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Cholinergic Interneurons:

Distribution and Synaptic Inputs in the Primate Putamen

By

Kalynda K. Gonzales

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

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Distribution and Synaptic Inputs in the Primate Putamen

By

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B.S. with Honors in Biology/Pre-Med

University of Massachusetts-Boston, 2005

Advisors: Yoland Smith, Ph.D. and Thomas Wichmann, M.D.

An abstract of a dissertation submitted to the Faculty of the
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Abstract

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By Kalynda K. Gonzales

Striatal cholinergic interneurons (ChIs) are central for the processing and reinforcement of goal-directed and habitual reward-related behaviors that can be negatively affected in states of altered dopamine transmission such as in Parkinson's disease. Although significant advances have been made in understanding the mechanisms by ChIs involvement in such behaviors, the development of potential therapeutic interventions that target ChI activity has been hampered by our limited knowledge about the network of connections that target ChIs. Anatomical, pharmacological, and electrophysiological studies in rodents and primates have demonstrated that striatal ChI activity is modulated by GABAergic and glutamatergic inputs. However, the source(s) and prevalence of these inputs have not been clarified. The main objective of this thesis was therefore to perform a quantitative ultrastructural analysis of the GABAergic inputs from direct (substance P-containing) and indirect (enkephalin-containing) striatofugal neurons, as well as inputs from GABAergic and glutamatergic parvalbumin-containing neurons, onto ChIs in the monkey putamen.

Electron microscopic observations from double-labeled (i.e., immunogold and immunoperoxidase) tissue revealed that approximately 60% of all synaptic inputs to ChIs originate from GABAergic terminals, where 24% of this innervation is derived from axon collaterals of direct and indirect striatal projection neurons, and 10% from striatal and/or pallidal GABAergic parvalbumin-containing neurons. Of the remaining synaptic inputs to ChIs, 21% are putatively glutamatergic, half of which originate from parvalbumin-containing neurons in the thalamus, and 19% from other (non-GABAergic) sources. The distribution of these synaptic inputs to striatal ChIs follows two major patterns: (1) GABAergic terminals that express substance P or enkephalin terminate on the entire somatodendritic domain of ChIs, whereas (2)

medium- and small-sized dendrites of ChIs are targets of parvalbumin-containing GABAergic and glutamatergic inputs.

Altogether, these studies have revealed an extensively diverse synaptic network between ChIs and their GABAergic and glutamatergic afferents, resulting in the first detailed map of ChI connectivity in the monkey striatum. Future studies in relation to the prevalence and pattern of additional ChI afferents, combined with a detailed description of changes in this afferent synaptic network in pathological conditions, will help to further characterize the substrate that underlies the role of ChIs in striatal functions in normal and diseased states.

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I am also very grateful for my social supports, which includes my family and friends. Each individual family member has supported me in one way or another and greatly contributed to my success. I honestly am not sure I could have tolerated what I did over the past seven years without their love and support. My partner, Todd, has possibly endured the most throughout my last few years of graduate school. However, he has continued to support me through the tough times and those that were good. Whenever I needed him, he was there for me, whether to let me cry on his shoulder or celebrate my successes. He is a wonderful person, and I thank him with all of my heart and soul. I also thank all of my science and lab colleagues, especially Jeff, Susan, Rosa, and Adriana. They were always patient and supportive in my training to learn extremely difficult techniques, which brought about much grief but also a lot of good memories! One thing I will never forget though is that they all taught me to love and enjoy science as well. I also greatly thank my neuroscience classmates, which are the most wonderful and talented group of friends and colleagues that could have been bestowed upon me.

Graduate school has not been an easy road for me, but with the love and support from my family, friends, advisors, committee members, and colleagues I have finally made it! Thanks to all of you from the bottom of my heart. I will be eternally grateful.

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Chapter 1 :

