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Glycinergic input to the mouse basal forebrain cholinergic neurons

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Abstract

The basal forebrain (BF) receives afferents from brain stem ascending pathways, which has been implicated first by Moruzzi and Magoun (Moruzzi and Magoun, 1949) to induce forebrain activation and cortical arousal/waking behavior; however, it is very little known about how brain stem inhibitory inputs affect cholinergic functions. In the current study, glycine, a major inhibitory neurotransmitter of brain stem neurons, and gliotransmitter of local glial cells, was tested for potential interaction with basal forebrain cholinergic (BFC) neurons in male mice. In the BF, glycine receptor α subunit-immunoreactive (GlyRα-IR) sites were localized in choline acetyltransferase (ChAT)-IR neurons. Glycine's effect on BFC neurons was demonstrated by bicuculline-resistant, strychnine-sensitive spontaneous inhibitory postsynaptic currents (IPSCs; $0.81 \pm 0.25 * 10^{-1}$ Hz) recorded in whole cell conditions. Potential neuronal, as well as glial sources of glycine were indicated in the extracellular space of cholinergic neurons by glycine transporter 1 and 2 (GLYT1 and 2)-IR processes found in apposition to ChAT-IR cells. Ultrastructural analyses identified synapses of GLYT2-positive axon terminals on ChAT-IR neurons, as well as GLYT1-positive astroglial processes, which were localized in the vicinity of synapses of ChAT-IR neurons. The brain stem raphe magnus was determined to be a major source of glycinergic axons traced retrogradely from the BF. Our results indicate a direct effect of glycine on BFC neurons. Furthermore, the presence of high levels of plasma membrane glycine transporters in the vicinity of cholinergic neurons suggests a tight control of extracellular glycine in the BF.

Significance Statement

BFC neurons receive various activating inputs from specific brain stem areas, and channel this information to the cortex via multiple projections. So far very little is known about inhibitory

67 brain stem afferents to the BF. The current study established glycine as a major regulator of BFC
68 neurons by (1) identifying glycinergic neurons in the brain stem projecting to the BF, (2)
69 showing GlyRα-IR sites in ChAT-IR neurons, (3) demonstrating GLYT2-positive axon terminals
70 synapsing on ChAT-IR neurons, and (4) localizing GLYT1-positive astroglial processes in the
71 vicinity of synapses of ChAT-IR neurons. Glycine's effect on BFC neurons was demonstrated by
72 bicuculline-resistant, strychnine-sensitive spontaneous IPSCs recorded in whole cell conditions.
73
74 **Introduction**
75 Forebrain activation and cortical arousal/waking behavior are thought to be critically influenced
76 by ascending pathways deriving from the brain stem (Buzsaki et al., 1988; Steriade and
77 Timofeev, 2002; Sutcliffe and de Lecea, 2002; Zaborszky and Duque, 2003; Saper et al., 2005;
78 Datta and Maclean, 2007; Fuller et al., 2011; Luppi et al., 2013). These axons form a ventral
79 pathway targeting the BF, including its cholinergic corticopetal neurons and hypocretin/MCH
80 neurons in the hypothalamus and a dorsal pathway innervating thalamic nuclei. The dorsal
81 pathway contains glutamatergic and cholinergic projections from the mesopontine tegmentum
82 (Luppi et al., 2013). Catecholaminergic and glutamatergic ascending axons have been suggested
83 to contact cholinergic neurons in the BF and GABAergic neurons in the ventrolateral preoptic
84 nucleus (Luppi et al., 2013; Zaborszky et al., 2015). In addition, it has been shown that
85 hypocretin axons synapse with septal cholinergic neurons and depolarize them (Wu et al., 2004).
86 Recently a glycinergic pathway has been described deriving from the pontine reticular formation
87 and targeting the intralaminar thalamic nuclei; optogenetic stimulation of the contributing cell
88 population in mice evoked behavioral arrest and transient interruption of awake cortical activity
89 (Giber et al., 2015).

A potential role for glycine in the BF has also been postulated in our previous study (Bardóczi et al., 2013) based on the presence of type 1 and 2 membrane glycine transporters (GLYT1 or 2). GLYTs, depending on their location, have distinct functions at glycinergic synapses. GLYT2 provides glycine for refilling of presynaptic vesicles of glycinergic neurons (Gomeza et al., 2003), whereas GLYT1 ensures the removal of glycine from the synaptic cleft into glial cells leading to the termination of glycine-mediated neurotransmission. In addition, GLYT1 is also present in certain glutamatergic neurons and regulates the concentration of glycine at excitatory synapses containing NMDA receptors, known to require glycine as a co-agonist (Eulenburg et al., 2005).

BF areas (i.e., the medial septum (MS), vertical, and horizontal limbs of the diagonal band (VDB and HDB), ventral pallidum (VP) and the substantia innominate (Si), contain a heterogeneous collection of cholinergic, GABAergic, glutamatergic projection neurons, and various interneurons (for ref. see (Zaborszky et al., 2015)). The large cholinergic neurons are wake-promoting neurons, as are the glutamatergic and parvalbumin-positive GABAergic neurons, which are more active during wakefulness and rapid eye movement (NREM) sleep (wake/REM active) than during non-REM (NREM) sleep. Optogenetic activation of these neurons rapidly induces wakefulness, contrasting with somatostatin-positive GABAergic neurons, the stimulation of which promotes NREM sleep (Xu et al., 2015). However, chemogenetic activation of BF cholinergic or glutamatergic neurons in behaving mice has no effect on total wakefulness. In contrast, similar chemogenetic activation of BF GABAergic neurons produces sustained wakefulness and high-frequency cortical rhythms (Anaclet et al., 2015). Other data suggest that specific types of BF neurons play distinct roles in cortical (Kim et al., 2015), and hippocampal oscillations (Sotty et al., 2003), or in inducing hippocampal LTP or

LTD (Gu and Yakel, 2011). Thus, it is very important to define which type(s) of BF neurons are regulated by glycine.

The aim of the present study was to define whether the function of BF cholinergic neurons is influenced by glycine. Confocal and electron microscopic studies were employed to characterize the morphological relationship of ChAT-IR neurons with GLYT1- and/or GLYT2-IR cellular processes. In addition, *in vitro* patch-clamp electrophysiology was used to demonstrate glycine effect on cholinergic neurons. Our results indicate that glycine has a direct influence on BF cholinergic neurons by demonstrating (1) bicuculline-resistant, strychnine-sensitive spontaneous IPSCs, (2) synaptic connections between glycinergic and cholinergic neurons and (3) strong presence of glycine transporters in glial and neuronal processes in the vicinity of cholinergic neurons. The primary origin of glycinergic axons of the BF is the raphe magnus and its neighboring reticular formation of the pons.

Materials and methods

Animals

Wild type (CD1, Charles River, Hungary, n=21) and transgenic male mice expressing the fluorescent reporter proteins eGFP (GLYT2-eGFP, (Zeilhofer et al., 2005), JCN, 2005, n=12 and ChAT^{BAC}-eGFP, Jackson Laboratories, Bar Harbor Maine, n=3), or tdTomato (ChAT^{CRE}-tdTomato, n=26, generated by crossbreeding of ChAT-CRE mice (n=2) ((Rossi et al., 2011); http://www.informatics.jax.org/reference/J:114556; JAX number: 006410) and floxed-stop-tdTomato reporter mice (n=6) ((Madisen et al., 2010), JAX mice accession number 007905), were used. Mice (1-3 month old, 25-30 g body weight) were housed under controlled lighting [12:12 light-dark cycle; lights on at 07:00h, Zeitgeber time 0] and temperature (22 ± 2°C), with

136 access to food and water *ad libitum*. Studies were carried out with permission from the Animal
137 Welfare Committee of the Institute of Experimental Medicine (No. 2285/003) and Debrecen
138 University (No. 6/2011/DE MÁB and 5/2015/DEMÁB) and in accordance with legal
139 requirements of the European Community (Decree 86/609/EEC). Surgery was performed on
140 animals under deep anesthesia induced by an intraperitoneally injected cocktail of ketamine (25
141 mg/kg b.w.), xylavet (5 mg/kg b.w.) and pipolphen (2.5 mg/kg b.w.) in saline.

