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Vglut1 and Vglut2 synapses on cholinergic neurons in the sublenticular gray of the rat basal forebrain: a double-label electron microscopic study

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Abstract

The basal forebrain (BF) comprises morphologically and functionally heterogeneous cell populations, including cholinergic and non-cholinergic corticopetal neurons that are implicated in sleep-wake modulation, learning, memory and attention. Several studies suggest that glutamate may be among inputs affecting cholinergic corticopetal neurons but such inputs have not been demonstrated unequivocally. We examined glutamatergic axon terminals in the sublenticular substantia innominata in rats using double-immunolabeling for vesicular glutamate transporters (Vglut1 and Vglut2) and choline acetyltransferase (ChAT) at the electron microscopic level. In a total surface area of 30,000 μm^2 , we classified the pre- and postsynaptic elements of 813 synaptic boutons. Vglut1 and Vglut2 boutons synapsed with cholinergic dendrites, and occasionally Vglut2 axon terminals also synapsed with cholinergic cell bodies. Vglut1 terminals formed synapses with unlabeled dendrites and spines with equal frequency, while Vglut2 boutons were mainly in synaptic contact with unlabeled dendritic shafts and occasionally with unlabeled spines. In general, Vglut1 boutons contacted more distal dendritic compartments than Vglut2 boutons. About 21% of all synaptic boutons ($n=347$) detected in tissue that was stained for Vglut1 and ChAT were positive for Vglut1, and 14% of the Vglut1 synapses were made on cholinergic profiles. From separate cases stained for Vglut2 and ChAT, 35% of all synaptic boutons ($n=466$) were positive for Vglut2, and 23% of the Vglut2 synapses were made on cholinergic profiles. On average, Vglut1 boutons were significantly smaller than Vglut2 synaptic boutons. The Vglut2 boutons that synapsed cholinergic profiles tended to be larger than the Vglut2 boutons that contacted unlabeled, non-cholinergic postsynaptic profiles. The presence of two different subtypes of Vgluts, the size differences of the Vglut synaptic boutons, and their preference for different postsynaptic targets suggest that the action of glutamate on BF neurons is complex and may arise from multiple afferent sources.

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Keywords

glutamate; nucleus basalis; vesicular glutamate transporter; acetylcholine; electron microscopy; substantia innominata

The basal forebrain (BF) comprises functionally and neurochemically heterogeneous cell populations along the medial and ventral surfaces of the telencephalon. A distinguishing feature of the BF is the continuous collection of aggregated and non-aggregated large cortically-projecting cells, many of which release the neurotransmitter acetylcholine (ACh) and serve as the primary source of cholinergic fibers that innervate the entire cortical mantle (Mesulam et al., 1983; Rye et al., 1984; Saper, 1984). Cholinergic projection neurons in the BF are distributed in the medial septum, nuclei of the vertical and horizontal limbs of the diagonal band of Broca, ventral pallidum, substantia innominata, bed nucleus of the stria terminalis, internal capsule, and globus pallidus. The space occupying cholinergic projection neurons also contains GABAergic, peptidergic, and glutamatergic neurons (Zaborszky and Duque, 2003; Hur and Zaborszky, 2005). Through its cholinergic and non-cholinergic projections to the cortex, these BF areas modulate complex functions such as arousal, sensory processing, motivation, attention, and the sleep-wake cycle (Buzsaki et al., 1988; Richardson and DeLong, 1988; Metherate et al., 1992; Sarter and Bruno, 1997; Szymusiak et al., 2000; Saper et al., 2005; Steriade and McCarley, 2005; Lee et al., 2005; Sarter et al., 2006; Datta and McLean, 2007; Parikh and Sarter, 2008; Lin and Nicolelis, 2008). BF cholinergic neurons are compromised in many diseases, including Alzheimer's disease, schizophrenia, Parkinson's disease, and Rett's syndrome (Bowen et al., 1976; Perry et al., 1978; Bartus et al., 1982; Wenk and Mobley, 1996). Due to the complex neuroanatomy of the BF, our understanding of the role of the BF in these functions and diseases remains incomplete.

Interaction of glutamate and ACh in the BF has long been studied in vitro (Khateb et al., 1995; Sim and Griffith, 1996). Furthermore, the release of ACh in the cortex following the local administration of glutamate in the BF suggests that glutamatergic inputs may excite BF cholinergic neurons. For example, administration of the glutamate agonists kainic acid and NMDA into the BF produces significant increases in ACh efflux in the cortex (Fadel et al., 2001; Fournier et al., 2004). However, it was not possible to determine if the increased ACh release was due to direct or indirect effects on BF cholinergic neurons in those experiments. The presence of glutamate receptors in the BF suggests that BF neurons receive direct glutamatergic inputs. Metabotropic, AMPA, and NMDA glutamate receptors are expressed in BF areas (Petrálie and Wenthold, 1992; Kiss et al., 1993; Martin et al., 1993; Page and Everitt, 1995; Standaert et al., 1996; De Souza Silva et al., 2006). However, the specific glutamate receptor subtypes expressed by cholinergic and non-cholinergic neurons have yet to be fully characterized.

