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GABAergic Inputs from Direct and Indirect Striatal Projection Neurons Onto Cholinergic Interneurons in the Primate Putamen

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Abstract

Striatal cholinergic interneurons (ChIs) are involved in reward-dependent learning and the regulation of attention. The activity of these neurons is modulated by intrinsic and extrinsic γ-aminobutyric acid (GABA)ergic and glutamatergic afferents, but the source and relative prevalence of these diverse regulatory inputs remain to be characterized. To address this issue, we performed a quantitative ultrastructural analysis of the GABAergic and glutamatergic innervation of ChIs in the postcommissural putamen of rhesus monkeys. Postembedding immunogold localization of GABA combined with peroxidase immunostaining for choline acetyltransferase showed that 60% of all synaptic inputs to ChIs originate from GABAergic terminals, whereas 21% are from putatively glutamatergic terminals that establish asymmetric synapses, and 19% from other (non-GABAergic) sources of symmetric synapses. Double pre-embedding immunoelectron microscopy using substance P and Met-/Leu-enkephalin antibodies to label GABAergic terminals from collaterals of “direct” and “indirect” striatal projection neurons, respectively, revealed that 47% of the indirect pathway terminals and 36% of the direct pathway terminals target ChIs. Together, substance P- and enkephalin-positive terminals represent 24% of all synapses onto ChIs in the monkey putamen. These findings show that ChIs receive prominent GABAergic inputs from multiple origins, including a significant contingent from axon collaterals of direct and indirect pathway projection neurons.

Indexing Terms

medium spiny neurons; substance P; enkephalin; GABA; striatum

The dorsal striatum receives glutamatergic inputs from all functional regions of the cerebral cortex and thalamus (Parent and Hazrati, 1995; Smith et al., 2004, 2009) and sends γ-
aminobutyric acid (GABA)ergic projections to the external and internal segments of the globus pallidus (GPe and GPi) and the substantia nigra pars reticulata (SNr) via the so-called “direct and indirect” pathways (Albin et al., 1989; Alexander et al., 1990; Gerfen et al., 1990). The basal ganglia output nuclei (i.e., the GPe and SNr) relay this information back to the cerebral cortex and striatum via the thalamus (Alexander et al., 1990; Smith et al., 2004, 2009). Processing of information within the dorsal striatum involves dopaminergic inputs from the substantia nigra pars compacta (SNc), GABAergic afferents from the GPe, and intrastriatal GABAergic and cholinergic interneurons (Galvan and Wichmann, 2007; Wilson, 2007; Tepper et al., 2008; Gerfen and Surmeier, 2011).

In rodents, the striatum comprises 95% projection neurons (i.e., medium spiny neurons; MSNs) and 5% interneurons (Kemp and Powell, 1971; Wilson and Groves, 1980; Graveland and DiFiglia, 1985; Bolam et al., 2000), whereas in primates, interneurons account for as much as 24% of the striatal neuron population (Pasik et al., 1976; Graveland and DiFiglia, 1985; Roberts et al., 1996; Wu and Parent, 2000). The large cholinergic interneurons (ChIs) have long been recognized as important constituents of the striatal microcircuitry. ChIs give rise to extensive intrastriatal axonal arborizations and harbor thick primary dendrites with highly branched and varicose distal dendritic processes (DiFiglia et al., 1980; Chang and Kitai, 1982; DiFiglia and Carey, 1986; Yelnik et al., 1991). ChIs are evenly distributed throughout all functional striatal territories and strongly influence the activity of striatal projection neurons (Graybiel, 1990; Bernacer et al., 2007; Pisani et al., 2007; Tepper et al., 2008; Ding et al., 2010; Bonsi et al., 2011; Crittenden and Graybiel, 2011). Their extensive dendritic and axonal arbors allow them to integrate and transmit information across functionally diverse striatal territories, subserving their known role in processing attentional salient stimuli in the context of reward-related behaviors (Kimura et al., 1984; Matsumoto et al., 2001; Pisani et al., 2001; Ravel et al., 2003; Morris et al., 2004; Joshua et al., 2008; Aosaki et al., 2010; Apicella et al., 2011; Bonsi et al., 2011).

Anatomical, electrophysiological, and neurochemical studies in rodents and primates have suggested that the excitability of ChIs is strongly modulated by GABAergic inputs (DiFiglia, 1987; Sullivan et al., 2008; Tepper et al., 2008; Ding et al., 2010; Bonsi et al., 2011; English et al., 2012). Although it is likely that most of these afferents originate from intrinsic striatal sources, including GABAergic interneurons and axon collaterals of striatal output neurons (Bolam et al., 1986, 2000; Martone et al., 1992; Tepper et al., 2004, 2008; Wilson, 2007), the relative abundance of these various GABAergic inputs upon ChIs is unknown. To address this issue, we quantitatively assessed the prevalence and relative distribution of GABAergic terminals onto ChIs in the monkey postcommissural putamen. Furthermore, to specifically examine the contribution of GABAergic axon collaterals from direct or indirect striatofugal neurons to this innervation, we quantified the synaptic relationships between substance P (SP)- or enkephalin (Enk)-containing terminals (representing terminals of the direct and indirect pathway, respectively) and ChIs. Our findings show that striatal ChIs are a major target of GABAergic terminals, and that many of these afferents originate from axon collaterals of direct and indirect striatal projection neurons.

Some of these data have been presented in abstract form (Gonzales et al., 2009, 2010, 2011).

**Materials and Methods**

**Animals**

Brain tissue from six adult (3–9 years old; 5–8 kg) female rhesus monkeys (*Macaca mulatta*) from the Yerkes National Primate Research Center colony was used in this study. At the time of sacrifice, these animals were deeply anesthetized with an overdose of
pentobarbital (100 mg/kg, i.v.) and transcardially perfusion-fixed with a cold oxygenated Ringer's solution and a fixative containing 0.1% glutaraldehyde and 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). All procedures were approved by the Institutional Animal Care and Use Committee at Emory University and conform to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (Garber et al., 2010).

Tissue processing

After perfusion, the brains were removed from the skull, cut in 10-mm-thick blocks in the coronal plane, and stored in cold phosphate-buffered saline (PBS; 0.01 M, pH 7.4) until sectioning. A vibrating microtome was used to cut the blocks into serial 60-μm-thick coronal sections that were collected in an antifreeze solution (1.4% NaH₂PO₄·H₂O, 2.6% Na₂HPO₄·7H₂O, 30% ethylene glycol, 30% glycerol dissolved in distilled water) and stored in a −20°C freezer. Prior to the immunocytochemical processing, the sections were placed into a 1% sodium borohydride/PBS solution for 20 minutes, followed by washes with PBS.

Primary antibodies

Sections from the postcommissural putamen were processed for light and electron microscopy immunoperoxidase localization of choline acetyl transferase (ChAT) or immunogold localization of SP, Leucine [Leu⁵]- and Methionine [Met⁵]-Enk, or GABA by using highly specific, well-characterized monoclonal and polyclonal antibodies (Table 1). Two ChAT antibodies were used in order to have primary antibodies raised in different species for the double immunocytochemical reactions.