Introduction & Background

1.1 Introduction

The basal ganglia are a group of tightly interconnected subcortical nuclei that play important roles in regulating various aspects of sensorimotor, cognitive and limbic functions (Alexander *et al.*, 1990; Parent, 1990; Parent & Hazrati, 1993). A basic principle of their functional organization, developed through a series of anatomical and functional studies, incorporates a number of parallel and primarily segregated basal ganglia-thalamocortical circuits (i.e., motor, oculomotor, cognitive, and limbic loops), through which these various modalities are transmitted and processed (Alexander *et al.*, 1986; Alexander *et al.*, 1990; Hoover & Strick, 1993; Groenewegen & Berendse, 1994), although support exists for a certain degree of cross-communication between these channels as well (Joel & Weiner, 1994; Zahm *et al.*, 1996; Joel & Weiner, 1997; Bolam *et al.*, 2000; Voorn *et al.*, 2004; DeLong & Wichmann, 2009; Humphries & Prescott, 2010; Haber, 2011). Cortical information gains access to these circuits through its substantial, highly topographic and segregated connections with particular sub-compartments in the striatum (Alexander *et al.*, 1986; Alexander *et al.*, 1990; Hoover & Strick, 1993; Groenewegen & Berendse, 1994; Parent & Hazrati, 1995), and more specifically, through a complex synaptic network between striatal projection neurons (also referred to as medium spiny neurons; MSNs) and various populations of striatal interneurons (Bolam *et al.*, 2000; Tepper *et al.*, 2010). Among those, the striatal cholinergic interneurons (ChIs), which display unique morphological characteristics, a heterogeneous and widespread distribution throughout the striatum, and an extensively diverse microcircuitry, have long been recognized as significant regulators of striatal and basal ganglia functions in normal and diseased states (Nicola *et al.*, 2000; Zhou *et al.*, 2002; Pisani *et al.*, 2007; Smith & Villalba, 2008; Williams & Adinoff, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Goldberg & Reynolds, 2011; Gutierrez *et al.*, 2011; Mark *et al.*, 2011; Threlfell & Cragg, 2011; Goldberg *et al.*, 2012; Schulz & Reynolds, 2013).

Within the striatum, extrinsic glutamatergic and dopaminergic systems, along with intrinsic GABAergic systems, form a tight network with striatal ChIs, where they have complementary roles in aversive, attentional, motivational and reward-related events, as well as in the regulation of synaptic plasticity, conditioned learning, and action selection (Nicola *et al.*, 2000; Zhou *et al.*, 2002; Pisani *et al.*, 2007; Smith & Villalba, 2008; Williams & Adinoff, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Crittenden & Graybiel, 2011; Goldberg & Reynolds, 2011; Gutierrez *et al.*, 2011; Mark *et al.*, 2011; Threlfell & Cragg, 2011; Goldberg *et al.*, 2012; Schulz & Reynolds, 2013). However, the diverse nature of ChI responses to afferent inputs, along with their highly complex downstream effects, obscures our understanding of the roles of the striatal cholinergic system under normal physiological conditions and in a wide range of neurological and psychiatric disorders (Nicola *et al.*, 2000; Zhou *et al.*, 2002; Pisani *et al.*, 2007; Smith & Villalba, 2008; Williams & Adinoff, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Crittenden & Graybiel, 2011; Goldberg & Reynolds, 2011; Gutierrez *et al.*, 2011; Mark *et al.*, 2011; Threlfell & Cragg, 2011; Goldberg *et al.*, 2012; Schulz & Reynolds, 2013). One approach to begin delineating this puzzle is to define the precise synaptic microcircuitry of striatal ChIs, leading to the identification of the chemical phenotype, distribution, and densities of their synaptic afferents. The main objective of this thesis, therefore, was to perform thorough quantification methods in double-immunolabeled tissue at the ultrastructural level in the monkey putamen, thereby providing the first detailed map of the synaptic microcircuitry of cholinergic neurons in the non-human primate striatum.

Before the presentation of these findings, an overview of the functional and cellular compartmentalization of the dorsal striatum will be described, followed by a depiction of ChIs highly diverse morphological characteristics, distribution patterns, regulation of striatal activity, and synaptic afferentation/efferentation in the caudate nucleus and putamen of non-primates and primates. Next, the experiments that were performed during my thesis and their results will be presented in Chapters 2 and 3. Afterward, a discussion will ensue on the limitations of previous

therapeutic approaches aimed at the striatal cholinergic systems for these disorders. This will be followed by the potential clinical relevance of new drugs developed to target specific cholinergic receptor subtypes as brain therapeutics, taking into consideration recent findings from this thesis and others highlighting new aspects of the synaptic connectivity between ChIs and their afferents.

1.2 Striatum: functional compartmentalization and afferent connections

1.2.1 Striatal compartmentalization

The striatum is as an entryway to the basal ganglia nuclei for the transmission and processing of extrinsic environmental signals related to various aspects of somatomotor function, motivated behavior, emotion, and cognition in order to generate context dependent, goal-directed and habitual behaviors (Bolam *et al.*, 2000; Belin & Everitt, 2008; Kreitzer, 2009; Balleine & O'Doherty, 2010; Redgrave *et al.*, 2010; Crittenden & Graybiel, 2011; Haber, 2011). It is often divided into two parts, the dorsal and ventral striatum, based on divergent functional connectivity. In primates, the dorsal striatum consists of the caudate nucleus and putamen separated from each other by fibers of the internal capsule, whereas in rodents it is a single mass of gray matter often referred to as the caudate-putamen complex (Kemp & Powell, 1970; 1971a; Carpenter, 1976; Chronister *et al.*, 1976). The ventral striatum consists of the nucleus accumbens and the striatal portion of the olfactory tubercle, along with the ventromedial extension of the caudate nucleus and putamen (Nauta *et al.*, 1978; Heimer *et al.*, 1997). The nucleus accumbens comprises core and shell sub-regions, two anatomically and functionally defined areas that have been well characterized in rodents (Záborszky *et al.*, 1985; Meredith *et al.*, 1989; Voorn *et al.*, 1989; Heimer *et al.*, 1991; Meredith *et al.*, 1992; Zahm & Brog, 1992; Voorn *et al.*, 2004; Haber, 2011), but a clear delineation between these accumbal areas is still a matter of debate in primates (Hurd & Herkenham, 1995; Meredith *et al.*, 1996; Voorn *et al.*, 1996; Haber & McFarland, 1999; Fudge & Haber, 2002; Prensa *et al.*, 2003). In general, the medial, lateral, and ventral parts of the accumbens are referred to as the shell, while its dorsal and central portions constitute the core