142 *Anatomical studies*

143 *Tissue preparation.* Mice were perfused transcardially with phosphate buffered saline
144 (PBS; 0.1M) containing 4% paraformaldehyde (PFA). The brains were post-fixed in 2%
145 PFA/PBS solution for 24h at 4 °C, cryoprotected overnight in 25% sucrose and 25µm-thick
146 coronal sections were cut from a block of the basal forebrain expanding between Bregma +1.3
147 and -1.3 mm on a freezing microtome. The sections were divided into three sequential pools
148 (approx. 30-35 sections in each set, with 50 µm gaps between consecutive sections) and stored in
149 antifreeze solution (30% ethylene glycol; 25% glycerol; 0.05 M phosphate buffer; pH 7.4) at -20
150 °C until use. For mapping the immunolabeled cells and cellular profiles, a set of sections from
151 each brain was Nissl-counterstained, photographed and matched with the corresponding atlas
152 figures. After the endogenous peroxidase activity had been quenched with 0.5% hydrogen
153 peroxide (10 min), sections were permeabilised with 0.5% Triton-X-100 (23,472-9, Sigma; 20
154 min). Finally, 2% normal horse serum was applied (20 min) to reduce non-specific antibody
155 binding. Subsequent treatments and interim rinses in PBS (3x5 min) were carried out at room
156 temperature, except for incubation in the primary antibody or fluorochrome conjugate, which
157 took place at 4°C.

158 *Double-labeling for GLYT1 or GLYT2 and ChAT immunoreactivity.* Sections from the
159 BF region of CD1 mice were incubated in goat anti-GLYT1 (Millipore Cat# AB1770
160 RRID:AB_90893, 1:10,000) or rabbit anti-GLYT2 (Masahiko Watanabe, Cat#N30aa (Hondo et
161 al., 2011), RRID:AB_2571606, 1µg/ml) primary antibodies. GLYT1 and GLYT2
162 immunoreactivities were visualized with biotinylated donkey anti-goat IgG (#705-065-147,
163 dilution 1:500, 2h; Jackson ImmunoResearch Laboratories) or biotinylated donkey anti-rabbit
164 IgG (#711-065-152, dilution 1:500, 2h; Jackson ImmunoResearch Laboratories), and Vectastain
165 ABC Elite solution (PK-6100, Vector Laboratories, dilution 1:1,000; 1.5h), then the signal was
166 detected with nickel (Ni)-intensified diaminobenzidine (DAB). The NiDAB reaction product was
167 further intensified by the application of a special physical developer producing silver precipitate
168 on NiDAB, which was then stabilized with gold toning (SGI-NiDAB) (Kallo et al., 2001),
169 similarly to the method described before for DAB (Liposits et al., 1984). Due to the relatively
170 large difference in the argyrophilia of Ni-DAB and the nervous tissue, and the minimal
171 immunohistochemical background, suppression of tissue argyrophilia with thioglycolic acid was
172 unnecessary and omitted from the protocol. Subsequently, the sections were incubated in goat
173 anti-ChAT (Millipore Cat# AB144P-1ML RRID: AB_262156, 1: 1,500), which was detected
174 with biotinylated donkey anti-goat IgG (dilution 1:500, overnight; Jackson ImmunoResearch
175 Laboratories), and ABC Elite solution (dilution 1:500; 1.5h), providing that the metallic shield
176 (i.e. SGI-NiDAB) formed around the antigen-antibody complex prevents unwanted binding of
177 immunoglobulins used for the detection of the second tissue antigen. (Liposits et al., 1986). The
178 ChAT-IR sites were visualized with DAB.

179 *Immunofluorescent detection and 3D reconstruction of GLYT1- and GLYT2-IR profiles.*
180 Sections containing the BF of $ChAT^{BAC}$-eGFP mice were incubated in a mixture of goat anti-

GLYT1 (Millipore Cat# AB1770 RRID: AB_90893, 1: 10,000) and rabbit anti-GLYT2 (N30aa, 1µg/ml) primary antibodies (for 48h). Then they were sequentially incubated in biotinylated donkey anti-goat IgG (#705-065-147, dilution 1:500, 2h; Jackson ImmunoResearch Laboratories), Vectastain ABC Elite solution (dilution 1: 1,000, 1h), biotinylated tyramide with H_2O_2 (dilution 1: 1,000 and 0.006%, respectively; 0.5 h). Finally, the GLYT1-linked labeling complexes and the GLYT2-anti-GLYT2 complexes were visualized, respectively, with Alexa 594-Streptavidin (S-11227, dilution 1:500, 12h; Molecular Probes, Carlsbad, CA, USA) and CY5 conjugated donkey anti-rabbit IgG (#711-175-152, dilution 1: 1,000, overnight; Jackson ImmunoResearch Laboratories). The double-labelled sections (i.e. for GLYT1 and GLYT2) containing the eGFP expressing ChAT neurons were imaged using a NIKON A1 confocal microscope. Multiple stacks of optical slices (1024x1024 pixels, z-steps 0.6 µm) were obtained by scanning selected areas with cholinergic neurons of the different subdivisions of BF using a 60x oil immersion objective. To detect eGFP, Alexa 594 and CY5, laser lines 488 nm, 594 nm, 633 nm, and dichroic/emission filters 560/500–530 nm, 650/565–625 nm, and a 660-nm-long pass filters were used, respectively. The emission images were recorded separately in green, red and blue channels, and displayed with the Image J software. Appositions were identified at the orthogonal view of the confocal Z-stacks, and the image generated by reconstructing the labelled profiles in 3D using the Amira software (FEI Visualization Sciences Group, Berlin, Germany).

Tissue preparation for electron microscopy. In order to investigate the morphological relationship of cholinergic neurons with plasma membrane glycine transporter-containing processes in the BF at the electron microscopic level, pre-embedding, double-label immunohistochemistry was carried out. CD1 mice were deeply anesthetized and perfused transcardially, first with 10 ml 0.01M M PBS (pH 7.4), followed by 30 ml 2% PFA/4% acrolein

in 0.1M M PB (pH 7.4) and then 10 ml 4% PFA in the same buffer. The brains were rapidly removed and fixed further in 4% PFA overnight at 4°C and then stored in PBS for 24h at 4 C°. Thirty µm-thick coronal sections were cut with a vibratome and treated with 1% sodium borohydride (30 min), 0.5% H_2O_2 (15 min) and permeabilised with three freeze-thaw cycles, as described previously (Jo et al., 2012).