In the SP and ChAT double-label experiments, we used rabbit polyclonal anti-ChAT antibodies. The specificity of this antiserum has been demonstrated through experiments showing that the overall pattern of immunostaining obtained with this antiserum is confined to brain areas known to express detectable levels of ChAT (German et al., 1985; Peterson et al., 1990; Smith ML et al., 1993, 1994), and that preadsorption immunohistochemical assays resulted in the reduction of ChAT staining in these regions (Shiromani et al., 1987, 1990; Smith et al., 1993; Holt et al., 1997). Furthermore, western blot analysis from human brain or placental tissue showed a distinct band at 68 kDa that corresponds to the molecular weight of enzymatically active ChAT (Bruce et al., 1985).

In double-label immunocytochemistry experiments with the rabbit anti-Enk antibodies, we used goat polyclonal anti-ChAT antibodies. The specificity of this antiserum has been demonstrated through western blot analysis in rat and human brain tissue (Karson et al., 1993). Control incubations from which the primary antibodies were omitted resulted in a complete lack of striatal cell body or fiber immunostaining (Holt et al., 1999). We found that the application of either of the two ChAT antisera on the tissue used in our study resulted in a pattern of staining that resembled that found in previous studies of ChAT immunoreactivity in nonhuman primates (Graybiel et al., 1986; DiFiglia, 1987; Bernacer et al., 2007).

Monoclonal antibodies against SP were used to identify the direct pathway MSN collaterals, whereas the indirect pathway MSN collaterals were identified with polyclonal antibodies against Met-Enk and Leu-Enk (Table 1). The specificity of the SP antibodies used in this study have been demonstrated by showing the equal displacement of labeled SP by five-, six-, and eight-amino acid COOH-terminal fragments of SP, as well as full-length SP, and by the specific binding of these antibodies with cell bodies and terminals located in well-defined SP-containing nuclei in the central nervous system (Cuello et al., 1979; Beach and McGeer, 1984; Bolam and Izzo, 1988; Smith et al., 1998; Reiner et al., 1999, Wolansky et al., 2007). The lack of tissue labeling following incubation with nonimmune rat serum or
primary antibodies preabsorbed with SP further confirmed the specificity of these antibodies (Cuello et al., 1979; Mai et al., 1986).

In order to maximize labeling of indirect (Enk-positive) pathway neurons, we used a cocktail of antibodies against Met- and Leu-Enk (Cuello, 1978; Brann and Emson, 1980; Del Fiacco et al., 1982; DiFiglia et al., 1982; Smith et al., 1998). The specificity of these antibodies was demonstrated by preincubation of the diluted antisera with various concentrations of peptides that resulted in a lack of immunoreactivity in the tissue (Elde et al., 1976; Williams and Dockray, 1983). In addition, we found an almost complete segregation of labeling for SP and Enk in the GPe and GPi, as previously described using other SP and Enk antibodies in the basal ganglia of rats, monkeys, and humans (see Results, Fig 5) (Haber and Elde, 1981; DiFiglia et al., 1982; Williams and Dockray, 1983; Beach and McGeer, 1984; Reiner et al., 1999).

Lastly, affinity-purified rabbit polyclonal antibodies against GABA-bovine serum albumin (BSA) were used to localize GABA-containing terminals in the putamen (Table 1). Details about the production, characterization, and specificity of this antiserum have been extensively described in previous studies (Hodgson et al., 1985; Somogyi and Hodgson, 1985; Smith et al., 1987). In brief, biochemical specificity tests with nitrocellulose paper strips and preadsorption of antibodies with excess of synthetic GABA demonstrated that these antibodies are highly specific for GABA and do not cross-react with related amino acids (Hodgson et al., 1985).

**Single immunoperoxidase labeling for light microscopy (LM)**

Sections from the postcommissural putamen were treated at room temperature (RT) with sodium borohydride for 20 minutes followed by a preincubation for 1 hour in a solution containing 1% normal horse serum or normal goat serum (NGS), 0.3% Triton-X-100, and 1% BSA in PBS. Sections were then incubated for 24 hours at RT in a solution containing goat anti-ChAT (1:100), rat anti-SP (1:100), or rabbit anti-Met/Leu-Enk (1:1,000) in 1% normal horse or goat serum, 0.3% Triton-X-100, and 1% BSA in PBS. On the following day, sections were thoroughly rinsed in PBS and then incubated in a PBS solution containing either (secondary) biotinylated horse anti-goat IgGs, goat anti-rat IgGs, or goat anti-rabbit IgGs (1:200; Vector, Burlingame, CA) combined with 1% normal horse or goat serum, 0.3% Triton-X-100, and 1% BSA for 90 minutes at RT. Sections were then incubated within a solution containing 0.025% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO), 10 mM imidazole, and 0.005% hydrogen peroxide in Tris buffer for 10 minutes at RT, rinsed with PBS, placed onto gelatin-coated slides, and coverslipped with Permount. Lastly, the sections were analyzed by using a Leica DMLB light microscope (Vienna, Austria) and photographed by using a ScanScope light microscope (Aperio Technologies, Vista, CA).

**Preparation for electron microscopic (EM) observations**

**Single pre-embedding immunoperoxidase labeling for electron microscopy**—

Sections were treated with a 1% sodium borohydride solution, placed in a cryoprotectant solution (PB 0.05 M, pH 7.4, 25% sucrose, and 10% glycerol), frozen at –80°C for 20 minutes each, before being returned to PBS-based solutions with decreasing gradients of cryoprotectant, and lastly, washed thoroughly in PBS. The tissue was then preincubated for 1 hour at RT in a solution containing PBS, 1% NGS, and 1% BSA, followed by a 48-hour incubation at 4°C in the primary antibody solution containing goat anti-ChAT with either rat anti-SP, or rabbit anti-Met/Leu-Enk antibodies in 1% NGS and 1% BSA in PBS. Sections were rinsed in PBS three times, incubated in a PBS solution containing 1% NGS, and 1%
BSA combined with secondary biotinylated horse anti-goat, goat anti-rat, or goat anti-rabbit IgGs (1:200; Vector), for 90 minutes at RT, rinsed in PBS, and then exposed to ABC and DAB as described above. After DAB exposure, the tissue was rinsed in PB (0.1 M, pH 7.4) and treated for 20 minutes with 1% OsO$_4$, returned to PB, and then dehydrated with decreasing concentrations of ethanol. To increase the tissue contrast at the electron microscope level, 1% uranyl acetate was added to the 70% ethanol solution for 35 minutes in the dark. After alcohol dehydration, sections were placed in propylene oxide and embedded in epoxy resin (Durcupan, Fluka, Buchs, Switzerland) for at least 12 hours, mounted onto slides, and placed in a 60 °C oven for 48 hours (Smith and Bolam, 1992). Tissue samples from the postcommissural putamen were cut out of large, resin-embedded sections and fixed onto resin blocks, before being cut into 60-nm ultrathin sections (Leica Ultracut T2). These sections were mounted onto Pioloform-coated copper grids, stained with a lead citrate for 5 minutes, and then examined with an electron microscope (EM; model 1011, Jeol, Peabody, MA). Digital micrographs of immunoreactive elements were collected with a Gatan CCD camera (Model 785; Warrendale, PA) controlled by Digital Micrograph software (version 3.11.1).