(Záborszky *et al.*, 1985; Voorn *et al.*, 1989; Zahm & Brog, 1992; Jongen-Rêlo *et al.*, 1994). Based on differential neurochemical expression as well as specific afferent and efferent connections, sub-regions called the patch (or striosome) and matrix compartments have been identified within the dorsal and ventral striatum (predominantly the caudate nucleus, anterior putamen, and core of the accumbens) in primate and non-primate species (Graybiel & Ragsdale, 1978; Graybiel *et al.*, 1981; Gerfen, 1984; Gerfen *et al.*, 1987a; Gerfen *et al.*, 1987b; Voorn *et al.*, 1989; Johnston *et al.*, 1990; Martin *et al.*, 1991; Zahm & Brog, 1992; Jongen-Rělo *et al.*, 1993; Meredith *et al.*, 1993; Meredith *et al.*, 1996).

For the sake of this introduction and the remaining chapters, the subsequent findings will mainly refer to those found in the dorsal striatum.

1.2.2 Corticostriatal system

Extrinsic topographically and functionally organized projections from the cerebral cortex, thalamus and ventral midbrain constitute the bulk of afferents to the striatum. Cortical glutamatergic afferents distribute widespread information to the whole striatum (Alexander *et al.*, 1986; Alexander *et al.*, 1990; Hoover & Strick, 1993; Groenewegen & Berendse, 1994; Parent & Hazrati, 1995). In primates, the post-commissural putamen (or lateral striatum in rodents) receives its main cortical inputs from sensorimotor cortices, while the pre-commissural putamen and the caudate nucleus (or medial striatum in rodents) are the main targets of cognitive afferents from associative prefrontal, temporal and parietal cortical regions (Alexander *et al.*, 1990; Bolam *et al.*, 2000; Joel & Weiner, 2000; Haber, 2011). These sensorimotor and cognitive inputs terminate predominantly within the matrix sector of the caudate nucleus and putamen, whereas the striosomes receive its main cortical innervation from limbic cortices (such as the orbitofrontal, anterior cingulate, and insular cortices) and the amygdala (Gerfen, 1984; Ragsdale & Graybiel, 1988; Eblen & Graybiel, 1995; Kincaid & Wilson, 1996; Levesque *et al.*, 1996; Crittenden & Graybiel, 2011; Novejarque *et al.*, 2011).

1.2.3 Thalamostriatal system

The thalamus is another major source of glutamatergic inputs to the striatum. Although rostral intralaminar (mainly the centrolateral nucleus), relay (ventral anterior/ventral lateral nuclei), associative (mediodorsal nucleus) and midline thalamic nuclei contribute to the thalamostriatal system, the main origin of these projections are the caudal intralaminar nuclei, namely the centre median (CM) and parafascicular (Pf) nuclei, that project primarily to the sensorimotor (CM inputs) and associative (Pf inputs) dorsal striatal regions in primates (McFarland & Haber, 2000; 2001; Smith *et al.*, 2004). In nonprimates, the intralaminar, and most thalamic nuclei, preferentially target the matrix region of the caudate-putamen complex, while the patch/striosomes receive their main thalamic innervation from the paraventricular (PV) nucleus (Herkenham & Pert, 1981; Ragsdale & Graybiel, 1991; Sadikot *et al.*, 1992b). Like the corticostriatal system, the thalamostriatal projections to the caudate nucleus and putamen are functionally topographic (Berendse & Groenewegen, 1990; Sadikot *et al.*, 1992a; Brog *et al.*, 1993; Groenewegen & Berendse, 1994; Giménez-Amaya *et al.*, 1995; Parent & Parent, 2005) and, in the case of CM/Pf, part of functionally-segregated basal ganglia-thalamostriatal loops that process sensorimotor, associative and limbic information (Smith *et al.*, 2004; Smith *et al.*, 2009; Galvan & Smith, 2011), although a certain level of integration and convergence exists within and between the various structures involved in these complex circuits, particularly at the level of associative and limbic loops (Joel & Weiner, 1994; 1997; Voorn *et al.*, 2004; Haber & Calzavara, 2009; Haber, 2011).