Pre-embedding detection of GLYT1 or GLYT2 and ChAT-IRs. Sections of the BF were double-labelled for ChAT and GLYT1 or GLYT2-IRs, as described above for bright field microscopy mapping. The double-labelled sections were then treated with 1% osmium tetroxide (1h) and 2% uranyl acetate (in 70% ethanol; 40 min), dehydrated in an ascending series of ethanol and propylene oxide, and flat-embedded in TAAB 812 medium epoxy resin between glass microscope slides pre-coated with a liquid release agent (#70880, Electron Microscopy Sciences, Fort Washington, PA). The resin was allowed to polymerize at 56 °C for 2 days. The flat-embedded sections were initially investigated by light microscopy at 60x magnification; areas exhibiting appositions of GLYT1- or GLYT2-IR processes on the somatodendritic region of ChAT-IR neurons were selected for further processing. Semithin (1 µm) and ultrathin (50-60 nm) sections were cut with a Leica ultracut UCT ultramicrotome (Leica Microsystems, Vienna, Austria). The ultrathin sections were collected in ribbons onto Formvar-coated single-slot grids, contrasted with 2% lead citrate and examined with a Jeol-100 C transmission electron microscope.

Demonstration of GlyR-IR in cholinergic neurons. To detect GlyR-IR, sections of CD1 or ChAT-eGFP mice (n=4) were subjected to pepsin (1 mg/ml in 0.2 N HCl; DAKO, Carpinteria, CA) pretreatment for 2–3 min at 37ºC. After blocking non-specific antibody binding with 2% normal horse serum (NHS; 20 min) sections were incubated (72 h) in a cocktail of the guinea pig

anti-GlyR (Masahiko Watanabe Cat#105-136aa, (Hondo et al., 2011) RRID:AB_AB_2571771, 1µg/ml) and goat anti-ChAT (Millipore Cat# AB144P-1ML RRID: AB_262156, 1:1,500) or rabbit anti-GFP (Millipore Cat# AB10145 RRID:AB_1587096, 1:2,500) primary antibodies. GlyR immunoreactivity was visualized with biotinylated donkey anti-guinea pig (#711-065-152, Jackson ImmunoResearch Laboratories, 1: 1,000, 2h), Vectastain ABC Elite solution (PK-6100, Vector Laboratories, 1: 1,000, 1h), biotinylated tyramide with H_2O_2 (1: 1,000 and 0.006%, respectively; 0.5h) and, finally, Alexa 594-streptavidin (S-11227, Molecular Probes, 1: 500, 12h). To visualize the cholinergic neurons, the sections were incubated in FITC-donkey anti-goat IgG (#711-095-152, dilution 1: 1,000, overnight; Jackson ImmunoResearch Laboratories). Full sets of sections (which means every fourth sections from each brain with 50 µm-gaps between consecutive sections) were processed for detection of panα-GlyR immunoreactivity in cholinergic neurons of the mouse BF. Each of the subdivisions were scanned in the confocal microscope and 5 to 10 cells per regions were investigated for co-localization.

Tract tracing, mapping of retrogradely labeled neurons. To label glycinergic neurons projecting to forebrain, GLYT2-GFP mice (n=12) were given cholera toxin B (CTB; 0.5% solution; #103, List Biological Laboratories, INC, Campbell, CA) or Fluorogold (2.5–5.0% FG, diluted in distilled water; Fluorochrome Inc., Englewood, CO; see (Schmued and Fallon, 1986) via unilateral iontophoresis (5µA, 7s on-off) into the BF for 20 minutes. For targeting different subdivisions the following stereotaxic coordinates were used, respectively, with reference to the Bregma (B) planes: (MS) antero-posterior: +0.61 mm, medio-lateral: + 0.0 mm, dorso-ventral: -4 mm; (HDB) antero-posterior: +0.37 mm or +0.02 mm, medio-lateral: +0.80 mm or +1.40 mm, dorso-ventral: -4.90 mm or -5.00 mm (Paxinos, 2013); (VP/SI) antero-posterior: +0.13 mm, medio-lateral: +1.20 mm, dorso-ventral: -4.25 mm. After a 6–10-day transport time, animals were

deeply anesthetized and perfused first with 20 ml PBS, followed by 0.1M PBS containing 4% PFA. The brains were removed, immersed in 30% sucrose for 1–2 days, and frozen on dry ice and 25-µm-thick coronal sections were cut from the blocks of the forebrain Bregma +1.3 and -1.3 mm and the brain stem expanding between Bregma -2.9 and -8 mm on a freezing microtome. The sections were divided into four sequential pools (approx. 20-22 sections of the forebrain and 45-50 sections of the brain stem in each sets with 75 µm gaps between consecutive sections). The location of CTB injection sites and the distribution of CTB-containing glycinergic neurons in the brain stem were studied in double-immunolabeled sections. A set of sections from each brain was pre-treated first with 0.5% H_2O_2 and 0.5% Triton X-100 in PBS for 15 minutes and then 2% normal horse serum in PBS for 20 minutes. They were then incubated in the goat anti-CTB antiserum (List Biological Cat# 703 RRID: AB_2313637) at 1:2,000 for 2 days at 4°C. After washes in PBS, they were immersed in CY3-donkey anti-goat IgG (Jackson ImmunoResearch; 1: 2,000) and incubated for 2 hours at room temperature. Sections containing labelled cells with Fluorogold were mounted and coverslipped with Vectashield mounting medium (Vector Laboratories), as described before. For mapping the glycinergic neurons retrogradely labeled from the different BF regions every fifth sections with 75 µm-gaps in between were processed, and the sections containing the anatomical region (e.g. 5 sections/brain for the raphe magnus; n=6) with double-labeled cells (GLYT2-GFP and CTB-IR) were selected and photographed in the confocal microscope. The border of the anatomical region was defined (based on the corresponding atlas image) and each single- or double-labeled cells were counted in the region of interest to avoid problems arising from a potential uneven distribution of retrograde-labeled cells. Perikarya with clear sign of nuclear presence were considered during the quantification, and co-localization of the signals were determined manually by turning the color channels alternatively on and off.

Immunohistochemical controls. The specificities of the ChAT, GLYT1, GLYT2, GlyR primary antisera have been reported previously, thus controls included preabsorption of the ChAT antibody with the corresponding protein antigen (Motts et al., 2008), immunoblot confirmation of GLYT2 and GlyR bands at the expected molecular weight in samples of transfected cells and mouse brain tissue (Hondo et al., 2011), and detection of GLYT1 mRNA signal in brain sections in comparative distribution to GLYT1 immunoreactivity (Kallo et al., 2008).

In the current study, the ChAT and GLYT2 antibody labeling did not reveal structures other than those detected by transgenic eGFP fluorescence expressed under the promoter of ChAT or GLYT2. The specific binding of the panαGlyR antibody to the glycine receptors was confirmed by a second, α2 subunit specific antiserum (Santa Cruz Biotechnology Cat# sc-17279 Lot# RRID: AB_2110230) detected in the same distribution in the BF. Increasing dilutions of the primary antisera resulted in a commensurate decrease and eventual disappearance of the immunostaining; omission of the primary antibodies or their preabsorption with corresponding peptide antigens resulted in complete loss of the immunostaining. The secondary antibodies employed here were designed for multiple labeling and pre-absorbed by the manufacturer with immunoglobulins from several species, including the one in which the other primary antibody had been raised.

