning the human gene encoding the muscarinic M₂ receptor (CHRM2), three SNPs were found that significantly associated with alcohol dependence and two SNPs associated with major depression (Wang et al., 2004). Furthermore, binding studies have shown that M₂ receptor binding decreased in the cingulate cortex of patients with bipolar disorder or major depressive disorder (Cannon et al., 2006; Gibbons et al., 2009). Although this *in vitro* study suggests M₂ receptor activation decreases the activity of BNST neurons, the functional effects of BNST M₂ receptor activation in emotional disorders remains unknown. Future studies will examine the behavioral effects of M₂ receptor activation or blockade in animal models of anxiety disorders.

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Research Highlights

- Endogenous ACh suppresses glutamate release in the BNST_{ALG}
- Effects are mimicked by muscarinic receptor agonists
- Effects are mediated by presynaptic M₂ receptors
- EM studies confirm presynaptic M₂ receptor expression in the BNST_{ALG}

Fig 1.

Close correlation of glutamate fibers and ACh fibers in the BNST_{ALG}. Photomicrographs showing *Thy-1*-eYFP expression in the BLA (**A**) and the BNST (**B**) of transgenic *Thy-1* mice (10x, scale bar = 100 μ m, aa: anterior commissure; CP: Caudate Putamen; ec: external capsule; ic: internal capsule; LV: lateral ventricle; st: stria terminalis). Arrows indicate stria terminalis bundle arising from the BLA (**A**) and entering the BNST (**B**). **C-C''** Dual immunofluorescence experiment demonstrated that both *Thy-1*-YFP-positive fibers (**C**, green) and choline acetyltransferase-positive (ChAT, **C'**, red) fibers are highly immunoreactive in the BNST_{ALG} and they course in parallel in the dorso-ventral direction. Occasionally, *Thy-1* and ChAT-positive fibers overlap and make point contacts which each other as indicated by arrows (**C''**, merged, 63x, scale bar 10 μ m).

Fig 2.

Endogenous ACh and the ACh analogue CCh decreased the amplitude of eEPSCs in the BNST_{ALG}. **A**, upper traces showing eEPSCs recorded before (1), during (2) and after (3) eserine application. Eserine decreased the amplitude of eEPSCs, an effect that was completely blocked by prior application of atropine (5 μM). *, p < 0.05 vs atropine. **B**, CCh (10 μM) application decreased the amplitude of eEPSCs, an effect that reversed after 10 min of wash with ACSF, **, p < 0.01. **C**, A dose-response curve for the CCh-mediated decrease in eEPSC amplitude revealed an EC₅₀ of 4.0 μM. The numbers of neurons tested at each CCh concentration were between 4 and 10. Scale bar: 10 ms, 100 pA.

Fig 3. The decrease of eEPSC amplitude during CCh application is associated with an increase of PPR. **A**, In the presence of CCh, an increased PPR was observed associated with the decrease of eEPSC amplitude. **B**, A bar chart showing group data for the mean increase in PPR during CCh application; **, paired t-test, $p < 0.01$.

Fig 4.

CCh decreased the frequency of mEPSCs. Spontaneous mEPSCs in BNST were recorded in the presence of TTX (1 μ M) and GABA_A antagonist SR 95531 (5 μ M). **A**, Representative traces showing mEPSCs recorded from BNST_{ALG} neurons before and during CCh (10 μ M) application (right). **B**, Group data indicated CCh reduced the frequency but not the amplitude of mEPSCs. $n=11$, **, paired t -test $p<0.01$. **C**, Cumulative data plots showing that CCh increased the inter-event interval but not the amplitude of mEPSCs.

Fig 5.

CCh has no effect on postsynaptic AMPA current induced by local pressure ejection of AMPA (1 mM). **A**, Representative traces showed postsynaptic currents induced by pressure application of AMPA, which were not affected by CCh application but were blocked by DNQX (20 μ M). **B**, Group data showing that no change of postsynaptic AMPA current was observed during CCh application in the presence or absence of TTX (1 μ M).

Fig 6. Uncoupling postsynaptic G-protein-coupled receptors with intracellular GDP-β-S had no effect on the CCh response. **A**, In neurons recorded with intracellular GDP-β-S, CCh reversibly decreased the amplitude of eEPSCs **B**, Group data showing that the CCh effect in the presence of GDP-β-S was not different from control neurons recorded with regular patch solution. **C,D**, Similarly, intracellular inclusion of GDP-β-S had no effect on the CCh-induced increase of PPR, which was similar to that of control neurons. *, $p < 0.05$, **, $p < 0.01$ vs baseline.

Fig 7.

Activation of muscarinic receptors, but not nicotinic receptors, mediates the CCh effect. **AB**, Histograms showing that the effect of CCh on the eEPSC amplitude and PPR was completely blocked by the muscarinic antagonist atropine (5 μ M), but not by the nicotinic antagonist tubocurarine (TC, 10 μ M). **C,D**, Histograms showing that the muscarinic receptor agonist, muscarine, mimicked the inhibitory effect the CCh on eEPSC amplitude, and increased the PPR. **E**, The CCh (10 μ M) effect was blocked or attenuated by M₁ or M₂ subtype selective antagonists, drug concentration (in μ M): TZP, telenzepine 1, 10; PZP, pirenzepine 1, 10; DMTD, dimethindene 1, 10; MTC, methoctramine 1, 10; but not by M₄ antagonist PD102807, 10. *, $p < 0.05$; **, $p < 0.01$.

Fig 8. CCh effects in wild type, $M_1^{-/-}$, and $M_2/M_4^{-/-}$ mice. **A**, Expression of muscarinic receptors mRNA in brain tissue from wild type (WT), $M_1^{-/-}$, $M_2/M_4^{-/-}$ mice. **B**, Representative traces showing the CCh effects on the amplitude of eEPSCs and PPR in these mouse lines. **C**, Group data showing that the effect of CCh on the eEPSC amplitude remained intact in $M_1^{-/-}$ mice, but was significantly attenuated in $M_2/M_4^{-/-}$ mice ($p < 0.01$, vs WT). **D**, Group data showing that the PPR was also significantly increased during CCh application in WT, $M_1^{-/-}$ mice but not $M_2/M_4^{-/-}$ mice. *, $p < 0.05$, ** $p < 0.01$.

Fig 9. Diaminobenzidine label for the M_2 acetylcholine receptor (arrows) identified in axon terminals in the BNST. These terminals were frequently observed to make asymmetric synaptic contacts with dendritic spines (**A** and **B**) as well as dendritic shafts (**B**). Immunoreactive axon terminals were sometimes noted to contain dense core vesicles (arrowheads, **B** and **C**). Scale bar is 500 nm.