In the present study, we provide morphological evidence for monosynaptic glutamatergic inputs to BF cholinergic and non-cholinergic neurons in the sublenticular substantia innominata—often referred to as basal nucleus of Meynert in primates - using correlated light and electron microscopy. BF sections were immunostained for choline acetyltransferase (ChAT) in combination with vesicular glutamate transporters (Vglut1 or Vglut2) to reveal two subtypes of glutamatergic synapses with cholinergic neurons in the sublenticular substantia innominata of rats. Some of these data were presented in abstract form (Zaborszky et al., 2003).

Experimental Procedures

Animals

All experiments were performed in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health *Guidelines for the Care and Use of Animals in Research*, and approved by the Rutgers University Institutional Review Board. All efforts were made to minimize the number of animals used and their suffering. Five male Sprague-Dawley rats (270–300 g; Zivic Miller Laboratories, Portersville, PA) were housed individually and maintained on a 12 h light/dark cycle with water and food ad libitum. Rats were given a lethal dose of urethane (2.8 g/kg, i.p.) and perfused transcardially with 0.9% saline, followed by a 200 mL mixture of 4% acrolein and 2% depolymerized paraformaldehyde, and then 200 mL of 2% depolymerized paraformaldehyde in 0.1M phosphate buffered saline (PBS, pH 7.4). The brains were removed and kept overnight at 4°C in 4% depolymerized paraformaldehyde. Six series of coronal sections (50 µm thick) were cut on a vibrating microtome and collected in PBS.

Immunocytochemistry for light and electron microscopy

Brain sections containing the BF were cryoprotected in 7.5%, 15%, and 30% sucrose for 15 min, 30 min, and 2 h, respectively, at 4°C. The sections were then placed flat in aluminum dishes and freeze-thawed sequentially three times in liquid nitrogen fumes for proper antigen retrieval. The sections were treated in 1% sodium borohydride (20 min), followed by 2% normal donkey serum (30 min), and incubated in primary antibodies against Vglut1 or Vglut2 for 2 nights at 4°C (1:2000; rabbit anti-Vglut1 and rabbit anti-Vglut2; antibody production and characterization described in Fremeau et al., 2001). The antibody diluent contained 0.1% thimerosal and 2% normal donkey serum in PBS. For light microscopy, the antibody diluent also contained 0.1% Triton X-100. The Vglut1 primary antibody was raised against a glutathione S-transferase (GST) fusion protein containing the 68 C-terminal amino acids (493–560) of rat Vglut1 and was previously shown to recognize a band of the correct molecular weight on Western blot of extracts of brain or COS cells transfected with the transporter. The Vglut2 primary antibody was raised against a GST fusion protein containing (519–582) of rat Vglut2. Western blots recognized a 50–62 kD band from rat brain (Fremeau et al., 2001). Vglut1 and Vglut2 immunolabeling was confined to axon terminals and was absent when the same antibody was preadsorbed with the fusion protein (Fremeau et al., 2001). After primary antibody incubation, sections were rinsed in PBS and incubated in biotinylated donkey anti-rabbit IgG (1:500; Vector Laboratories, Burlingame, CA) in antibody diluent overnight at 4°C. The sections were then incubated in Vectastain® peroxidase ABC (1:500 in PBS, 2 h) and rinsed in tris-buffered saline (TBS, pH 7.60). The peroxidase reaction was carried out using a nickel-enhanced diaminobenzidine chromogen (Ni-DAB; 0.4 mg/ml 3,3'-diaminobenzidine tetrahydrochloride, 0.4 mg/ml nickel ammonium sulfate, 0.4 mg/ml ammonium chloride, and 0.0006% hydrogen peroxide dissolved in TBS) to reveal Vglut1 or Vglut2 fibers. The Ni-DAB precipitate was silver-gold intensified as described by Liposits et al. (1984) without thioglycolic acid pretreatment.

Following silver-gold intensification, the sections were treated in 1% hydrogen peroxide (in PBS, 10 min), incubated in an avidin/biotin blocking kit solution (1:25; Vector; 30 min), and then processed overnight at 4°C in a sheep polyclonal anti-ChAT antibody solution (1:5,000, Chemicon; Temecula, CA, USA; catalog number AB1582). The anti-ChAT antibody was raised against a 22 amino acid peptide from porcine ChAT, coupled to KLH. The peptide sequence is H-GLFSSYRLPGHTQDTLVAQKSS-NH₂. The secondary antibody was a biotinylated donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA, 1:500 in antibody diluent, overnight) followed by Vectastain ABC (1:500 in PBS, 2 h at room temperature), and biotin-tyramide amplification (Adams, 1992; Kim et al., 2003).