In vitro electrophysiology

Animals, slice preparation, chemicals. Mice expressing tdTomato fluorescent proteins in a ChAT-dependent way (n = 26) were used from both sexes. After decapitation of the animal and removal of the brain, 200 μm-thick coronal forebrain slices were prepared using a Microm HM 650V vibratome (Microm International GmbH, Walldorf, Germany) in ice-cold low Na artificial

cerebrospinal fluid (aCSF) with the following ingredients (in mM): sucrose, 130, glycerol, 60, NaCl, 25; KCl, 2.5; $NaHCO_3$, 26; glucose, 10; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgCl_2$, 1; myo-inositol, 3; ascorbic acid, 0.5; and sodium-pyruvate, 2; pH 7.2. After cutting, slices were incubated in normal aCSF for 60 minutes on 37°C. Concentration of NaCl was elevated in the normal aCSF to 120 mM, while sucrose and glycerol were omitted from the solution. All chemicals were purchased from Sigma (St. Louis, MO, USA), unless stated otherwise. Brain slices were visualized with a Zeiss Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany). The microscope was equipped with a fluorescent imaging system (Till Photonics GmbH, Gräfeling, Germany) containing a xenon bulb-based Polychrome V light source, a CCD camera (SensiCam, PCO AG, Kelheim, Germany), an imaging control unit (ICU), and the Till Vision software (version 4.0.1.3). Patch pipettes with 5 MΩ pipette resistance were fabricated, and filled with a solution containing (in mM): KCl, 120; NaCl, 5; 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES), 10; EGTA, 2; $CaCl_2$, 0.1; Mg-ATP, 5; Na_3-GTP, 0.3; Na_2-phosphocreatinine, 10; biocytin, 8; pH 7.3. In a set of experiments, KCl was replaced with equimolar K-gluconate. To detect postsynaptic currents, whole-cell patch-clamp recordings were performed using an Axopatch 200A amplifier (Molecular Devices, Union City, CA, USA) in voltage clamp configuration at holding potentials -120, -100, -60, -40 and 0 mV. Action potential firing pattern was recorded in current clamp configuration; the resting membrane potential was set to -75 mV and current injections were employed from -30 to +120 pA in 10 pA steps. All recordings were performed in normal aCSF at room temperature. Data acquisition was achieved using the Clampex 10.0 software (Molecular Devices, Union City, CA, USA), while data analysis was performed using the Clampfit 10.0 (Molecular Devices) and MiniAnalysis (Synaptosoft, Decatur, GA, USA) programs. When the latter software was employed for event detection,

traces, where all events were pharmacologically inhibited, were used for setting detection parameters; from cell to cell, the detection threshold of the events varied from 7 to 15 pA (12.3 ± 0.5 pA). Other parameters were set as follows: period to search a local maximum: 80 ms; time before a peak for baseline: 20 ms; area threshold: 5 fC; period to search a decay time: 40 ms; period to average a baseline: 1 ms.

Pharmacology, visualization of the labeled neurons. When recording glycinergic IPSCs, slices were continuously perfused with 10 µM 2,3–dihydroxy–6–nitro–7–sulfamoyl-benzo[f]quinoxaline–2,3–dione (NBQX), 50 µM D–2–amino–5–phosphonopentanoate (D-AP5; Tocris Cookson Ltd., Bristol, UK), and 10 µM bicuculline in order to block ionotropic glutamatergic and GABAergic neurotransmissions (GG blocking cocktail). This cocktail was later completed with one µM strychnine, in order to show that the observed IPSCs are strychnine-sensitive. The neurons were filled with biocytin during the electrophysiological recordings. The slices accommodating the filled neurons were fixed overnight (4% PFA in 0.1M phosphate buffer; *pH* 7.4; 4 °C). Permeabilization was achieved in Tris buffered saline (in mM, Tris base, 8; Trisma HCl, 42; NaCl, 150; *pH* 7.4) supplemented with 0.1% Triton X-100 and 10% bovine serum (60 min). The slices were incubated in phosphate buffer containing streptavidin-conjugated Alexa488 (1:300; Molecular Probes Inc., Eugene, OR, USA) for 90 min. The cells were visualized using a Zeiss LSM 510 confocal microscope (Carl Zeiss AG).

Experimental design and statistical analysis.

Anatomical studies:

Wild type (n=21) and transgenic male mice (GLYT2-eGFP, n=12 or ChATBAC-eGFP, n=3) were used to characterize the cellular interaction in the BF potentially providing glycinergic input to cholinergic neurons. Efforts were made to keep animal usage at minimum while

ensuring representation of the different subdivisions of the BF in the analysis of morphological interactions of receptors or inputs on cholinergic neurons. Every fourth (with 50µm tissue gaps) sections were processed from each brain for a certain type of immunostaining and for each of the BF subdivisions confocal microscopy was used to scan 5 to 10 cholinergic cells for co-localization of receptor on cholinergic neurons. In order to better define anatomical regions, immunostained sections were compared with their matching counter-stained pairs. For synaptic interactions of glycine axons, 32 synapses were identified with cholinergic profiles from randomly selected close appositions (n=84). For assessing the importance of brainstem sites projecting to the cholinergic forebrain, each single and double-labeled neuron were counted in sets of 1:5 series, with 75 µm gaps between consecutive sections. A set of sections from brains with different BF injection sites (see Fig. 7) of the retrograde tracer were analyzed. The percentages of the retrogradely labeled glycinergic neurons in the nucleus raphe magnus of 6 animals were averaged from counts of 30 sections (5 sections/brain). Immunohistochemical controls involved tests to exclude false negativity and positivity potentially generated either by the animal models, reagents or methods used (in details see "Immunohistochemical controls" above.

In vitro electrophysiology:

ChAT-tdTomato mice (n=26) were used from both sexes. Slices were cut from the basal forebrain region, and used to record from cholinergic neurons (n=46) of the different subdivisions of the basal forebrain. One neuron was patched in each slice. All data represent mean ± SEM. Postsynaptic currents detected by whole-cell patch-clamp recordings in voltage clamp configuration were characterized for amplitude and frequency under control conditions, and pharmacological interventions. To distinguish early and late-firing cholinergic neurons,

365 action potential firing pattern was recorded in current clamp configuration. Statistical
366 significance was determined using Student's t-test. Two-sample Kolmogorov-Smirnov test was
367 employed for comparison of amplitude, rise and decay time of IPSCs in different neuronal
368 populations. The level of significance was $p < 0.05$.

369 ## Results

370 **Cholinergic neurons are immunoreactive for glycine receptors**

371 Using a panα-GlyR antibody, immunoreactive puncta were detected in all subdivisions of BF.
372 The ventral pallidum showed the weakest panα-GlyR immunoreactivity; no obvious difference
373 could be observed in the intensity of staining in the MS, VDB/HDB, SI and NBM. The deposited
374 immunohistochemical reaction product often delineated cellular borders even in weakly labelled
375 areas (Fig. 1A-C). Confocal microscopic analyses of double-labeled sections identified GlyR-IR
376 sites at the periphery of most cholinergic neurons (Fig. 1D-E) indicating that glycine can directly
377 influence BF cholinergic functions.