1.2.4 Nigrostriatal and mesostriatal systems

In addition to glutamatergic inputs, the mammalian striatum is also the target of prominent dopaminergic afferents from the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) (Lynd-Balta & Haber, 1994a; b; Haber & Fudge, 1997; Joel & Weiner, 2000; Haber, 2011). The VTA mainly projects to the limbic striatum, the lateral SNc innervates

the associative and sensorimotor striatum, while the medial SNc sends afferents to all three functional regions of the dorsal and ventral striatum in all species (Lynd-Balta & Haber, 1994a; b; Haber & Fudge, 1997; Joel & Weiner, 2000; Haber, 2011). In rats, the medial and lateral regions of the dorsal SNc innervate the matrix compartment of the associative and sensorimotor striatum, respectively, whereas the patches receive their main dopaminergic innervation from the medial and lateral aspects of the ventral SNc (Gerfen *et al.*, 1987b; Jimenez-Castellanos & Graybiel, 1987a; Joel & Weiner, 2000; Prensa & Parent, 2001; Crittenden & Graybiel, 2011). Despite this general topographical arrangement, it is noteworthy that single dopaminergic axons often innervate neurons in both striatal compartments (Matsuda *et al.*, 2009). It is currently unknown if a similar relationships exist between the patch-matrix compartments and the mesostriatal dopaminergic systems in primates (Jimenez-Castellanos & Graybiel, 1987b; 1989; Langer & Graybiel, 1989; Langer *et al.*, 1991).

1.2.5 Other striatal afferents

Additional inputs to the dorsal striatum originate from GABAergic neurons in the globus pallidus (GP) (Beckstead, 1983; Oertel *et al.*, 1984; Kita & Kitai, 1991; Bevan *et al.*, 1998; Kita *et al.*, 1999; Sato *et al.*, 2000; Mallet *et al.*, 2012), along with those from serotonergic neurons in the dorsal raphe (Gaspar *et al.*, 1985; Ikemoto *et al.*, 1996; Olvera-Cortés *et al.*, 2008; Parent *et al.*, 2011; Mathur & Lovinger, 2012), noradrenergic neurons in the locus coeruleus (mainly to the ventral striatum) (Ikemoto *et al.*, 1996; Delfs *et al.*, 1998; Tong *et al.*, 2006), and glutamatergic neurons in the subthalamic nucleus (Smith & Parent, 1986; Groenewegen & Berendse, 1990).

1.3 Striatum: cellular organization and efferent connections

1.3.1 Dorsal striatum

In rodents, 90-95% of dorsal and ventral striatal neurons are GABAergic projection MSNs, while the remaining striatal neuronal population consists of interneurons (Kemp &

Powell, 1971a; Wilson & Groves, 1980; Graveland & DiFiglia, 1985; Hussain *et al.*, 1996; Meredith, 1999). A similar cellular organization makes up the primate dorsal striatum, although the proportion of interneurons appears to be slightly larger in primates than nonprimates (Pasik *et al.*, 1976; Graveland & DiFiglia, 1985; Roberts *et al.*, 1996; Wu & Parent, 2000). Two populations of dorsal striatal MSNs have been categorized on the basis of their projection sites, neuropeptide expression and dopamine receptor content. The MSNs that project directly to the basal ganglia output nuclei (i.e., the internal segment of the globus pallidus, GPi, and the substantia nigra pars reticulata, SNr), referred to as "direct pathway" neurons, express predominantly substance P (SP), dynorphin and D1 dopamine receptors, whereas striatal MSNs that project to the GPe, called "indirect pathway" neurons contain enkephalin (Enk) and express preferentially the D2 dopamine receptors (Gerfen, 1988; Gerfen *et al.*, 1990; Reiner *et al.*, 1999a; Aubert *et al.*, 2000; Bolam *et al.*, 2000). Although this segregation of striatal output neurons is the basis for functional models of information flow through the basal ganglia circuits, it is somewhat oversimplified due to the fact that a small, albeit significant, subset of striatal MSNs co-express D1 and D2 dopamine receptors (possibly including D3 and D4 receptors), and a number of striatal MSNs send axonal projections to both GPe and GPi (or SNr) in rats and monkeys (Surmeier *et al.*, 1996; Kawaguchi, 1997; Nicola *et al.*, 2000; Lévesque *et al.*, 2003; Wilson, 2007; Tepper *et al.*, 2008).