The peroxidase reaction in cholinergic profiles were developed with 0.4 mg/mL DAB and 0.0006% hydrogen peroxide dissolved in TBS. Sections for light microscopy were mounted onto glass slides and cover slipped with DePeX (Electron Microscopy Sciences, Hatfield, PA, USA) mounting medium.

Tissue processing for electron microscopy

Sections for electron microscopy were further treated with 1% osmium tetroxide in PBS (Electron Microscopy Sciences, Fort Washington, PA) for 40 minutes and increasing concentrations of ethanol (30%, 50%, 70%, 90%, and 100%). To enhance contrast, the tissue was treated for 30 minutes with 1% uranyl acetate (Electron Microscopy Sciences) in 70% ethanol. Following two rinses in propylene oxide (Electron Microscopy Sciences, 3 min) the sections were soaked overnight in Durcupan (Fluka Chemie AG, Buchs, Switzerland), and flat embedded between glass slides and coverslips pretreated with liquid release agent (Electron Microscopy Sciences). After embedding, small regions from the sublenticular substantia innominata (at the border of the ventral globus pallidus and substantia innominata) at the level of the crossing of the anterior commissure were photographed with a digital camera under brightfield microscopy. The BF areas were chosen based on the presence of cholinergic dendrites and Vglut1 or Vglut2 axons (Figs. 1-2).

The selected tissue pieces were cut and mounted onto blank Durcupan blocks. Serial ultrathin sections were cut on a Reichert Ultracut E ultramicrotome and then transferred onto formvar-coated single-slot grids (Electron Microscopy Sciences). The ultrathin sections were analyzed using a Phillips Tecnai 12 transmission electron microscope. We selected three ultrathin sections from tissue stained for Vglut1/ChAT and four ultrathin sections from tissue stained for Vglut2/ChAT comprising a total surface area of 30,000 μm^2 (Table 1). The blocks used for ultrathin sectioning were obtained from a total of 5 rats (03009, 03027, 03043, 03062, and 03063). The three blocks for Vglut1 staining came from case 03009 (n=1) and case 03062 (n=2). The four blocks for Vglut2 staining came from one series of 03043, one series from 03027, and two series from 03063. About 25 continuous photos were taken under 4800 $\times$ magnification and developed on photographic film (Kodak EM film 4489) for each ultrathin section. The total surface area analyzed from the photos was not statistically different (Table 1). The films were then digitized using a Polaroid Sprints can i45 scanner at 1000 dpi in grayscale mode and assembled into seven photomontages, one for each ultrathin section. Photomontages from digitized film scans were assembled in Photoshop. Brightness and contrast were adjusted.

Image analysis and quantification of synapses

All electron microscopic images were imported into Reconstruct software (version 1.03.0; <http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm>). We traced all the unlabeled and immunolabeled synaptic boutons, dendrites, somata, and glia present in the 7 photomontages. Using Reconstruct on the calibrated EM images we extracted the surface values of each traced profile. Surface areas were calculated from an algorithm based on Green's Theorem in Reconstruct (Fiala and Harris, 2001; Fiala, 2005). We classified all the synapses in the photomontages as belonging to one of four categories: 1) Vglut boutons synapsing on cholinergic profiles, 2) Vglut boutons synapsing on non-cholinergic (unlabeled) profiles, 3) non-Vglut (unlabeled) boutons synapsing on cholinergic profiles, and 4) non-Vglut (unlabeled) synapses on non-cholinergic (unlabeled) profiles. The morphometry data presented in Tables 1-4 were derived from the photomontages using Reconstruct software. Synaptic contacts were identified by clusters of vesicles at the presynaptic membrane, parallel arrangement of pre- and postsynaptic membranes with a slight widening of the intercellular space, and filamentous material within the synaptic cleft (Peters et al., 1991). Asymmetric synapses typically exhibited larger zones of contact and thicker postsynaptic densities than symmetric synapses.

Asymmetric synapses were often characterized by the presence of subsynaptic dense bodies beneath the postsynaptic membrane. Dendritic spines were identified as small dendritic profiles without mitochondria that often contained the spine apparatus (Peters et al., 1991). The term “contacts” in this study refers exclusively to positively identified synapses as defined above. The term “profiles” denotes postsynaptic cellular components (dendritic spines, shafts and cell bodies).