Double post-embedding immunogold for GABA and pre-embedding immunoperoxidase for ChAT—The methods for post-embedding GABA immunostaining used in this study are similar to those described by Somogyi and Hodgson (1985) as modified by Phend et al. (1992). A series of adjacent ultrathin sections of striatal tissue immunostained with the goat anti-ChAT antibody (1:100 dilution) with the pre-embedding immunoperoxidase method mentioned above were cut with an ultramicrotome and placed onto gold grids. Once the grids were dry, they were preincubated in a solution containing Tris-buffered saline (TBS; 0.5 M, pH 7.6) and 0.01% Triton X-100 (TBS-T) and then, incubated overnight at RT with primary rabbit anti-GABA IgGs (1:1,000; Sigma) in TBS-T. On the next day, the grids were rinsed in a series of TBS-T washes, followed by a rinse in TBS (0.05 M, pH 8.2, 10 minutes,) and incubated with 15 nm gold-conjugated goat anti-rabbit IgG (1:50 in TBS 0.05 M, pH 8.2; British Biocell, Cardiff, UK, 90 minutes). The sections were then rinsed in TBS (0.05 M, pH 8.2) for 20 minutes, washed in distilled water for 5 minutes, and stained with 1% uranyl acetate (in distilled water, 90 minutes). Lastly, the grids were washed in distilled water and stained with lead citrate for 5 minutes before being examined with the electron microscope.

Double pre-embedding immunoperoxidase for ChAT and pre-embedding immunogold for SP or Met/Leu-Enk—Following sodium borohydride treatment, sections were processed with the cryoprotectant protocol described above followed by rinses in PBS and preincubation for 30 minutes in a PBS solution containing 5% dry milk. Sections were then rinsed in a TBS-gelatin buffer (0.02 M, 0.1% gelatin, pH 7.6), and incubated in an antibody solution (either rat anti-SP with rabbit anti-ChAT or rabbit anti-Met-/Leu-Enk with goat anti-ChAT) with 1% dry milk in TBS-gelatin buffer for 24 hours at RT. Additional control sections for each experiment were incubated in solutions from which either of the two primary antibodies was omitted in order to assess specificity of the immunogold and immunoperoxidase labeling. One day later, sections were rinsed in TBS-gelatin and then treated for 2 hours at RT with the following secondary antibody solutions prepared with TBS-gelatin in 1% milk. To visualize SP and ChAT, we used secondary goat anti-rat Fab’ fragments conjugated to 1.4-nm gold particles (1:100; Nanoprobes, Yaphank, NY) and secondary biotinylated goat anti-rabbit IgG (1:200; Vector), respectively. To visualize Met-/Leu-Enk and ChAT, we used secondary goat anti-rabbit Fab’ fragments conjugated to 1.4-nm gold particles (1:100; Nanoprobes) and secondary biotinylated horse anti-goat IgG (1:200; Vector). Sections were washed in TBS-gelatin and 2% sodium acetate buffer before incubation with the HQ Silver Kit (Nanoprobes) for 4–10 minutes to increase gold particle.
sizes to 30–50 nm through silver intensification. Following this reaction and TBS-gelatin washes, ABC and DAB procedures were carried out as described above to localize ChAT.

Sections were rinsed in PB (0.1 M, pH 7.4) and treated with the same protocols for osmification, dehydration, embedding, and tissue selection described above, with the exception that sections were kept in 0.5% OsO₄ for 10 minutes, and the tissue was stained with 1% uranyl acetate for 10 minutes in the dark.

Analysis of material

GABA inputs onto ChAT-positive interneurons—To determine the proportion of GABA-positive terminals onto ChIs, ultrathin sections of double immunostained tissue (ChAT/GABA) from the postcommissural putamen of four animals were randomly examined to localize ChAT-immunoreactive (ir) dendrites and somata. A series of large-, medium-, and small-sized ChAT-positive dendrites were selected for this analysis (classification scheme described below), irrespective of their type and extent of synaptic innervation. We calculated an index of the density of gold particles in every terminal bouton that formed clearly identifiable symmetric or asymmetric synapses (Peters et al., 1991; Peters and Palay, 1996) with ChAT-positive elements, by dividing the number of gold particles for each individual terminal by the cross-sectional area of that terminal (Rashband, 1997–2012). A bouton was categorized as “symmetric/GABA-positive” if it formed a symmetric synapse, and if the gold particle density in the bouton was three times greater than the average gold particle density associated with terminals forming asymmetric synapses. Terminal boutons forming symmetric synapses that contained gold particle densities below this cutoff were categorized as “symmetric/unknown.” Because of interindividual variability in the absolute number of gold particles associated with GABA-positive and GABA-negative terminals across animals and postembedding reactions, these measurements were collected for each run of postembedding reaction in the striatal tissue from each monkey (Fig. 3E). Once the gold particle densities were measured for each individual animal, the mean percentage of the different subtypes of terminals in contact with different parts of ChAT-positive neurons was determined in that animal and compared across groups by using a one-way analysis of variance (ANOVA) with a post hoc Tukey’s test (significance level set at \( P < 0.05 \); Fig. 4B).

SP or Met-/Leu-Enk-positive terminals onto ChAT-positive interneurons—

Sections from the postcommissural putamen double-labeled with antibodies for rat anti-SP (gold-labeled) and goat anti-ChAT (peroxidase-labeled; five animals) or rabbit anti-Met-/Leu-Enk (gold-labeled) and goat anti-ChAT (peroxidase-labeled; four animals) were used to quantify the proportion of SP- or Enk-positive terminals in contact with ChAT-ir neurons. In order to avoid subjective bias, the tissue was randomly scanned for gold-labeled SP or Met-/Leu-Enk terminals and their postsynaptic target (i.e., ChAT-labeled or unlabeled) was determined irrespective of their possible association with ChAT-positive or ChAT-negative elements. The tissue was randomly scanned to determine the proportion of gold-labeled SP or Met-/Leu-Enk terminals that formed synapses with ChAT-ir neurons. The postsynaptic target of approximately 100 immunostained boutons per animal was categorized as ChAT-positive (or not) for each double-label experiment (Fig. 6F,G). The average proportion of SP- or Met/Leu-Enk-positive terminals forming synapses onto ChAT-labeled elements was compared by using Student’s \( t \)-test (significance level set at \( P < 0.05 \); Fig. 6F) and between the postsynaptic targets within each group by using Student’s \( t \)-test (significance level set at \( P < 0.05 \); Fig. 6G).