1.3.2 Striatal interneurons

GABAergic neurons that express parvalbumin, somatostatin/neuropeptide Y/nitric oxide synthase, or calretinin and non-GABAergic acetylcholine-expressing cells account for 5-10% of all striatal neurons and represent the main populations of dorsal striatal interneurons in primates and nonprimates (Hussain *et al.*, 1996; Kawaguchi *et al.*, 1997; Meredith, 1999; Tepper *et al.*, 2010). In the rodent caudate-putamen complex, ChIs account for approximately 1-2% of the total neuronal population (Phelps *et al.*, 1985), whereas the proportion of ChIs in the primate dorsal

striatum has not been thoroughly quantified, as is the case for parvalbumin- and calretinin-positive GABAergic interneurons (Selden *et al.*, 1994; Cicchetti *et al.*, 1998; Wu & Parent, 2000).

1.4 Heterogeneity of striatal cholinergic interneurons

1.4.1 Cholinergic interneurons in the dorsal striatum

1.4.1.1 Morphological, ultrastructural, and cytological features

Immunoreactive cell bodies for choline acetyltransferase (ChAT) or acetylcholinesterase (AchE) have an average diameter of 35 µm and display various shapes in primates (***Figure 1.1***A-C) (Mesulam *et al.*, 1984; DiFiglia, 1987; Yelnik *et al.*, 1991; Bernacer *et al.*, 2007), while their cell bodies are slightly smaller (around 25 µm) and mostly oval in rodents (***Figure 1.2***A,B) (Woolf & Butcher, 1981; Bolam *et al.*, 1984a; Bolam *et al.*, 1984b; Phelps *et al.*, 1985). In addition to these somatic differences, ChIs in rats are characterized by the presence of more somatic and dendritic spines, less primary dendrites, and a sparser ramification of their distal dendritic trees (***Figure 1.2***A-E) (Woolf & Butcher, 1981; Bolam *et al.*, 1984a; Bolam *et al.*, 1984b; Phelps *et al.*, 1985; Dimova *et al.*, 1993; Goldberg & Reynolds, 2011) compared with monkeys (***Figure 1.1***A-C and ***Figure 1.2***G) and humans (***Figure 1.2***F) (Difiglia *et al.*, 1980; Mesulam *et al.*, 1984; Graveland *et al.*, 1985; DiFiglia & Carey, 1986; DiFiglia, 1987; Yelnik *et al.*, 1991; Yelnik *et al.*, 1993; Bernacer *et al.*, 2007; Gonzales *et al.*, 2013). Through correlations between the morphology of Golgi-filled neurons and ChAT- (or AchE)-positive cells in the primate dorsal striatum, ChIs have been characterized as having a "spidery" appearance because of their large cell bodies from which emerge thick primary dendrites that give rise to profuse, "spider-like", dendritic trees and widespread intrinsic axonal arborizations (***Figure 1.1***A-C and ***Figure 1.2***F-H) (Difiglia *et al.*, 1980; Mesulam *et al.*, 1984; Graveland *et al.*, 1985; DiFiglia & Carey, 1986; DiFiglia, 1987; Yelnik *et al.*, 1991; Yelnik *et al.*, 1993; Bernacer *et al.*, 2007; Gonzales *et al.*, 2013). However, "spidery" cells correspond to only a small proportion of putative ChIs (AchE-