378 **Cholinergic neurons exhibit strychnine-sensitive IPSCs**

379 Whole cell patch clamp recordings were carried out on 36 genetically identified cholinergic
380 neurons from the medial septal nucleus, the horizontal diagonal band, the ventral pallidum,
381 substantia innominata and the lateral nucleus of the diagonal band. In order to increase the
382 amplitude of chloride currents, a KCl-based internal solution was applied in the recording
383 pipettes. Thirty-one of the neurons displayed bicuculline-resistant, strychnine-sensitive
384 spontaneous IPSCs with an average frequency of $0.81 \pm 0.25 * 10^{-1}$ Hz (ranging between 0.04
385 and $9.22 * 10^{-1}$ Hz; $t(36) = 4.68$, $p = 0.0008$, paired t-test, compared to control) and an average
386 amplitude of 15.23 ±1.38 pA (ranging between 8.4 and 29.9 pA; $t(39) = 5.28$, $p = 0.0009$, paired
387 t-test, compared to control; Fig. 2A-D). 11.2 ± 2.7% of all synaptic events remained detectable

after application of the GG blocking cocktail, but were completely eliminated by strychnine added in the incubating medium (for frequency: $t(23) = 3.39$, $p = 0.001$; for amplitude: $t(23) = 18.47$, $p = 1{,}35823 * 10^{-15}$, paired t-test). The number of putative glycinergic IPSCs analyzed per cells varied from 6 to 153 events during the recording periods. However, the distribution of the IPSC frequency was not uniform: among the recorded cells, there was a slight ventrolateral-dorsomedial gradient of IPSC frequency (Fig. 2E).

To ensure that the strychnine-sensitive events were chloride currents, the reversal potential had also be examined. First, recordings were performed by using KCl-filled pipettes at -60 and -100 mV holding potentials. In order to avoid bias in determining the average amplitude of events by involving low amplitude events appearing at -100 mV due to higher driving force for chloride, amplitudes of the three largest events were considered from each recordings; their average values were plotted against the holding potentials. Recordings were also done on -40mV and 0 mV holding potentials, but no events could be detected very likely because amplitude of events at this range of holding potentials fell under the detection threshold. Reversal potential was determined by the intersection of the linear fit of the averaged data with the abscissa of membrane potentials. The extrapolated value (+5.5 mV; n =8) was close to chloride's calculated equilibrium potential (-0.6 mV; Fig. 2G). Based on these reversal potential values, it is very likely that the recorded bicuculline-resistant events were not based on potassium currents, as the calculated reversal potential for potassium would be -99.1 mV at these conditions. Of note, mixed cationic conductances (Kandel, 2013) could have still served as a background of the observed events. To exclude this possibility, KCl was replaced with K-gluconate in the internal solution of the recording pipette (n = 10), resulting in a calculated equilibrium potential for chloride at -83.1 mV. Contrasting the traces recorded with KCl-containing pipettes that show

411 negative-going events at -60 mV holding potential in the presence of the GG blocking cocktail
412 (Fig.2 B), negative-going events were almost completely absent (0.01 ± 0.007 $*10^{-1}$ Hz) when K-
413 gluconate-filled pipettes were used (Fig.2 H). Application of the GG blocking cocktail
414 significantly reduced the appearance of such events ($t(6) = 2.97$, $p = 0.01$, paired t-test; from 1.41
415 ± 0.47 $*10^{-1}$ Hz seen at control conditions). In turn, bicuculline-resistant, positive-going events
416 were seen in 7 cases with an average amplitude of 12.6 ± 1.1 pA (Fig. 2 H). The frequency of
417 these positive-going events (0.47 ± 0.13 $*10^{-1}$ Hz) and the negative-going events recorded with
418 the KCl-containing pipettes were not significantly different ($t(29) = 0.74$, $p = 0.25$, two-sample t-
419 test). These experiments support the hypothesis that the strychnine-sensitive events recorded
420 were truly chloride currents. By using the K-gluconate-filled pipettes, bicuculline-resistant
421 events were recorded at -120, -60 and -40 mV holding potentials (n=5), and the average
422 amplitudes were plotted against the holding potentials, as described above. The linear fit crossed
423 the abscissa at -86.9 mV, which is close to the reversal potential of chloride calculated with the
424 Nernst equation (-83.1 mV), thus mixed cationic currents as background of these events were
425 clearly excluded (Fig 2 I).

426 The firing pattern of the neurons was also recorded in 28 cases, using current clamp mode
427 and depolarizing current injections in 10 pA steps and the action potential firing pattern was
428 evaluated at the step using 60 pA current injection. Twenty-two of the neurons proved to be
429 early-firing (EF; Fig. 3A, C), the other 6 were late-firing (LF; Fig. 3B, C). In accordance with
430 Unal et al. (Unal et al., 2012), these neurons possessed similar input resistance values (606.6 ±
431 89 MΩ for the EF and 628.3± 79 MΩ for the LF, $t(12) = 0.174$, $p = 0.43$, two sample t-test;), but
432 showed significant differences in the delay of the first action potential (43.4±5.4 ms for EF and
433 247.9±19.8 ms LF; $t(16) = 10.06$, $p = 1.26 * 10^{-8}$, two sample t-test). When the glycinergic

sIPSC ($sIPSC_G$) were compared in EF and LF neurons, significantly longer inter-event intervals were seen in LF neurons ($p = 0.018$; Kolmogorov-Smirnov test). In contrast, EF (n=20) neurons showed $sIPSC_G$ with significantly higher amplitude ($p = 3.15 * 10^{-7}$), shorter rise- (p=0.008) and longer decay ($p = 2.19 * 10^{-4}$) time than LF (n=6) cholinergic neurons (Kolmogorov-Smirnov test; two of the early firing neurons did not show $IPSC_G$ and were excluded from evaluation of these parameters) (Fig. 3D-F). When neurons lacking $sIPSC_G$s were included in the statistical analysis, due to the variability of $sIPSC_G$ frequency, no significant difference was revealed between EF and LF neurons in this parameter ($0.33\pm0.18 \times 10^{-1}$ Hz for EF and $0.5\pm0.16 \times 10^{-1}$ Hz for LF).

Distribution of glycinergic (GLYT2-IR) axons in the BF

Although the location of glycinergic cell bodies is mainly restricted to the spinal cord and the brain stem, the axonal projections from the brain stem reach most of the forebrain regions, including the BF. By immunohistochemically labeling GLYT2, the plasma membrane transporter of glycine, we detected a high density of glycinergic axons in the mouse BF, including the areas where ChAT-IR neurons are clustered i.e. GLYT2-IR fibers were observed in the MS, VDB, HDB, the lateral nucleus of the diagonal band (LDB), VP, SI and the extension of the amygdala (EA; Fig.4A-E). Bright field (Fig. 4F & 5A-C) and electron microscopic approaches (Fig.5D-G) revealed axo-somatic and axo-dendritic connections between GLYT2-positive axon terminals and ChAT-IR neurons. Analyses of high power images often revealed GLYT2-IR fiber varicosities with a central non-labelled area indicating embedded processes of target cells and concave joined surfaces (Fig. 5B-C insets). At the ultrastructural level 32 synapses were identified with cholinergic profiles; GLYT2-IR axon terminals were found to surround smaller as well as larger diameter ChAT-IR dendrites (Fig. 5E-F) and formed

457 symmetric synapses with dendritic shafts (Fig. 5D-F; n=20) and perikarya (Fig. 5G; n=1). In
458 addition, the correlated bright field (Fig.6A) and electron microscopic (Fig.6B-D) approach and
459 analysis of serial ultrathin sections also revealed axo-spinous connections on somatic (Fig.6C;
460 n=2) and dendritic spines (Fig.6D; n=9).

461 **Brainstem nuclei provide the BF with glycinergic input**

462 To identify the source of glycinergic input to the BF, the retrograde tracers CTB or Fluorogold
463 were injected into the MS, HDB, VP and SI (Fig. 7A-D) of mice expressing GFP under the
464 control of GLYT2 promoter. Although the distribution of retrogradely labeled GFP-positive
465 neurons varied from brain to brain depending on the exact location and size of injection sites,
466 there were brainstem areas and nuclei commonly labeled for the tracer (Fig.7E). The highest
467 number of double-labeled neurons were in the raphe magnus (Fig.7E-I; 25±7.4% of all GFP
468 neurons, n=6). Retrogradely labeled GFP-positive neurons were also commonly present in the
469 different parts of the pontine reticular nucleus and the gigantocellular reticular nucleus. A few
470 cells could also be detected in the periaqueductal gray (Fig. 7E).