Statistical analysis

To compare differences in the postsynaptic cholinergic dendrite size from Vglut1/Ch and Vglut2/Ch stained material, we used the two-tailed Student's t-test with $p < 0.05$ regarded as significant. To compare differences in the Vglut1/ChAT and Vglut2/ChAT synapse parameters, we used Kruskal-Wallis nonparametric test followed by Dunn's post-hoc test for multiple comparisons ($p < 0.05$). The parameters we examined included the average sizes of Vglut synaptic boutons, total numbers of Vglut1 vs. Vglut2 boutons, and densities of Vglut1 and Vglut2 synaptic boutons in the BF.

Results

Light microscopy

In general, Vglut1 and Vglut2 immunoreactivity displayed a complementary distribution pattern in BF areas that are known to be rich in cholinergic neurons, in agreement with previous studies (Freneau et al., 2001; Fujiyama et al., 2001; Kaneko and Fujiyama, 2002; Härtig et al., 2003). Vglut1 and Vglut2 immunolabeled elements consisted of small punctate staining representing putative axon terminals. Strong Vglut1 immunoreactivity was observed in the piriform cortex, olfactory tubercle, nucleus accumbens, and caudate putamen. In contrast, Vglut1 immunoreactivity was markedly less abundant in BF areas that contained cholinergic projection neurons (Fig. 1A-C). At higher magnification, a small patch of Vglut1 immunoreactivity was detected at the border between the substantia innominata and the ventral part of the globus pallidus, behind or at the level of the crossing of the anterior commissure. This area, the sublenticular gray, was processed for electron microscopy (Figs. 1C, 2A). Vglut2 immunoreactivity produced a different pattern of staining compared to Vglut1. BF areas rich in cholinergic neurons, including the ventral pallidum, substantia innominata, medial septum, vertical and horizontal nuclei of the diagonal band showed strong Vglut2 immunolabeling (Fig. 1D-F). Vglut2 varicosities richly surrounded cholinergic cells within the sublenticular gray (inset to Fig. 2B).

Electron microscopy

The material processed for electron microscopy was sampled at the level of the crossing of the anterior commissure at the border of the ventral globus pallidus and substantia innominata, as outlined by the small box in Figs. 1C and 1F. The surface fraction of glia in the sampled tissue, cholinergic and non-cholinergic profiles, were similar for the Vglut1 and Vglut2 stained cases (Table 1). The total number of synaptic boutons counted in all of the samples was 813 (Table 2). The total number of synaptic boutons adjusted for surface area were similar ($275/10,000 \mu\text{m}^2$ in Vglut1 cases and $268/10,000 \mu\text{m}^2$ in Vglut2 cases).

The highly electron-dense silver-gold grains in the Vglut terminals and the flocculent DAB precipitate in the cholinergic profiles were easily distinguishable in the electron micrographs (Figs. 3-4). From the Vglut1/ChAT material, Vglut1 axon terminals accounted for 21% of all synapses examined but only about 14% of these Vglut1 synapses were with cholinergic profiles (Table 2). Vglut1 boutons contained clear, spherical synaptic vesicles and formed asymmetrical synapses with cholinergic dendrites (Fig. 3A), non-cholinergic dendritic shafts and unlabeled dendritic spines (Fig. 3B-E).

From the Vglut2/ChAT double labeled material (Tables 1-2), Vglut2 synapses accounted for over 45% of all synapses made on cholinergic profiles. For non-cholinergic BF neurons, Vglut2 synapses constituted 33% of all synapses in the tissue sampled from the sublenticular gray. Vglut2-positive terminals synapsed on cholinergic and non-cholinergic cell bodies and dendritic shafts as well as on unlabeled dendritic spines (Table 3, Fig. 4). Vglut2 stained material revealed often complex synaptic arrangements. For example, in Fig. 4B, one cholinergic dendrite received two Vglut2 synapses. In Fig. 4D, a single cholinergic dendrite is completely surrounded by Vglut2 terminals, with one of them also contacting another small cholinergic dendritic branch. Figure 5 displays an unlabeled dendritic shaft with prominent subsynaptic dens bodies in serial sections, receiving input from a large Vglut2 bouton.

Table 3 shows the distribution of Vglut synapses according to their cellular targets. The number of Vglut2 synapses on cholinergic dendritic shafts was almost three times higher than for Vglut1 synapses on cholinergic dendrites. Vglut1 boutons synapsed equally frequently with dendritic shafts and spines of non-cholinergic BF neurons. On the other hand, over 91% of the Vglut2 boutons that synapsed on non-cholinergic profiles were on dendritic shafts.