In order to ascertain whether the overall pattern of ChAT labeling was similar between series of double immunoreactions, we determined the relative density of ChAT-ir elements...
in each block of tissue used from double immunostained sections. Ultrathin sections were also taken from tissue that was labeled for ChAT alone in one monkey to compare the overall pattern of ChAT immunoreactivity in this tissue with that found in the double-labeled material. To determine the density of ChAT-labeled elements in each of these tissue blocks, we took 25 digital micrographs of random tissue locations from the surface of the blocks at 25,000×. ChAT-ir elements were categorized as cell bodies, dendrites, or terminals, based on ultrastructural features described in Peters et al. (1991). The cross-sectional diameter of each dendrite was measured by using Image J (Rasband, 1997–2012), and dendritic profiles were categorized as large-sized (diameter > 0.99 μm, presumably representing proximal dendrites), medium-sized (diameter 0.5-0.99 μm), and small-sized (diameter < 0.5 μm, presumably representing distal dendrites) (DiFiglia and Carey, 1986; DiFiglia, 1987; Peters et al., 1991; Yelnik et al., 1991). The double-labeled sections were used for further quantification only if the proportion of ChAT-labeled proximal and distal dendrites were comparable to that in singly labeled sections (see Fig. 6E).

**Total synaptic innervation of ChAT-positive neurons from SP- or ENK-positive terminals**

In other measurements on the same double-immunostained striatal tissue in three monkeys, we determined the proportion of synaptic inputs onto ChIs accounted for by SP- or Enk-positive terminals. To do so, we characterized the synaptic innervation of approximately 100 ChAT-containing dendrites in each animal. The percentage of total boutons in contact with these dendrites was categorized as follows: 1) symmetric/SP-positive or symmetric/Enk-positive; 2) symmetric/SP-negative or symmetric/Enk-negative; and 3) asymmetric/SP-negative or asymmetric/Enk-negative. The results were then statistically analyzed with a Student's *t*-test (significance set at *P* < 0.05) (Fig. 7B,C).

In order to ensure that the quantitative analysis of the double-labeled material was not affected by poor tissue penetration of antibodies or gold particles, we compared the density of SP-and Enk-ir boutons in the single-labeled (immunoperoxidase) and double-labeled (immunogold) tissue, with the expectation that they would be in the same range. For this analysis, single- or double-labeled ultrathin sections from the surface of blocks with suitable ultrastructural preservation were randomly scanned for areas of tissue that had terminal immunostaining for SP or Met-/Leu-Enk, and electron micrographs were taken of every field visible in that area at 25,000×. To calculate the bouton density index, the number of labeled terminals in these micrographs was counted and divided by the total surface area represented by the examined micrographs. If the density of labeled boutons in the double-labeled tissue was 10% lower than that calculated in single-labeled sections, the tissue was not considered for further analysis.

**Results**

**GABAergic inputs onto cholinergic interneurons**

At the light microscopic level, the neuropil in the caudate nucleus and putamen was moderately immunoreactive, consisting of a meshwork of fine ChAT-labeled processes and large immunoreactive cell bodies with prominent primary dendritic shafts from which emerged thinner dendritic processes that often extended over long distances from their parent cell bodies (Fig. 1). These morphological characteristics are consistent with those described for ChAT-positive neurons in previous primate studies (DiFiglia, 1987; Bernacer et al., 2007).

The GABAergic innervation of cholinergic cells was characterized from postcommissural putamen tissue that was double immunostained for ChAT (pre-embedding immunoperoxidase) and GABA (postembedding immunogold). At the electron microscopic...
level, ChAT-immunostained cell bodies, dendrites, and axon terminals displayed the electron-dense amorphous DAB reaction product (Fig. 2), whereas GABA-ir terminals were recognized by their large density of gold particles (Fig. 2A,B). To assess the specificity of the immunogold reaction for GABA, we analyzed the relative density of gold particles associated with terminals forming symmetric (putatively GABAergic) or asymmetric (putatively glutamatergic) synapses in the double-immunostained striatal tissue (Fig. 3). We performed this analysis separately for each of the four animals used in this part of the study (animals 1–4 in Fig. 3), because the absolute number of gold particles over labeled and unlabeled elements across animals may vary when localized with the postembedding immunogold method. In each animal, the average density of gold particles associated with terminals forming symmetric synapses outnumbered that in terminals forming asymmetric synapses (Figs. 2, 3) by a factor of 4:1 (Fig. 3E). As described in Materials and Methods, we used these data to categorize the neurochemical phenotype of terminals forming synapses onto ChAT-positive elements. As shown in Figure 4A, the analysis of a total of 709 afferent terminals in synaptic contact with ChAT-ir profiles (soma = 26; large-sized dendrites = 133; medium-sized dendrites = 328; small-sized dendrites = 222) revealed that 60 ± 4% were GABAergic, 19 ± 4% were categorized as symmetric/unknown, and 21 ± 4% were asymmetric/non-GABAergic (putatively glutamatergic).

When the postsynaptic ChAT-ir elements were categorized as soma or dendrites of various sizes based on their cross-sectional diameter, the medium-sized (0.5–0.99 μm in diameter) and large-sized (diameter > 0.99 μm) dendrites received a significantly greater proportion of synaptic inputs from symmetric GABAergic boutons (N terminals = 418; 29 ± 1% and 13 ± 2% of total synapses onto ChAT-positive elements, respectively) than from the symmetric unknown (N terminals = 137; 10 ± 2% and 4 ± 1%, respectively) and asymmetric non-GABAergic boutons (N terminals = 155; 7 ± 2% and 1 ± 0.3%, respectively) (P < 0.001, one-way ANOVA with a post hoc Tukey’s test; Fig. 4B). A significant difference was also found between the percentage of symmetric GABAergic inputs versus symmetric unknown inputs onto small (<0.5 μm in diameter) ChAT-positive dendrites (P < 0.001, one-way ANOVA with a post hoc Tukey’s test; Fig. 4B).

**“Direct” and “indirect” striatofugal inputs onto cholinergic interneurons**—Experiments were carried out to determine the proportion of GABAergic inputs onto ChAT-labeled neurons that originate from axon collaterals of striatal projection neurons. To do so, we took advantage of the segregation of SP and Enk into two populations of striatofugal neurons to label GABAergic terminals that originate from direct or indirect pathway projection neurons, respectively (Brann and Emson, 1980; Cuello et al., 1981; Del Fiacco et al., 1982; Beach and McGeer, 1984; Reiner et al., 1999). In agreement with previous reports (Haber and Elde, 1981; DiFiglia et al., 1982; Williams and Dockray, 1983; Beach and McGeer, 1984; Reiner et al., 1999), the striatum and GPi showed strong positive immunostaining for SP (Fig. 5A), whereas the antibodies for Met-/Leu-Enk positively labeled the striatum and GPi (Fig. 5B,C).