471 **GLYT1 is also present in the BF**

472 In BF regions, immunoreactivity for GLYT1 was very strong (Fig.8A-F). Immunohistochemical
473 co-labeling with the astroglial marker Glutamine Synthetase revealed, that GLYT1 is primarily
474 present in astrocytes (not shown). Confocal microscopic analysis detected GLYT1-IR thin
475 astrocytic processes in the vicinity of axon varicosities in apposition to cholinergic neurons (Fig.
476 9A-D) in all studied BF regions. At the ultrastructural level, the presence of GLYT1
477 immunoreactivity in thin glial processes was confirmed, often adjacent to axon terminals
478 establishing asymmetric or symmetric synapse with the cholinergic neurons (Fig. 10A-D).
479

480 **Discussion**

481 According to the classical concept of the ascending arousal system (Nauta and Kuypers, 1957), a
482 major branch of the complex pathways from the rostral pons and caudal midbrain reaches the
483 hypothalamus and the BF and mediates activating function for these brain regions. Subsequent
484 studies identified inputs from the locus coeruleus, from the adrenergic, dopaminergic brainstem
485 neurons and from the parabrachial nucleus to synapse with the BF neurons, including
486 cholinergic, glutamatergic and parvalbumin-containing neurons using electron microscopy
487 (Zaborszky et al., 1993; Gaykema and Zaborszky, 1997; Zaborszky et al., 1999; Hajszan and
488 Zaborszky, 2002), or viral tracing (Fuller et al., 2011; Kaur et al., 2013; Anaclet et al., 2014).
489 The current morphological and functional analyses identified for the first time an ascending
490 glycinergic pathway from the nucleus raphe magnus and neighboring reticular formation of the
491 pons to the BF cholinergic neurons. Glycine exhibits bicuculline-resistant, strychnine-sensitive
492 spontaneous IPSCs on BFC neurons.

493 **Glycine is a major transmitter in the BF**

494 The current data support a substantial glycinergic input to cholinergic neurons in all subdivisions
495 of the BF. About 80 % of the recorded neurons, selected randomly from the medial septal
496 nucleus, the horizontal diagonal band, the ventral pallidum, substantia innominata and the lateral
497 nucleus of the diagonal band displayed bicuculline-resistant, strychnine-sensitive spontaneous
498 IPSCs. Based on the reversal potential calculations of the events, these IPSCs were very likely
499 chloride currents, as they were clearly distinguished from potassium currents and mixed cationic
500 conductances. Although a slight difference in the frequency range of IPSCs could be observed in
501 dorsomedially versus ventrolaterally distributed cells, the frequent appearance of cholinergic
502 neurons showing panαGlyR-immunoreactivity, or close relationship with GLYT1 or GLYT2-IR

503 cell processes indicates a non-selective, general role for glycine in all major subdivisions of the
504 BF. However, these do not exclude the possibility of prevailing differences within glycinergic
505 input of specific cholinergic cell populations. For example, early (EF) and late firing (LF)
506 cholinergic neurons were distinguished (Unal et al., 2012) with putative functional
507 consequences. It was suggested, that the EF neurons are more suitable for phasic changes in
508 acetylcholine release associated with attention, and the late firing neurons could support general
509 arousal by maintaining tonic acetylcholine levels. Comparison of the glycinergic sIPSCs of these
510 subtypes in the current study revealed a significantly higher amplitude and longer decay time in
511 EF than in LF neurons, indicating a potential difference in the somato-dendritic, proximo-distal
512 location of their glycinergic synapses or in the general membrane properties determining
513 propagation characteristics of IPSCs. Responses of EF and LF cholinergic neurons to glycine
514 raises a possibility for the involvement of this inhibitory neurotransmitter in both attention
515 regulation and arousal.

516 **Origin of glycine input and putative co-localization with GABA**

517 The BF does not contain glycinergic neurons; they are primarily located in the brain stem and
518 spinal cord. Only the axonal projections reach the forebrain areas, as it was shown in transgenic
519 animals expressing GFP under the control of GLYT2 promoter (Zeilhofer et al., 2005). In the
520 current study, the same animal model was used to localize the glycinergic neurons projecting to
521 the BF. Relatively few areas in the brainstem were found to exhibit cells double-labelled for GFP
522 and the retrograde tracer Choleratoxin B subunit or Fluorogold. The majority of glycine-
523 containing projecting neurons were found in the nucleus raphe magnus and the gigantocellular
524 reticular nucleus. These nuclei have been reported to establish a descending pathway responsible
525 for muscle atonia during REM sleep (Holstege and Bongers, 1991; Kato et al., 2006). Although

526 the projection of GABA/glycinergic neurons from these nuclei to the spinal cord has been
527 demonstrated, recent findings emphasize the primary role of glutamatergic neurons in these
528 nuclei in indirectly inhibiting the motoneurons via spinal cord interneurons during REM sleep
529 (Vetrivelan et al., 2009). Further studies are required to clarify the function of the ascending
530 glycinergic pathways from these REM sleep active nuclei to the BF. It would be interesting to
531 find out whether bifurcating collaterals of the same cell bodies project to the spinal cord and BF
532 cholinergic neurons, as such scenario was implicitly suggested by demonstrating bifurcating
533 axons originating from cell bodies in the pontine reticular formation reaching the anterior horn of
534 the spinal cord and the hypothalamus using the classical Golgi-technique (Scheibel, 1958).
535 During embryonic development nearly all inhibitory neurons in caudal brain regions express
536 GLYT2, indicating that glycinergic neurons are capable of co-releasing GABA. After birth the
537 level of co-transmission falls, but still remains at about 40% in adulthood (Rahman et al., 2015).
538 It remains to be determined whether glycinergic neurons projecting to the BF contain GABA in
539 their axons, since a mixed-glycinergic/GABAergic input and cell-specific expression of glycine
540 receptors and $GABA_A$ receptors would indicate the capability for cholinergic neurons to shape
541 their inhibitory control.

542 **Functional considerations**

543 GLYT2-IR axon terminals were found on ChAT-IR perikarya, as well as smaller and larger
544 diameter cholinergic dendrites, including spines on both the soma and dendrites of cholinergic
545 neurons. Inhibitory synapses often appear on the soma and the proximal dendrites of target cells,
546 in a position to efficiently block the generation of action potentials. Presence on more distal
547 dendritic branches, frequently found in the current study, was indicated to exert a less powerful
548 but still significant inhibitory influence on target cells, involving plasticity (Chiu et al., 2013).