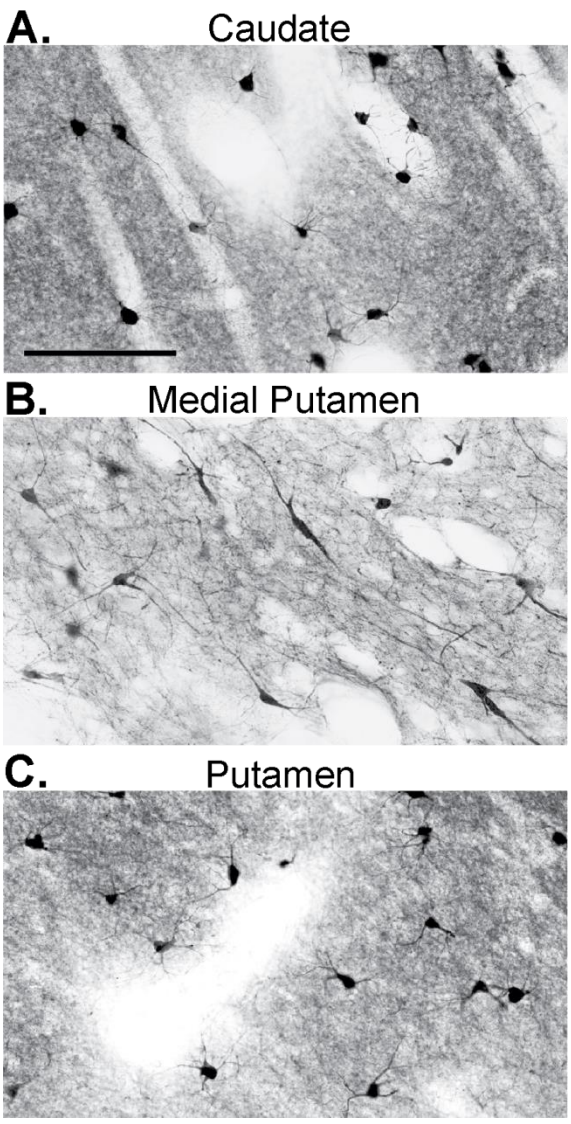

***Figure 1.1*. Morphological characterization of striatal cholinergic interneurons.**
A-C: Under high power views at the light microscopic level, the morphological characteristics (i.e., the distribution, size, and shape) of cholinergic interneurons located in the caudate and putamen of rhesus monkeys are compared and contrasted. Note the varying levels of the size and shape of the ChAT-labeled cell bodies and dendritic arborizations between the different areas of the striatum, as well as the diverse neuropil immunolabeling. Scale bar = 200 µm in A (also applies to B and C).

positive) in the rodent striatum, and even for these so-called "spidery" neurons (***Figure 1.2***C-E), major morphological differences exist between rodents and primates (***Figure 1.2***) (Woolf & Butcher, 1981; Bolam *et al.*, 1984a; Bolam *et al.*, 1984b; Takagi *et al.*, 1984; Yelnik *et al.*, 1993). For example, the "spidery" neurons in rats have a smaller number of dendritic tips (21-28 versus 129) and a significantly shorter total dendritic length (1,400-2,500 µm versus 23,400 µm) than in monkeys (Yelnik *et al.*, 1991; Yelnik *et al.*, 1993), suggesting that the extent of synaptic innervation and the processing of extrinsic information by ChIs are far more complex in primates than in rodents. Such robust inter-species morphological differences must be taken into account when anatomical and functional data gathered from rodent ChIs are translated to the primate striatum. However, despite these striking differences, there appears to be a general assumption across studies achieved in different species that ChAT-positive neurons belong to a single cell population in the dorsal striatum of primates and nonprimates, mainly characterized by their large-sized soma (Bolam *et*

al., 1984b; Phelps *et al.*, 1985; DiFiglia & Carey, 1986; Bernacer *et al.*, 2007), similar subcellular features such as deeply indented nuclei and richly embedded cytoplasm with rough endoplasmic reticulum, subsurface cisternae, lipofuscin granules and large dense bodies (Satoh *et al.*, 1983; Bolam *et al.*, 1984a; Bolam *et al.*, 1984b; Phelps *et al.*, 1985; DiFiglia & Carey, 1986; DiFiglia, 1987; Dimova *et al.*, 1993; Contant *et al.*, 1996), and the absence of a GABAergic phenotype (Kosaka *et al.*, 1988; Kawaguchi *et al.*, 1997; Kubota & Kawaguchi, 2000).