In double-label experiments, the pre-embedding immunogold method was used to localize SP or Enk immunoreactivity, whereas ChAT-positive neurons were labeled with the immunoperoxidase method (Fig. 6A–D). Consistent with previous reports on the ultrastructural localization of SP and Enk immunoreactivity in the primate striatum (DiFiglia et al., 1982; Hutcherson and Roberts, 2005), most immunoreactivity was found in terminals forming symmetric synapses. Less labeling was observed in dendrites, cell bodies, and dendritic spines (Fig. 6A–D). Gold-containing boutons were only considered as being positively labeled if they contained two or more gold particles. The gold particles were often found in large densecore vesicles, a known vesicular storage site of neuropeptides in central nervous system (CNS) neurons (Merighi et al., 2011) (Fig. 6A–D). The pattern of ChAT
immunostaining in the double-labeled tissue was the same as described above for the ChAT/GABA-immunostained material (Fig. 6E). Very few neuronal elements exhibited dual labeling for gold and peroxidase in the double-immunostained material, indicating the lack of SP/ChAT or Enk/ChAT colocalization (Fig. 6A–D).

**SP-positive inputs onto ChAT-immunoreactive neurons**

In the first series of double-label experiments carried out using postcommissural putamen tissue from five monkeys, the proportion of SP-positive terminals in contact with ChAT-labeled neurons was determined. Ultrathin sections taken from the surface of blocks of striatal tissue that contained elements labeled with either gold (i.e., SP-positive) or peroxidase (i.e., ChAT-positive) deposits were examined to determine whether the postsynaptic targets of SP-ir elements contained ChAT immunoreactivity. The analysis of 527 SP-ir terminals revealed that 36 ± 4% of these boutons formed symmetric synapses with ChAT-ir dendrites (Fig. 6A,B), which were preferentially of the medium (52 ± 6%) or small (29 ± 3%) size diameter (Fig. 6G). We also determined the proportion of the total synaptic innervation of ChAT-positive neurons that was accounted for by SP-ir terminals in three of the five monkeys used in the previous analysis. In areas that contained both gold-and peroxidase-labeled structures, the tissue was randomly scanned under the electron microscope for ChAT-ir dendrites. The analysis of 219 ChAT-ir dendrites (large-sized = 21; medium-sized = 140; small-sized = 58) revealed that 16 ± 1% of their total synaptic innervation was from SP-ir terminals forming symmetric synapses, whereas 68 ± 3% originated from SP-negative boutons forming symmetric synapses (Fig. 7B), and 16 ± 2% came from putatively glutamatergic terminals forming asymmetric synapses (Fig 7B). The proportion of SP-negative terminals forming symmetric synapses (i.e., Symm/SP(−)) with medium-sized ChAT-labeled dendrites was significantly larger than the two other populations of terminals in contact with these ChAT-positive elements (Fig. 7B). To ensure that the overall density of gold-containing SP-ir terminals in the putamen of monkeys used in these double-label reactions (n = 3) was not underestimated because of the double-labeling procedure, we compared it with the density of SP-ir boutons found in single SP-labeled sections processed with the immunoperoxidase reaction (n = 1; Fig. 7A). We found that these values were in the same range, thereby showing that our results were not affected by a limited penetration of the gold-conjugated secondary antibodies used to label SP in this tissue.

**Enk-positive terminals onto ChAT-positive neurons**

In the other series of double-label experiments, we studied the synaptic relationships between Enk-positive terminals and ChAT-ir elements in the postcommissural putamen of four of the five animals that were used for the SP/ChAT analysis described in the previous section. The analysis of 454 Enk-positive terminals revealed that 47 ± 2% of these terminals formed symmetric synapses with ChAT-positive dendrites and soma (Fig. 6F), a proportion significantly greater (P < 0.05, Student’s t-test) than that found for SP-positive terminals in contact with ChAT-positive cells (Fig. 6F). This difference is accounted for by a significantly larger proportion of Enk-ir terminals in contact with large ChAT-positive dendrites (Fig. 6G; P = 0.012; Student’s t-test).

In the same material, the analysis of the synaptic innervation of 208 ChAT-positive dendrites (large-sized = 45; medium-sized =118; small-sized = 45) revealed that 11 ± 1% of all boutons forming synapses with ChAT-positive dendrites were immunoreactive for Enk, whereas 79 ± 3% and 10 ± 1% of the total synaptic innervation of ChAT-containing dendrites was accounted for by Enk-negative symmetric or asymmetric synapses, respectively (Fig. 7C). Large- and medium-sized ChAT-positive dendrites received a
significantly larger synaptic innervation from Enk-negative boutons forming symmetric synapses (i.e., Symm/Enk(−)) than from symmetric Enk-positive and asymmetric Enk-negative terminals ($P < 0.001$, one-way ANOVA with a post hoc Tukey’s test; Fig. 7C). In contrast, small-sized ChAT-ir dendrites also received a significantly greater synaptic innervation from the symmetric Enk-negative terminals than the symmetric Enk-positive terminals ($P < 0.001$, oneway ANOVA with a post hoc Tukey's test; Fig. 7C), but it did not reach significance when compared with putatively glutamatergic boutons (i.e., Asymm/Enk(−)). As was the case for the SP/ChAT double immunostaining, the average relative density of gold-containing Enk-positive terminals seen in our double-immunostained tissue was the same as that assessed in striatal sections single-stained for Enk by using the immunoperoxidase method (Fig. 7A).

Altogether, findings from the double-immunostaining striatal sections for SP or Enk with ChAT revealed that 24 ± 1% of the total synaptic innervation of ChIs in the monkey postcommissural putamen originate from axon collaterals of GABAergic direct and indirect striatal projection neurons, whereas 63 ± 2% arise from other subpopulations of terminals that form symmetric synapses, and 13 ± 2% from putatively glutamatergic boutons (Figs. 7D, 8).

Discussion

Our findings demonstrate that ChIs are a major target of GABAergic inputs, and that a significant proportion of this innervation originates from axon collaterals of direct and indirect medium spiny projection neurons in the monkey postcommissural putamen. Through these anatomical connections, the intrinsic axon collaterals of GABAergic striatal projection neurons may exert a powerful control of ChI activity in primates.

Synaptic GABAergic regulation of striatal cholinergic interneurons

It is believed that the feedforward and feedback connections between intrinsic striatal GABAergic neurons and ChIs (DiFiglia, 1987; Sullivan et al., 2008; Tepper et al., 2008; Ding et al., 2010; Bonsi et al., 2011; English et al., 2012) may play a role in behaviors such as attention and reward-based learning (Kimura et al., 1984; Matsumoto et al., 2001; Ravel et al., 2003; Morris et al., 2004; Joshua et al., 2008; Aosaki et al., 2010; Apicella et al., 2011; Bonsi et al., 2011). Many in vitro studies have characterized the synaptic influences of intrastriatal GABAergic connections on the excitability and/or spontaneous activity of cholinergic cells in rodents. For example, activation of GABA$_A$ receptors, located postsynaptically on ChIs (DeBoer and Westerink, 1994; Yan and Surmeier, 1997; Fujiyama et al., 2000; Waldvogel et al., 2004), readily evoked inhibitory postsynaptic potentials (IPSPs) in tonically active neurons (TANs) (Yan and Surmeier, 1997; Bennett and Wilson, 1998; Pisani et al., 2000), and decreased striatal acetylcholine release (Anderson et al., 1993; DeBoer and Westerink, 1994) in rodents.