549 Spine-like processes were observed in most biocytin-filled mouse cholinergic neurons (Fig. 2F),
550 and can also be demonstrated in juxtacellularly filled or immunostained rat BFC neurons at
551 ultrastructural level (unpublished observation). It remains to be determined whether glycinergic
552 synapses detected on the spines of the soma and the proximal dendrites (Fig. 6) counterbalance
553 excitatory inputs impinging on adjacent dendritic segments. Spine morphology and synaptic
554 plasticity ~~is~~ are regulated by the cytoskeletal and scaffolding protein actin (dos Remedios et al.,
555 2003) and its dysfunction is thought to be critical in Amyloid β induced cholinergic cell loss in
556 Alzheimer's disease (Song et al., 2002; Maloney et al., 2005). Organophosphate insecticides
557 were shown to induce a concentration-dependent reduction of spine-like processes in BFC
558 neurons (Del Pino et al., 2016) which is accompanied by the alteration of NMDA receptors.
559 Unbalanced NMDA receptor-mediated signaling results in cognitive and memory dysfunctions,
560 and it is supposedly the leading pathological process in many neurological and
561 neurodegenerative diseases (Gu et al., 2014). It seems that either hyper- or hypofunction of this
562 receptor can evoke glutamate toxicity in vulnerable parts of the CNS (Newcomer et al., 2000);
563 the reason for that may be in the different signaling pathways recruited by the two major
564 subpopulations, the synaptic and extrasynaptic pools of NMDA receptors. Although there are
565 some contradictory data in this regard, the apparent agreement is that besides the physiological
566 signaling, glutamatergic transmission via synaptic NMDA receptors is neuroprotective, whereas
567 transmission via non-synaptic NMDA receptors is excitotoxic (Papouin et al., 2012). Glycine is
568 also a co-agonist on NMDA receptors, although D-serine released by astrocytes can also occupy
569 the glycine-binding site on the NMDA receptors. However, D-serine acts at synaptic NMDA
570 receptors and glycine acts at their extrasynaptic counterparts (Papouin et al., 2012). The high
571 level of GLYT1 in BF (Fig.10.; (Kallo et al., 2008) suggests a very tight control on the

extracellular levels of the co-agonist, glycine. It still needs to be clarified whether glycine influences NMDA mediated currents on cholinergic neurons and thereby contributes to BF plasticity and learning.

Concluding remarks

In summary, detection of (1) a rich network of glycinergic axons in connection with the cholinergic neurons in the different subregions of the BF, (2) symmetric glycinergic synapses on the perikaryon, and both proximal and distal dendritic branches of BFC neurons, and (3) bicuculline-resistant, strychnine-sensitive postsynaptic events, indicates a general, inhibitory role for glycine in the regulation of BFC functions. It remains for future studies to explore whether specific behavioral conditions are linked with glycine release in the BF and whether glycine could act as a modulator of NMDA receptor function s and thus could contribute to the vulnerability of cholinergic neurons in Alzheimer's or related neurodegenerative diseases.

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Legends:

Fig. 1. Distribution of glycine receptor (GlyR) - immunoreactive (IR) sites in the basal forebrain detected by a panαGlyR antibody. (A) The punctate appearance of GlyR-immunoreactivity (revealed by nickel intensified DAB) often delineates cellular borders in the strongly labeled accumbens core, in the medium labeled HDB, as well as in the weakly labeled ventral pallidum (Bregma level 0.73). The boxed area of HDB is further magnified in (B) showing GlyR-immunoreactivity in the perikaryon, as well as in the processes of cells. (C) A high power photograph of a single cell, with GlyR-IR sites primarily distributed at the periphery of the cell (arrows). (D-E) Confocal microscopic images of two optical slices (0.75 µm) of a ChAT-eGFP neuron exhibiting GlyR-IR sites at the contour of the cell (arrows). aca: anterior commissure, anterior part; AcbC and AcbSh: accumbens nucleus, core and shell regions; HDB and VDB: nuclei of the horizontal and vertical limbs of the diagonal band; VP: ventral pallidum; Scale bars: A, B: 100 µm; C: 10 µm; D, E: 5 µm (Contrast and brightness were adjusted by the Curve function of Adobe Photoshop 5.1)

Fig. 2. Glycinergic IPSCs recorded on cholinergic neurons in the basal forebrain. **(A)** A 10-s-long representative trace of spontaneous (excitatory and inhibitory) postsynaptic currents recorded in normal ACSF. **(B)** Inhibitors of ionotropic glutamate (NBQX, D-AP5)- and GABAA-receptors (bicuculline) did not fully abolish postsynaptic currents. **(C)** Adding strychnine to the recording cocktail blocked all events. **(D)** Bar chart diagrams of the frequency and amplitude of the recorded events in normal aCSF (N, hollow), with NBQX, D-AP5 and bicuculline (C; green) and with additional strychnine application (C+S; gray). All differences were statistically significant ($p<0.001$). Cumulative histogram of the inter-event interval and amplitude of synaptic events in normal aCSF (black) or in the presence of the NBQX-, D-AP5-

776 and bicuculline-containing blocking cocktail (green). All differences were statistically
777 significant (Kolmogorov-Smirnov test; $p<0.001$). **(E)** Positions of the recorded neuronal somata
778 in the basal forebrain shown by dots. Differences in the frequency of the strychnine-sensitive
779 events are color-coded. The mouse forebrain is reconstructed and the distribution of recorded
780 cells is shown from two different angles (axial and lateral views; based on the mouse brain atlas
781 (Paxinos, 2013). **(F)** Confocal image of a biocytin-filled cholinergic neuron from the HDB. The
782 inserts are magnified images of the boxed areas and show secondary dendritic processes with
783 scattered spines (arrows and asterisks). **(G)** Determination of the reversal potential of the
784 strychnine-sensitive events with KCl-based pipette solution (E_{Cl}^-= -0.6 mV). Black dots:
785 average±SEM of the 3 largest events of each trace. Dashed line: linear fit of the average values.
786 Gray dot: calculated equilibrium potential of chloride. Representative current traces belonging
787 to different holding potentials are also included in the inserts. **(H)** Current traces recorded with
788 KCl- (E_{Cl-}= -0.6 mV) and K-gluconate-based (E_{Cl-}= -83.1 mV) pipette solutions. **(I)**
789 Determination of the reversal potential of the strychnine-sensitive events with K-gluconate-based
790 pipette solution (E_{Cl-}= -83.1 mV). Black dots: average±SEM of the 3 largest events of each
791 trace. Gray line: linear fit of the average values. Gray dot: calculated equilibrium potential of
792 chloride. Current traces recorded with K-gluconate-based (E_{Cl-}= -83.1 mV) pipette solutions at
793 different holding potentials (-120 mV, -60 mV, -40 mV; n=7) are also shown in the inserts. Scale
794 bars: E: 1 mm; F: 20 µm, small insert: 10 µm

795 **Fig. 3.** Differences between the glycinergic sIPSCs recorded from early- and late-firing BF
796 cholinergic neurons. **(A)** Firing pattern of an early-firing neuron, elicited by 60 pA depolarizing
797 current injection. **(B)** Firing pattern of a late-firing neuron, elicited by 60 pA depolarizing
798 current injection. The dashed line indicates -75 mV. **(C)** Statistical summary of the delay (time

between the beginning of the rheobase current injection and the first action potential, from -75 mV holding potential) of early- (E, black) and late-firing (L, red) neurons. **(D)** Representative trace of glycinergic events from an early-firing neuron (black trace) and a late firing neuron (red trace). **(E)** Glycinergic IPSCs from an early firing (left) and a late firing (right) cholinergic BF neuron. (Gray traces: individual IPSCs, black trace: average of spontaneous glycinergic IPSCs from an early firing neuron, red trace: average of spontaneous glycinergic IPSCs from a late firing neuron). **(F)** Statistical summary of the observed differences between early (black) and late-firing (red) neurons with bar charts (black, E = early firing; red, L = late firing; average ± SEM) and cumulative histograms (inter-event interval = time between two glycinergic IPSCs in s; amplitude = amplitude differences of the glycinergic events in pA; rise = rise time; decay = decay time in ms)