The morphometric data related to the size of various synaptic boutons are summarized in Table 4. On average, Vglut1 boutons were significantly smaller than Vglut2 synaptic boutons. The Vglut2 boutons that contacted cholinergic profiles tended to be larger than the Vglut2 boutons that contacted unlabeled, non-cholinergic postsynaptic profiles. Vglut1 synapses tended to contact more distal cholinergic (average surface area = $2.33 \mu\text{m}^2$; $n=13$) and non-cholinergic dendritic profiles (average surface area = $1.1 \mu\text{m}^2$; $n=61$), while Vglut2 synapses tended to contact larger, more proximal dendritic segments (cholinergic surface area: $3.64 \mu\text{m}^2$; $n=31$; non-cholinergic $1.60 \mu\text{m}^2$, $n=112$), although these differences were not significant. The larger surface fraction of Vglut2 synaptic boutons (0.58%) compared to Vglut1 boutons (0.20%) in the sublenticular gray may be explained by the larger average size of Vglut2 boutons and the larger number of Vglut2 boutons per surface area (58 Vglut1 boutons vs. 94 Vglut2 boutons per $10,000 \mu\text{m}^2$, calculated from Table 2).

Discussion

The results of this study provide anatomical evidence for two subtypes of glutamatergic synaptic terminals on cholinergic and non-cholinergic neurons of the rat BF. From a small sample in the sublenticular substantia innominata, the Vglut input to cholinergic cells accounted for 14% of all Vglut1 synapses and 23% of all Vglut2 synapses. The Vglut1 synapses represented 11% of all synapses made on cholinergic profiles while Vglut2 synapses represented 45% of the synaptic input to cholinergic profiles. Vglut1 and Vglut2 inputs represented about 25% and 33% of the total innervation of non-cholinergic profiles, the neurotransmitter content of these unlabeled postsynaptic neurons, however was not identified. The morphological differences between the Vglut1 and Vglut2 glutamatergic synaptic boutons included differences in their average sizes and the cellular topography of the synapses. These differences suggest that the action of glutamate on BF cells is complex and may arise from multiple afferent sources. The results of the present study, together with previous pharmacological studies, are compatible with a mechanism for direct synaptic effects of glutamate on BF cholinergic function.

Methodological considerations

Vglut1 and Vglut2 immunostaining are heterogeneously distributed across areas of the BF, as reported previously (Härtig et al., 2003) and confirmed in the present study (Fig. 1). Using the characteristic staining pattern of Vglut1 ("patch") in the sublenticular substantia innominata as the target area, we sampled tissue from the same general area stained for both Vglut1/ChAT and Vglut2/ChAT. We intentionally chose this area because at the light microscopy level,

Vglut1 terminal labeling is maximal in this region. The uniqueness of this area is underlined by the fact, that the number of catecholaminergic appositions per cholinergic cell bodies is the highest among BF areas in this particular region (Zaborszky and Cullinan, 1996; Hajszan and Zaborszky, 2002). There were no statistical differences in the tissue compartments of the samples for Vglut1/ChAT and Vglut2/ChAT (Table 1). Therefore, it is likely that the sampled areas represent the same region of the BF, even though the individual thin sections originated from different animals and were stained with different antibodies (Vglut1 or Vglut2). Since the same methodology was used for both Vglut1/ChAT and Vglut2/ChAT processing, it is likely that the data represent with good accuracy the real proportion of Vglut1 and Vglut2 synapses in this particular area. Because we did not test for the presence of colocalization of Vglut1 and Vglut2 (see below), and due to the small sample size, our data can only indicate that Vglut2 synapses represent a larger proportion of synapses than Vglut1 synapses. It is unclear if individual BF cholinergic neurons receive both Vglut1 and Vglut2 inputs and whether such inputs still follow a 3:1 (Vglut2 to Vglut1) ratio (Table 2) in the respective BF area. The quantitative data reported in our study pertain to the sampled area only (sublenticular substantia innominata) and by no means should be taken as characteristics for the entire cholinergic BF area. Based on light microscopic screening, the contribution of Vglut1 synapses maybe even lower in other areas, and the percentage of Vglut2 synapses from the respective total synaptic pool is likely to be different in different BF areas, since the neuropil organization show considerable changes across BF areas.

If there would be a colocalization of Vglut1 and Vglut2, this would reduce our estimates of the total glutamatergic innervation of this area of the BF. However, apparently the Vglut1 staining is absent in many of the Vglut2 positive BF regions, suggesting a preferential Vglut2 innervation for most of the cholinergic BF areas (Fig. 1). Moreover, in most regions of the brain, Vglut1 and Vglut2 colocalization at the mRNA level seldom occurs. For example, colocalization of Vglut1 and Vglut2 mRNA was reported in layer IV neocortex, and thalamic relay and association nuclei (Freneau et al., 2004; Barroso-Chinea et al., 2007), but these regions do not provide input to the BF. It is therefore unlikely that there is substantial colocalization, if any, of Vglut1 and Vglut2 in the presynaptic terminals in our sample.