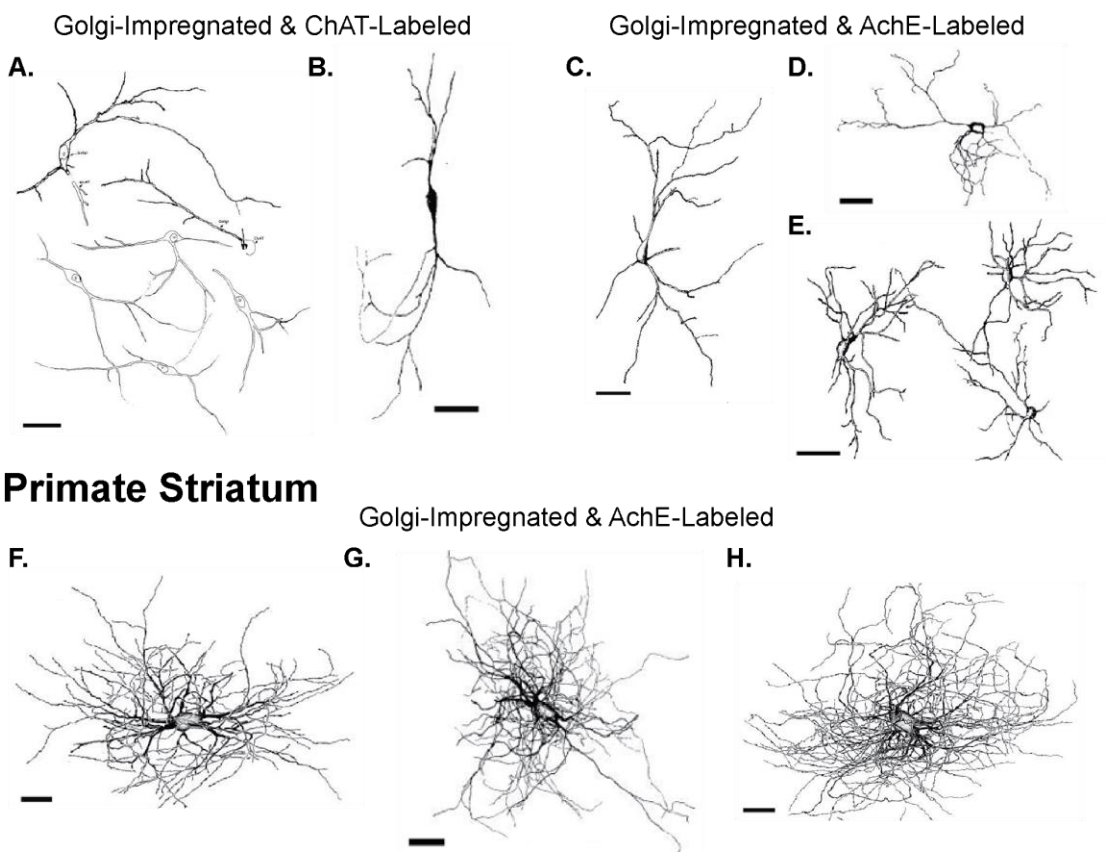

Figure 1.2. **Morphological differences between cholinergic interneurons from varying species.** **A,B:** Representations of Golgi-impregnated neurons that were immunopositive for ChAT in the rat striatum from Bolam et al. (1984) (**A**) and Phelps et al. (1985) (**B**). **C,D:** Examples of Golgi-impregnated neurons in the rat striatum that were immunopositive for AchE from Bolam et al. (1984) (**C**) and Yelnik et al. (1993) (**D**). **F-H:** Three examples of primate neurons from Yelnik et al. (1991) that were Golgi-impregnated and AchE immunoreactive in the human (**F**), monkey (**G**) and baboon (**H**). Note the smaller cell body size and dendritic trees of putative cholinergic interneurons of rodents in comparison to the primate species. Scale bars = 50 µm.

Bernacer et al. (2007) has performed the most comprehensive stereological counts of ChAT-immunoreactive (ir) neurons across all striatal territories in the human caudate and putamen. In brief, they found that ChI cell bodies display a different prevalence and pattern of distribution when compared across functionally segregated regions of the striatum, based on its cortical inputs (Parent, 1990; Haber, 2003; Haber *et al.*, 2006). For instance, ChIs are most abundant in the "associative" striatum (dorsomedial sector of the caudate head, body and gyrus) compared with the "sensorimotor" (post-commissural putamen, except its ventromedial portion) and "limbic" (posteroventral putamen) striatal regions. However, ChIs in all functional regions of the human striatum follow a positive rostrocaudal gradient in their pattern of distribution, i.e., the density of ChIs in pre-commissural striatal regions is lower than in post-commissural sectors of the striatum. No obvious difference was found in the cell body shape and the total number of primary dendrites per ChI neuron from different functional territories of the human dorsal striatum (Bernacer *et al.*, 2007). Although similar types of quantitative analyses have not been carried out in the monkey and rodent striatum, it has been suggested that ChAT-labeled cell bodies are more densely distributed in the rostral than in the caudal regions of the caudate-putamen complex in rats (Phelps *et al.*, 1985), a markedly different pattern of distribution than in the human (Bernacer *et al.*, 2007) and monkey striatum (***Figure 1.3***). However, in both rats and primates, a similar characteristic feature of ChIs is their predominant localization within the striatal matrix compartment, or at the borders of striosomes, where their dendrites cross over the patch-matrix boundaries, providing them a unique position to facilitate cross-communication between MSNs of the different striatal sub-compartments (Mesulam *et al.*, 1984; Hirsch *et al.*, 1989; Lehéricy *et al.*, 1989; Kubota & Kawaguchi, 1993; Aosaki *et al.*, 1995; Holt *et al.*, 1996; Bernacer *et al.*, 2007; Crittenden & Graybiel, 2011).

1.4.1.2 Dorsal striatal cholinergic neuropil

In addition to ChAT-ir cell bodies and dendrites, the whole striatum is filled with a dense meshwork of fine ChAT-ir processes which most likely represent thin axons, small distal

dendritic processes and axon terminals of cholinergic cells (***Figure 1.1*** and ***Figure 1.3***) (Bolam *et al.*, 1984b; Mesulam *et al.*, 1984; Phelps *et al.*, 1985; Contant *et al.*, 1996; Holt *et al.*, 1996; Gonzales *et al.*, 2013). Three main features characterize this rich cholinergic neuropil in the monkey and human striatum. First, it displays a patchy appearance made up of pockets of light immunostaining, reminiscent of the striatal patch compartment, embedded within a field of denser immunoreactivity (***Figure 1.3***) (Mesulam *et al.*, 1984; Graybiel *et al.*, 1986; Hirsch *et al.*, 1989; Lehéricy *et al.*, 1989; Bernacer *et al.*, 2007). Second, it follows a positive rostrocaudal gradient in labeling intensity (***Figure 1.3***) (Mesulam *et al.*, 1984). Third, areas of denser ChAT immunostaining lay within the medial parts of the pre-commissural and commissural caudate and putamen (***Figure 1.3***A,B) and the lateral borders of the post-commissural putamen (***Figure 1.3***C)