Fig. 4. Dual immunohistochemical labeling to identify the relationship between GLYT2-IR fibers and ChAT-IR neurons in the basal forebrain (A-E). Distribution of GLYT2/ChAT- IR cellular profiles in the basal forebrain shown in coronal sections at five different rostro-caudal levels. Rich network of GLYT2-immunoreactive fibers is present in regions of the basal forebrain where cholinergic neurons are located i.e. medial septum (MS, Ch1), ventral diagonal band of Broca (VDB, Ch2), horizontal diagonal band of Broca (HDB, Ch3), ventral pallidum (VP, Ch4) and substantia innominata (Si, Ch4) (Mesulam et al., 1983). The rostro-caudal levels are given in mm from the Bregma, based upon the mouse brain atlas (Paxinos, 2013). A medium power image shows GLYT2-IR fiber varicosities adjacent to ChAT-IR soma (white arrowheads) and dendrites (black arrowheads) in the HDB. The inset highlights one of these appositions. AA: anterior amygdaloid area; ACo: anterior cortical amygdaloid nucleus; acp: anterior commissure, posterior limb; B: basal nucleus (Meynert); CeM:central amygdaloid nu, medial; Cpu: caudate

putamen (striatum); CxA: cortex-amygdala transition zone; EA: extension of the amygdala; f: fornix; GP: globus pallidus; ic: internal capsule; IPACL: interstitial nucleus of the posterior limb of the anterior commissure, lateral part; IPACM: interstitial nucleus of the posterior limb of the anterior commissure, medial part; LDB: lateral nucleus of the diagonal band; LH: lateral hypothalamic area; LPO: lateral preoptic area; MeA: medial amygdaloid nucleus, anterior part; MPA: medial preoptic area; ns: nigrostriatal bundle; och: optic chiasm; opt: optic tract; PLH: peduncular lateral hypothal; Rt: reticular nucleus (prethalamus); SIB: substantia innominata, basal part; sm: stria medullaris; Tu: olfactory tubercle; VLH: ventrolateral hypothalamic nucleus; VLPO: ventrolateral preoptic nucleus; ZI: zona incerta Scale bars: A-E: 500 µm; F: 25 µm (Contrast and brightness were adjusted by the Curve function of Adobe Photoshop 5.1)

Fig. 5. Bright field and electron microscopic images of GLYT2-IR fibers in apposition to ChAT-IR neurons of the basal forebrain. Varicose GLYT2-IR axons establish axo-somatic and axo-dendritic connections with ChAT-IR neurons in the medial septum (A), nucleus of the horizontal limb of the diagonal band (B) and basal nucleus (C). The axon varicosities often surround or embed neural profiles, as demonstrated by the insets, showing central lighter areas in certain axon varicosities. The appositions between GLYT2-IR axons and ChAT-IR dendrites (D-F) or soma (G) often proved to be synaptic connections (black arrows) showing characteristics of the symmetric types. Scale bars: A-C: 10 µm; D-G: 500 nm

Fig. 6. GLYT2-IR axon terminals synapse with somatic (C) and dendritic (D) spines of cholinergic neurons in the HDB. (A) High power micrograph of the immunohistochemically double labeled and epoxy-embedded section shows multiple contacts (arrowheads) between GLYT2-IR axon varicosities and a ChAT-IR neuron (highlighted in red). (B) The outlined areas (labeled with C and D) of the low power electron micrograph are shown in further magnified

images of consecutive ultrathin sections (C'-C'' and D'-D''''). Dendritic spines are embedded in and synapsing (black arrows) with GLYT2-IR axon terminals; the spine neck identifies the dendritic spine (stars) in connection with the ChAT-IR neuron. Scale bars: A: 5 µm; B: 2 µm; C'-C", D'-D"": 500 nm

Fig. 7. Location of basal forebrain-projecting glycinergic neurons revealed by iontophoretic injection of the retrograde tracer CTB or Fluorogold into different subdivisions of the BF. Representative CTB-IR (#2875, #2874, and #2877) and Fluorogold (#3030) injection sites plotted in basal forebrain section images (A-D) and the corresponding atlas figures (based on (Paxinos, 2013)). (E) General overview of glycinergic projections from the brainstem to basal forebrain showing the most abundant input from the raphe magnus and its neighboring nuclei (red arrow) and the least number of retrogradely labeled cells locating in the periaqueductal grey matter (PAG; light read arrow). (F) Retrogradely labeled cells detected in the pons, containing GLYT2-GFP expressing cells in the raphe magnus nucleus (RMg), pontine reticular nucleus ventral and caudal part (PnV and PnC), and raphe interpositus nucleus (RIP). The outlined area is further magnified in G, H and I to demonstrate double-labeled neurons projecting to the BF. The single- (white arrow) and double-labeled cells (black arrows) are shown in corresponding single (G and H) and dual (I) color images. Gi: gigantocellular reticular nucleus; GiA: gigantocellular reticular nucleus, alpha part; GiV: gigantocellular reticular nucleus, ventral; PnO: pontine reticular nucleus, oral part; py: pyramidal tract; Rpa: raphe pallidus nucleus; Tz: nucleus of the trapezoid body. Scale bars: A-D, F: 100 µm; G-I: 25 µm

Fig. 8. Dual immunohistochemical labeling of GLYT1-IR structures (arrowheads) in the vicinity of ChAT-IR neurons. (A-E) Distribution of GLYT1- and ChAT-IR cellular profiles in the basal forebrain shown in coronal sections at five different rostro-caudal levels. Cholinergic neurons are

868 embedded in GLYT1-IR puncta present in all major regions of the basal forebrain. The rostro-
869 caudal levels are given in mm from the Bregma, based upon the mouse brain atlas (Paxinos,
870 2013). (F) The medium power reveals a patchy arrangement of GLYT1-IR puncta resembling the
871 shape of astrocytes. Many IR puncta are in close vicinity of ChAT-IR neurons (arrowheads). st:
872 stria terminalis; STLP: bed nucleus of the stria terminalis, lateral division, posterior part; STLV:
873 bed nucleus of the stria terminalis, lateral division, ventral part; STMV: bed nucleus of the stria
874 terminalis, medial division, ventral part. Scale bars: A-E: 500 µm; F: 50 µm (Contrast and
875 brightness were adjusted by the Curve function of Adobe Photoshop 5.1)
876 **Fig. 9.** Three-dimensional reconstruction from the confocal image of neural structures in a
877 section immunohistochemically labeled for ChAT (green), GLYT1 (red) and GLYT2 (blue). (A)
878 GLYT2-IR varicose processes are in apposition to the ChAT-IR cell body or processes (arrows).
879 GLYT1-positive elements get in association with surface areas of ChAT-IR neurons including
880 areas near apposing GLYT2-IR terminals. The outlined areas are further magnified and rotated in
881 (B) and (C). In addition, the GLYT2-IR processes are made semi-transparent to demonstrate
882 apposing surfaces. The inset in (C) shows the overlapping structures from the side and the color
883 generated by the differently labelled processes at the white line.
884 **Fig. 10.** Electron microscopic images of thin GLYT1-IR astrocytic processes (labeled with
885 silver-gold particles) in the vicinity of non-labeled (A) and ChAT-IR neurons (labeled with
886 DAB; B, C, D). The GLYT1-IR processes (highlighted in blue) appear adjacent to non-labeled
887 axon terminals synapsing (arrows) with a ChAT-IR perikaryon (C) or dendrite (B&D). Scale
888 bars: A: 1 µm; B-D: 250 nm
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