Previous electron microscopic studies in rodents and monkeys have shown that most synaptic inputs to the somatodendritic domain of ChIs are symmetric (Chang and Kitai, 1982; Bolam et al., 1984; Phelps et al., 1985; DiFiglia, 1987; Sizemore et al., 2010). Although it is frequently assumed that such symmetric synapses are GABAergic (Gerfen, 1988; Pasik et al., 1988; Ribak and Roberts, 1990; Roberts et al., 1996), the chemical composition of terminals that form symmetric synapses in the striatum is heterogeneous, including, for instance, GABAergic, dopaminergic and cholinergic terminals (Haber, 1986; Semba et al., 1987; Gerfen, 1988; Graybiel, 1990; Smith Y et al., 1994; Bolam et al., 2000; Tepper et al., 2010; Crittenden and Graybiel, 2011).
Our data collected from material immunostained for GABA and ChAT provide direct ultrastructural evidence that most synaptic inputs onto ChIs in the monkey post-commissural putamen are, in fact, GABAergic. This pattern of synaptic connectivity is strikingly different from that of projection neurons, which receive most of their dendritic innervation from glutamatergic and dopaminergic inputs, except for the proximal somatodendritic domain that is innervated by GABAergic afferents from interneurons (Sadikot et al., 1992a,b; Bolam et al., 2000; Kubota and Kawaguchi, 2000; Wilson, 2007). The GABAergic inputs onto ChIs may originate from a variety of sources, including GABAergic collaterals of striatal projection neurons (Oertel et al., 1983; Aronin et al., 1984; Bradley et al., 1984; Christensson-Nylander et al., 1986; Penny et al., 1986; Surmeier et al., 1988; Graybiel, 1990; Bolam et al., 2000; Wilson, 2007), GABAergic parvalbumin- and calretinin-containing interneurons (Augood et al., 1995; Kawaguchi et al., 1997; Bolam et al., 2000; Kubota and Kawaguchi, 2000; Tepper et al., 2010), or extrinsic GABAergic inputs from the GPe (Beckstead, 1983; Oertel et al., 1984; Smith et al., 1987; Kita and Kitai, 1991; Bevan et al., 1998; Kita et al., 1999; Sato et al., 2000; Mallet et al., 2012). The prevalence of these respective inputs onto ChIs cells remains to be established. As discussed below, MSN axon collaterals are a significant source of this innervation.

It is noteworthy that a significant proportion of terminals involved in symmetric synaptic connections with ChIs did not, however, display a level of GABA immunoreactivity high enough to be categorized as GABAergic. The most likely source of these terminals may be the striatal neuropeptide-Y/somatostatin-containing interneurons, known to express a low level of GABA (Aoki and Pickel, 1989; Chesselet and Robbins, 1989; Vuillet et al., 1992; Kubota et al., 1993; Catania et al., 1995; Kubota and Kawaguchi, 2000). However, low levels of nonspecific gold labeling in dopaminergic SNc inputs (Kubota et al., 1987; Pickel and Chan, 1990; Dimova et al., 1993) or cholinergic terminals from striatal ChIs themselves (Calabresi et al., 1998; Sullivan et al., 2008) are other potential sources of these boutons.

Finally, we found that the distal dendrites of ChIs receive a significant innervation from putative glutamatergic terminals that form asymmetric synapses. Some of these terminals may originate from the cerebral cortex (Dub et al., 1988; Lapper and Bolam, 1992; Sadikot et al., 1992a; Dimova et al., 1993; Bennett and Wilson, 1998; Sidibe and Smith, 1999; Thomas et al., 2000), although most of them are likely to arise from projections of the centromedian/parafascicular thalamic nuclei (Meredith and Wouterlood, 1990; Lapper and Bolam, 1992; Bennett and Wilson, 1998; Sidibe and Smith, 1999).

Although the functional effects of GABAergic and putatively glutamatergic afferents on the activity of ChIs do not merely rely on their preferential distribution along the proximal and distal dendrites of these neurons, respectively, it is important to note that synaptic inputs onto primary dendrites are often seen as more powerful than distal synaptic afferents (Wilson, 2007). Thus, the pattern of synaptic organization of GABAergic and putatively glutamatergic inputs onto ChIs described in our study suggests that primate ChIs may be more strongly regulated by GABAergic than by glutamatergic inputs (Bennett and Wilson, 1998; Wilson, 2007; Tepper et al., 2008). However, rodent and monkey in vitro and in vivo electrophysiological data suggest that GABAergic and glutamatergic inputs to ChIs do not compete with one another but tend to be active in sequence. For instance, activation of glutamatergic inputs from the cerebral cortex or the thalamus often result in an early, presumably monosynaptic, excitation of ChIs, followed by a robust inhibition, which may be mediated by GABAergic intrastriatal axon collaterals or interneurons (Matsumoto et al., 2001; Suzuki et al., 2001; Reynolds and Wickens, 2004; Nanda et al., 2009; Ding et al., 2010). It is noteworthy that additional phenomena not studied here, such as spike backpropagation, dendritic calcium entry, or the type and density of postsynaptic receptors, may also contribute significantly to the synaptic strength of GABAergic and glutamatergic
synapses onto ChIs, as they do in striatal projection neurons (Banks et al., 1998; Kerr and Plenz, 2002, 2004; Plenz, 2003; Carter and Sabatini, 2004; Tepper and Bolam, 2004; Wilson, 2007; Day et al., 2008; English et al., 2012).

**Axon collaterals of striatal projection neurons are a main source of GABAergic inputs to striatal cholinergic interneurons**

We found that ChIs receive massive GABAergic inputs, but the exact source(s) of these afferents could not be determined solely based on GABA immunoreactivity. DiFiglia and Carey (1986) recognized up to eight morphologically different types of terminals forming symmetric synapses with ChIs, implying that these neurons receive GABAergic innervation from multiple sources. The phenotype of these neuronal sources can be identified by staining for neuropeptides and neurochemicals that coexist with GABA in these cells (Oertel et al., 1983; Aronin et al., 1984; Bradley et al., 1984; Christensson-Nylander et al., 1986; Penny et al., 1986; Surmeier et al., 1988), while not being significantly expressed in other striatal neurons or extrinsic afferents (Taniyama et al., 1980; Fonnum et al., 1981; Young et al., 1981; Kosaka et al., 1988; Kawaguchi et al., 1997; Kubota and Kawaguchi, 2000). In this study, we used the neuropeptides SP and Enk as markers of intrinsic axon collaterals of the two populations of striatal projection neurons (Cuello, 1978; Cuello et al., 1979; Brann and Emson, 1980; Del Fiacco et al., 1982; DiFiglia et al., 1982; Beach and McGeer, 1984; Smith et al., 1998; Reiner et al., 1999). Previous rodent studies have provided qualitative evidence that SP-expressing terminals form symmetric synapses with the somatodendritic domain of ChAT-labeled neurons (Bolam et al., 1986; Martone et al., 1992; Kuramoto et al., 2007). However, attempts at providing evidence for Enk- or parvalbumin-containing inputs onto ChIs in rats resulted in negative data (Chang and Kita, 1992; Martone et al., 1992), suggesting specific interactions between ChIs and the direct pathway MSNs.