For this study, we used single ultrathin section analysis. Therefore, the data remain inconclusive regarding the exact proportion of symmetric/asymmetric Vglut1 and Vglut2 synapses in the BF. However, most of the synapses investigated, we found that Vglut1 synapses were of the asymmetric type. This is in agreement with data showing that Vglut1 synapses in other brain areas are asymmetric (Freneau et al., 2001; Alonso-Nanclares et al., 2004; Fujiyama et al., 2006; Kubota et al., 2007; Omelchenko and Sesack, 2007). Although we found many symmetric synapses in the material stained for Vglut2, from our limited serial reconstruction, we observed that some of these Vglut2 synapses actually established asymmetric contacts (Fig. 5). On the other hand, symmetric Vglut2 synapses were observed in the serial thin sections of the ventral tegmental area (Omelchenko and Sesack, 2007) and in young ferret cortex (Nahmani and Erisir, 2005). Omelchenko and Sesack (2007) suggested that many of symmetric synapses formed by Vglut2 afferents may be associated with the coexpression of multiple neurotransmitters in glutamatergic axons. This is an interesting proposition that may apply to the BF, due to the presence of neurochemically heterogeneous afferent fibers (Zaborszky and Duque, 2003).

BF areas rich in cholinergic neurons also contain various non-cholinergic neurons, including calbindin, calretinin, or parvalbumin-containing neurons (Kiss et al., 1997; Zaborszky et al., 2005). Glutamatergic neurons (Hur and Zaborszky, 2005) and various peptidergic neurons (Zaborszky and Duque, 2000; 2003) have also been described in BF areas rich in cholinergic neurons. However, there are no detailed ultrastructural studies on BF non-cholinergic neurons, and it is unclear whether or not the unlabeled spines receiving Vglut1 or Vglut2 synapses (see

Table 3.) belonged to the same or different cell population that received glutamatergic synapses on their dendritic shafts.

Sources of Vglut1 and Vglut2 input to the basal forebrain

Although we have provided evidence for Vglut1 and Vglut2 synapses on BF cholinergic cells, the sources of these glutamatergic inputs remain to be investigated. In a previous electron microscopy study, it has been shown that the prefrontal cortex was the only cortical area that sent projections to the BF, synapsing mainly with parvalbumin-containing dendritic shafts and unlabeled dendritic spines (Zaborszky et al., 1997). Because prefrontal cortical glutamatergic axons do not seem to synapse on BF cholinergic cells (Zaborszky et al., 1997), the present paper is the first study to demonstrate that there are glutamatergic synapses on BF cholinergic neurons.

Since Vglut1 is heavily expressed in the cortex (Freneau et al., 2001), the inputs from the prefrontal cortex may account for some of the Vglut1 boutons that synapsed on non-cholinergic BF neurons reported in the present study. However, due to the absence of prefrontal cortical input to BF cholinergic dendrites (Zaborszky et al., 1997), it is unlikely that the Vglut1 input to BF cholinergic dendrites reported in the present study derives from the prefrontal cortex. Vglut1 input to BF cholinergic cells may originate, at least in part, from the basolateral nucleus of the amygdala, which is high in Vglut1 expression and has known projections to BF cholinergic cells (Zaborszky et al., 1984; Carnes et al., 1990; Jolkonnen et al., 2002; Poulin et al., 2008). Other possible sources of Vglut1 input to the BF are orexin neurons of the lateral hypothalamic area, many of which have been shown to express Vglut1 (Rosin et al., 2003). Orexin axons project to BF areas rich in cholinergic neurons, provide synapses to cholinergic neurons in the medial septum, and the peptides orexin A and B depolarize cholinergic neurons in vitro (Eggerman et al., 2001; Wu et al., 2004). Vglut2 expression is especially high in the intralaminar and midline thalamic nuclei (Hur and Zaborszky, 2005; Barroso-Chinea et al., 2007). These nuclei project to the caudate putamen and some basal forebrain areas rich in cholinergic cells (Carnes et al., 1990; Groenewegen and Witter, 2004), but no studies to date have established direct synaptic connections from these sources to cholinergic BF cells.

Vglut2 mRNA, but not Vglut1, is abundantly expressed in the BF (Hur and Zaborszky, 2005). Vglut2 neurons originating in the BF may project to cholinergic and non-cholinergic neurons in distant BF compartments (Hur and Zaborszky, unpublished observations) or act as local interneurons as has been suggested in the septum (Hajszan et al., 2004).

The brainstem may provide an additional source of glutamate input to BF cholinergic neurons. Stimulation of the area of the mesopontine tegmentum increases ACh release in the cortex, which can be blocked by the infusion of the nonspecific NMDA blocker kynurenin into the BF (Rasmusson et al., 1994). Because the BF, not the mesopontine tegmentum, is the source of ACh to the cortex, it is suggested that the brainstem activation of the BF elicits ACh release in the cortex via a polysynaptic pathway (Rasmusson et al., 1994). Other potential brainstem sources of glutamate to the BF include the ventral tegmental area, the periaqueductal gray, and the parabrachial nucleus, since these areas send projections to the BF (reviewed in Zaborszky and Duque, 2003) and contain Vglut2 cell bodies (Hur and Zaborszky, 2005).

Glutamate is a major synaptic input to BF cholinergic cells

The presence of a dense postsynaptic membrane thickening, a characteristic of asymmetric synapses, is associated with excitatory functions, while symmetric synapses are generally associated with inhibitory synapses (Uchizono, 1965). Most of Vglut1 and Vglut2 synapses reported in the present study were of the asymmetric type.

In earlier electron microscopic studies, 30-35% of the synaptic contacts on BF cholinergic corticopetal cells were classified as asymmetric and suggested to be glutamatergic (Ingham et al., 1985, 1988). In the present study, Vglut1 and Vglut2 axons accounted for 11% and 45%, respectively, of all the synapses made with cholinergic neurons in the sublenticular gray. From these data, one may estimate that together Vglut1 and Vglut2 synaptic boutons supply over 50% of the total synaptic input to BF cholinergic neurons in ventral globus pallidus and substantia innominata. However, we did not stain Vglut1 and Vglut2 in the same sections with ChAT and did not account for potential colocalization of Vglut1 and Vglut2 in the same neurons (Barroso-Chinea et al., 2007). Moreover, the cholinergic neuropil is not evenly distributed in the BF (Zaborszky, 1992). The estimated percentage of Vglut input to BF cholinergic cells may depend on the areas that are sampled. For example, it was reported that in the ventral pallidum, GABAergic synapses accounted for 86% of the synaptic input to BF (non-cholinergic and cholinergic) neurons but only 65% in the substantia innominata (Chang et al., 1995). In another electron microscopy study, 72% of the synaptic inputs to BF corticopetal neurons in the globus pallidus were GABAergic (Ingham et al., 1988). Therefore, it is likely that the percentage of glutamatergic synapses on cholinergic neurons show regional differences. Nevertheless, the results of our study indicate that glutamate provides a substantial direct input to BF cells. Our results together with those from previous electron microscope studies indicate that the majority of synaptic inputs to BF cholinergic cells are either glutamatergic or GABAergic.

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Abbreviations

ACh	acetylcholine
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BF	basal forebrain
ChAT	choline acetyltransferase
DAB	3,3'-diaminobenzidine

GABA	gamma-aminobutyric acid
Ni-DAB	nickel enhanced 3,3'-diaminobenzidine
NMDA	N-methyl-D-aspartic acid
PBS	phosphate buffered saline
TBS	tris buffered saline
Vglut	vesicular glutamate transporter

Fig. 1. Complementary distribution of Vglut1 (A-C) and Vglut2 (D-F) immunoreactivity in the rat basal forebrain. Strong Vglut1 immunoreactivity is present in the caudate-putamen (CPu), olfactory tubercle and the nucleus accumbens. In contrast, Vglut2 immunoreactivity is abundant in the shell of the nucleus accumbens (Acbs), anterior commissure (ac), horizontal limb of the diagonal band (HDB), bed nucleus of the stria terminalis (BSt), and ventral globus pallidus (GP). The small box in the substantia innominata outlines the tissue area processed for electron microscopy. Scale bar = 1.0 mm.

Fig. 2.

Light micrographs of sections stained for Vglut1/ChAT (**A**) or Vglut2/ChAT (**B**) at the level of the crossing fibers of the anterior commissure (ac) show the distribution of glutamatergic axonal varicosities around cholinergic neurons in the substantia innominata (boxed area). Boxed areas are enlarged in the lower right insets. Inset to panel (A) shows three cholinergic cell bodies and a few Vglut1 varicosities dispersed among the cell bodies. The enlargement of panel (B) displays an area of heavy staining of Vglut2 varicosities. Cholinergic cell bodies (arrowheads) are embedded in an area of dense Vglut2 neuropil immunostaining. Note in (A) the globus pallidus (GP) and the area of cholinergic neurons in the horizontal limb of the diagonal band (HDB) are devoid of immunostaining for Vglut1, although the caudate putamen (CP) and bed nucleus of the stria terminalis (BST) are much darker due to abundant expression of Vglut1. Scale bar = 500 μ m in (A), 15 μ m in inset to (A).

Fig. 3.