In striking contrast with these studies, we found that 35.6% of SP-positive terminals and 47% Enk-labeled boutons form symmetric synapses with ChI dendrites in monkeys, indicating that ChIs are a major target of intrinsic axon collaterals of both direct and indirect pathway GABAergic MSNs in the primate postcommissural putamen. This discrepancy may be explained, in part, by the fact that the proportion of interneurons over the total striatal neuronal population is much larger in primates than in rodents (24% vs. approximately 5%; see Pasik et al., 1976; Graveland and DiFiglia, 1985; Roberts et al., 1996; Wu and Parent, 2000), and that striatal ChIs in primates have a denser and larger dendritic tree than rodents (DiFiglia et al., 1980; Bolam et al., 1984; Phelps et al., 1985; DiFiglia and Carey, 1986; Yelnik et al., 1991, 1993; Bernacer et al., 2007). Technical differences cannot be ruled out, especially the increased sensitivity of the ultrastructural double immunocytochemical technique used in our study. In regard to the SP innervation, although there appears to be a significant synaptic interaction between SP-positive terminals and ChIs in rodents, the supporting evidence for such connections is qualitative, making these findings difficult to compare with our quantitative data (Bolam et al., 1986; Kuramoto et al., 2007).

It is important to realize that our data lay the foundation for a potentially important, but unexplored, function of the local MSN axon collaterals. Although it has long been believed that these collaterals primarily reach other MSNs, it is now clear that these inter-MSN connections are sparse and confined to their distal dendrites, so that their functional impact is likely to be weak (Jaeger et al., 1994; Stern et al., 1998; Czubayko and Plenz, 2002; Tunstall et al., 2002; Tecuapetla et al., 2005; Wilson, 2007; Blomeley and Bracci, 2008, 2011; Taverna et al., 2008; Tepper et al., 2008; Blomeley et al., 2009). Our findings suggest that one of the main targets of MSN collaterals in primates could instead be ChIs, and that such interactions could permit MSNs to regulate the activity of ChIs.
Peptidergic modulation of ChIs by striatal projection neurons?

In addition to their GABAergic influences, MSN collaterals could also modulate the activity of ChIs through the release of their coexpressed neuropeptides, SP and Enk. It has, indeed, been shown that these neuropeptides participate in the synaptic modulation of striatal neuronal activity (Aosaki and Kawaguchi, 1996; Miura et al., 2007, 2008; Blomeley et al., 2009; Govindaiah et al., 2010; Merighi et al., 2011). Thus, in vitro and in vivo studies have demonstrated that SP and Enk are released in the striatum (Lindefors et al., 1985, 1989; Llorens-Cortes et al., 1990; Ruzicka et al., 1991; Bell et al., 1998; Jabourian et al., 2004). In addition, rat and primate ChIs display strong mRNA and protein expression for tachykinin NK1 receptors (Gerfen et al., 1991; Kaneko et al., 1993; Aubry et al., 1994; Parent et al., 1995; Richardson et al., 2000; Perez et al., 2007) along with mu- and delta-opioid receptors (Pasquini et al., 1992; Lendvai et al., 1993; Le Moine et al., 1994; Jabourian et al., 2005; Perez et al., 2007), which, upon activation, lead to an increase or decrease in the striatal acetylcholine (ACh) release, respectively (Mulder et al., 1984; De Vries et al., 1989; Lapchak et al., 1989; Izquierdo, 1990; Arenas et al., 1991; Lendvai et al., 1993; Perez et al., 2007). In vitro studies from rat brain slices demonstrated that the release of striatal SP from MSN axon collaterals, or bath-applied SP, leads to the depolarization of TANs, but not fast-spiking interneurons or other MSNs (Aosaki and Kawaguchi, 1996; Kawaguchi et al., 1997; Bell et al., 1998; Govindaiah et al., 2010), whereas Enk activation of opioid receptors results in the hyperpolarization of ChIs (Jiang and North, 1992; Miura et al., 2007). Therefore, the prominent synaptic interactions between SP-positive and Enk-positive terminals and cholinergic neurons shown in our study also open up the possibility for peptidergic modulation of primate ChIs.

Conclusions

The strong association between MSNs and ChIs shown in this study provides further evidence that the reciprocal functional interactions between these two neuronal populations are important elements of striatal processing. Cholinergic modulation of MSNs has long been established (Phelps et al., 1985; DiFiglia, 1987; Izzo and Bolam, 1988; Graybiel, 1990; Pisani et al., 2007; Tepper et al., 2008), but the exact role(s) of MSN-ChI connections remains to be determined. It is possible, however, that an additional role of striatal ChIs, besides feedback modulation of directly coupled MSNs, is to provide feed-forward information from one group of MSNs to another through MSN-ChI-MSN chains. In primates, such links may exist between direct and indirect striatofugal neurons situated in functionally specific striatal territories or between MSNs located in striatal patch and matrix compartments, supported by the fact that cholinergic cell bodies are often located along the borders of striatal patches, whereas their dendrites extend beyond the boundaries of each striatal compartment (Graybiel et al., 1986; Kubota and Kawaguchi, 1993; Aosaki et al., 1995; Kawaguchi et al., 1995; Bernacer et al., 2007).

Interactions between MSNs and ChIs may also be relevant in disease states, specifically in those known to respond to cholinergic medications, such as Parkinson’s disease or dystonia (Pisani et al., 2007; Bonsi et al., 2011; Goldberg et al., 2012). Although the functional significance of MSN-ChI neuron connections under normal or diseased states remains poorly understood, it is noteworthy to mention that the metabolic and physiological activity of both populations of MSNs, as well as MSN-MSN synaptic interconnections, are significantly altered in the dopamine-depleted state (Ingham et al., 1991; Taverna et al., 2008; Surmeier, 2010; Gerfen and Surmeier, 2011). The overall firing frequency of striatal ChIs does not change in parkinsonism (Raz et al., 1996, 2001; Aosaki et al., 2010). However, significant abnormal cholinergic function in the dopamine-depleted striatum has been noted (Pisani et al., 2007; Bonsi et al., 2011; Goldberg et al., 2012), but may not be solely dependent upon the removal of dopamine from the system (MacKenzie et al., 1989;...
Joyce, 1991, 1993; Ding et al., 2006). Thus, in light of our data showing that inputs from MSN collaterals are located to subserve control over ChIs, a detailed characterization of the electrophysiological properties of MSN-ChI synaptic connections in normal and parkinsonian states is warranted.

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J Comp Neurol. Author manuscript; available in PMC 2014 August 01.