Electron micrographs showing Vglut1-labeled axon terminals in the basal forebrain. **(A)** Vglut1 terminals in asymmetric contact with a cholinergic dendrite (CD). The arrow points to the subsynaptic dense bodies. Adjacent to the Vglut1 bouton, an unlabeled bouton (asterisk) forms a symmetric synapse with the same cholinergic dendrite. **(B)** A Vglut1 terminal synapses with a dendritic spine (sp) adjacent to a cholinergic cell body (CB). Arrowheads point to the postsynaptic membrane. **(C-E)** Serial ultrathin sections of a Vglut1 axon terminal surrounding an unlabeled dendritic shaft (UD) adjacent to a cholinergic cell body (CB). **(D)** Arrowheads point to the asymmetric postsynaptic thickening. The black asterisk in Fig. 2C marks an unlabeled axon terminal into which another Vglut1 terminal invaginates (white asterisk in Figs. 2D-E). The Vglut1 staining was revealed with Ni-DAB followed by silver gold intensification. Cholinergic profiles contain flocculent DAB end-product. The black particles in the Vglut1 boutons are silver-gold granules. Scale bar = 0.5 μ m.

Fig. 4.

Electron micrographs showing Vglut2-labeled axon terminals. **(A)** A cholinergic cell body (CB) receives a Vglut2-synapse (black arrow). Another Vglut2-labeled bouton synapses with a small cholinergic dendritic branch. White arrowheads point the asymmetric synapse. **(B)** A cholinergic dendrite (CD) is surrounded by two Vglut2 synapses. The arrows point to the subsynaptic dense material. **(C)** An enlarged view of the axosomatic synapse from panel A. **(D)** A cholinergic dendrite is completely surrounded by Vglut2 axon terminals. The Vglut2 terminal in the lower left is also in a synapse with another strongly-labeled cholinergic dendrite (asterisk). The arrows point to the dense postsynaptic membrane. Scale bar: A= 1.0 μm ; B-D= 0.5 μm .

Fig. 5. Serial thin sections showing an unlabeled large dendritic shaft (**A-C**) in synaptic contact with a Vglut2-positive axon terminal. Note the presence of subsynaptic dense bodies in (**C**). The synapse in (**C**) is a typical perforated synapse. Scale bars = 0.5 μm .

TABLE 1

Surface fractions of neuropil components

Tissue Processing	Total Surface, μm^2	Surface area as a percentage of the total sampled area				
		Glia	Cholinergic profiles	Non-Cholinergic profiles	Vglut1 synaptic boutons	Vglut2 synaptic boutons
Vglut1/ChAT	12,614	1.7%	3.7%	23.1%	0.20%	--
Vglut2/ChAT	17,353	1.9%	2.8%	17.5%	--	0.58%

TABLE 2
Percentages of Vglut1 and Vglut2 synaptic boutons from all synaptic boutons

Tissue Processing	Total number of synaptic boutons	Vglut boutons on cholinergic profiles	Unlabeled boutons on cholinergic profiles	Vglut boutons on unlabeled profiles	Unlabeled boutons on unlabeled profiles
Vglut1/ChAT	347	2.8%	23.0%	18.1%	55.9%
Vglut2/ChAT	466	8.2%	9.9%	27.0%	54.9%

Numbers of Vglut1 and Vglut2 synapses in the sublenticular substantia innominata according to postsynaptic target

TABLE 3

	Cholinergic		Non-cholinergic		Total
	Soma	Dendrite/Spine	Soma	Dendrite/Spine	
Vglut1	0	13	1	32	27
Vglut2	1	32	4	116	7
					160

TABLE 4

Average sizes of Vglut1 and Vglut2 synaptic boutons, μm^2

Presynaptic bouton - postsynaptic profile	Vglut1/ChAT cases			Vglut2/ChAT cases		
	Mean	SD	SEM	Mean	SD	SEM
Unlabeled-Cholinergic	0.444 (n=80)	0.356	0.040	0.511 (n=46)	0.298	0.044
Unlabeled-Unlabeled	0.431 (n=194)	0.265	0.019	0.409 ^a (n=256)	0.274	0.017
Vglut-Cholinergic	0.495 (n=10)	0.468	0.148	0.799 (n=38)	0.562	0.091
Vglut-Unlabeled	0.329 ^c (n=63)	0.235	0.030	0.561 ^{a,c} (n=126)	0.284	0.025
Total Vglut (on Ch and Unlabeled postsynaptic profiles)	0.351 ^{b,d} (n=73)	0.280	0.033	0.616 ^{b,e,f} (n=164)	0.379	0.030
Total Unlabeled (on Ch and Unlabeled postsynaptic profiles)	0.435 ^{d,f} (n=274)	0.294	0.018	0.424 ^e (n=302)	0.280	0.016

Superscripts denote comparisons with p<0.05

^a Vglut2-Unlabeled vs. Unlabeled-Unlabeled (both groups from Vglut2 stained cases)

^b Total Vglut1 vs. Total Vglut2

^c Vglut1-Unlabeled vs. Vglut2-Unlabeled

^d Total Vglut1 vs. Total Unlabeled (both groups from Vglut1 cases)

^e Total Vglut2 vs. Total Unlabeled (both groups from Vglut2 cases)

^f Total Vglut2 (from Vglut2 cases) vs. Total Unlabeled (from Vglut1 cases)