Figure 1.
General pattern of distribution and morphology of cholinergic interneurons in the monkey putamen at the light microscopic level. A: Low-power view of ChAT-positive neurons and neuropil in the postcommissural putamen. B,C: High-power views of typical ChIs from the monkey sensorimotor putamen. Note that the ChAT-labeled somata have thick primary dendrites from which emerge multiple dendritic branches that extend over long distances in the surrounding neuropil. Scale bar = 100 μm in A; 25 μm in B (applies to B,C).
Figure 2.
Electron micrographs of tissue double immunostained for GABA (immunogold) and ChAT (immunoperoxidase) in the monkey putamen. A, B: Photomicrographs demonstrating GABA-positive (G+) terminals forming symmetric synapses (arrows) with medium-sized ChAT-labeled dendrites. Note that a GABA-negative (G−) terminal in each micrograph is forming an asymmetric synapse (black arrowheads) with a spine. C: Example of a terminal categorized as “symmetric/unknown” (Unk.) that forms a symmetric synapse (black arrows) with a large-sized ChAT-positive dendrite. A GABA-negative (G−) terminal forming an axo-spinous synapse (arrowhead) is also visible. D: Example of a GABA-negative terminal forming an asymmetric synapse onto a small-sized ChAT-labeled dendrite and an unlabeled spine (arrowheads). Other GABA-negative (G−) terminals are also visible in this tissue. Scale bar = 0.2 μm in A (applies to A,B); 0.5 μm in D (applies to C,D).
Figure 3.
Frequency distribution histograms of the relative gold particle density in terminals forming synapses with ChAT-labeled elements in the four animals used for the GABA (immunogold) and ChAT (immunoperoxidase) reactions in the monkey putamen. A–D: Histograms showing the overall distribution of gold particle densities over terminals forming symmetric (Symm) or asymmetric (Asymm) synapses with Chls in the postcommissural putamen of each of the animals. The average surface density of gold particles (± SEM) measured in each category of terminals is provided in the legend at the upper right corner of each panel and is indicated by arrows in the histograms pointing to the non-GABA and...
GABA means for each animal. The symmetric and asymmetric terminals analyzed in each monkey were collected from blocks of striatal tissue processed from the same double-label immunoreaction. E: Comparison of the average ratio of gold particle densities over terminals forming Symm versus Asymm synapses with cholinergic elements in each monkey. A gold particle density ratio of 3 (dashed line in E) between Symm and Asymm terminals served as an (arbitrary) cutoff to separate confirmed GABAergic from “unknown” symmetric terminals.
Figure 4.
Proportion of GABAergic and non-GABAergic terminals in contact with ChAT-labeled neurons in the putamen of four monkeys. A: Mean percentage (± SEM) of terminals categorized as “symmetric/GABAergic,” “symmetric/unknown,” or “asymmetric/non-GABAergic.” These categories were based on the relative density of gold particle labeling in terminals forming synaptic contact with ChAT-containing neurons. The gold particle density criteria for each terminal category are described in the legend of Figure 3 and in the text. B: Comparison of the distribution of the different categories of terminals along the entire somatodendritic domain of striatal cholinergic interneurons. The legend key used in A also applies here. *, P < 0.001 Symm, GABAergic versus Symm, Unknown and Asymm, non-GABAergic; #, P < 0.001 Symm/GABAergic versus Symm, Unknown.
Figure 5.
Light microscopic distribution of the immunoreactivity for SP (A), Met-Enk (B), and Leu-Enk (C) in the monkey striatopallidal complex. SP labeling is intense in the caudate nucleus (CD), putamen (Put), and internal globus pallidus (GPi), whereas strong Met- and Leu-Enk immunostaining is found in the CD, Put, and external globus pallidus (GPe). Scale bar = 2 mm in A (applies to A–C).
Figure 6.
Synaptic relationships between terminals immunostained for SP or Met-/Leu-Enk (immunogold) and ChAT-immunoreactive dendrites (immunoperoxidase) in the monkey putamen. A, B: Photomicrographs showing SP-ir boutons (+Ter) forming symmetric synapses (black arrows) with a small-sized and a medium-sized ChAT-labeled dendrite (A and B, respectively). C, D: Examples of Enk-ir terminals (+Ter) forming symmetric synapses (arrows) with a large-sized (C) and a medium-sized (D) ChAT-labeled dendrite. Note examples of unlabeled boutons (U Ter) forming asymmetric synapses (arrowheads) with spines (Sp) in the same fields of view. E: Mean percentages (± SEM) of ChAT-ir dendrites of different sizes in the double-immunostained tissue for SP/ChAT (n = 5 animals) or Enk/ChAT (n = 4 animals). F: Mean percentages (± SEM) of SP-positive and Enk-positive terminals in contact with ChAT-ir elements in the monkey putamen. *, P < 0.005.
**G:** Mean percentages (± SEM) of SP- and Enk-ir terminals in contact with ChAT-immunoreactive soma and dendrites of various sizes. *, $P = 0.012$ Enk-labeled versus SP-labeled on large dendrites. Abbreviations: Den, dendrite. Scale bar = 0.5 μm in A,C; 0.2 μm in B,D.
Figure 7.
Proportion of total synaptic inputs onto ChIIs from SP- and Enk-ir terminals. A: Comparison of the mean densities of SP- or Enk-positive terminals as revealed with the pre-embedding immunogold method in double-immunostained striatal tissue of three monkeys (SP/ChAT-left gray bar; Enk/ChAT-right gray bar) with the density of these terminals in striatal tissue from one monkey single labeled for each peptide with the immunoperoxidase method. The density of labeled terminals for either SP or Enk is the same irrespective of the marker used in single or double immunolabeling reactions. B,C: Mean percentages (± SEM) of total terminals in synaptic contact with ChI dendrites in the monkey putamen. Terminals are categorized based on their type of synaptic specializations (symmetric vs. asymmetric) and neuropeptide immunoreactivity (SP+ vs. SP− in B; Enk+ vs. Enk− in C). In B, *, P < 0.001 Symm/SP(−) versus Symm/SP(+) and Asymm/SP(−) on medium dendrites. In C, *, P < 0.001, Symm/Enk(−) versus Symm/Enk(+) and Asymm/Enk(−) on large or medium dendrites; #, Symm/Enk(−) versus Symm/Enk(+) on small dendrites. D: Mean percentages of synaptic inputs onto ChIIs that originate from SP+ and Enk+ collaterals of GABAergic striatal projection neurons (Symm/SP+ or Enk+) or other sources of putatively GABAergic terminals (Symm/SP− and Enk−) and putatively glutamatergic boutons (Asymm/SP− and Enk−).
Figure 8.
Schematic of the distribution of various chemically characterized synaptic inputs onto ChIIs in the monkey putamen based on results shown in this study. The relative location and proportion of the different terminal subtypes along the somatodendritic domain of ChIIs are represented. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
### Table 1

**Primary Antibodies Used in This Study**

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<td>1:2,000</td>
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