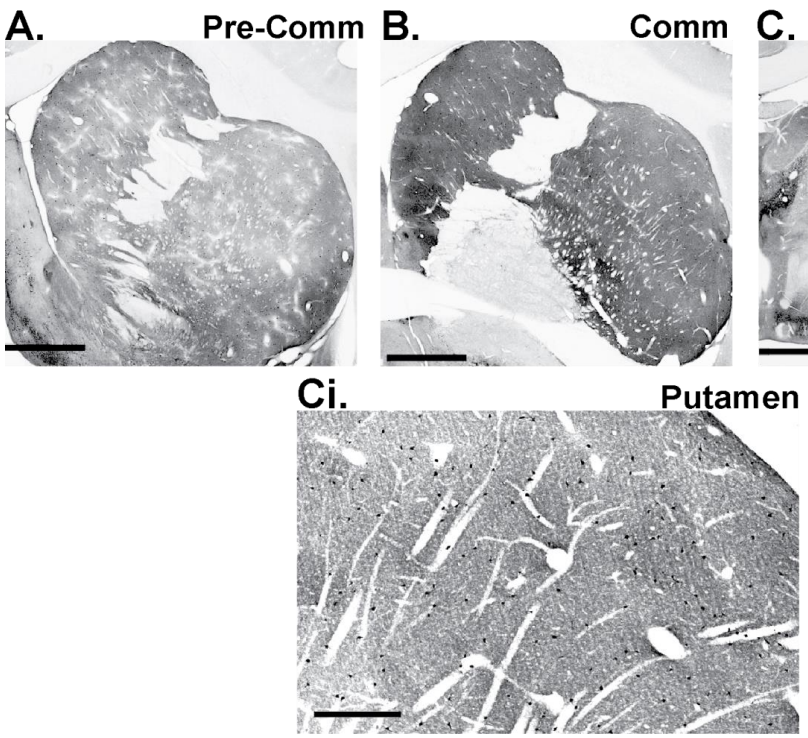

Figure 1.3. **Distribution of ChAT immunolabeling in the dorsal striatum of monkeys.**
At the light microscopic level, the distribution of ChAT immunoreactivity is shown at various levels throughout the caudate nucleus and putamen. **A-C:** Low-power views of ChAT immunolabeling at various levels in accordance with the location of the anterior commissure: pre-commissural (Pre-Comm; A), commissural (Comm; B), and post-commissural (Post-Comm; C). **Ci:** High-power view of the typical "patchy" neuropil observed with ChAT immunolabeling in the putamen (taken from (C); represented by an asterisk). Scale bar = 3 mm in A,B;1 4mm in C; 600 µm in Ci.

(Holt *et al.*, 1996; Bernacer *et al.*, 2007). In contrast, the rodent ChAT-immunolabeled neuropil is homogeneously stained (Bolam *et al.*, 1984b; Phelps *et al.*, 1985), except for an increased labeling intensity (includes both somata and neuropil) in the lateral border and rostral-most regions of the striatum (Phelps *et al.*, 1985), distinguishing the intrastriatal arborizations of the primate cholinergic striatal network from that in rodents.

Table 1.1. Comparison of the morphology and distribution of ChIs in the dorsal striatum of rodents and primates

Characteristic	**Rats**	**Primates**
Soma size (diameter)	25-35 µm	35-50 µm
Soma shape	Mainly oval	Highly diverse
Dendritic tree size	Moderate, Infrequently branched	Large, Highly branched
Somata densities	Highest rostrally	Highest caudally
Neuropil densities	Mostly homogeneous	Moderately patchy
Ultrastructure	Indented nucleus, organelle-rich cytoplasm, subsurface cisternae, and lipofuscin granules	Indented nucleus, organelle-rich cytoplasm, subsurface cisternae, and lipofuscin granules

1.4.2 Physiological activity of cholinergic interneurons in the dorsal striatum

Tonically active neurons (TANs) in the dorsal striatum frequently respond to sensory salient events during behavioral conditioning with a triphasic response that includes an early excitation, a pause in activity, and a rebound excitation (Kimura, 1990; Aosaki *et al.*, 1994a; Aosaki *et al.*, 1994b; Aosaki *et al.*, 1995; Matsumoto *et al.*, 2001; Morris *et al.*, 2004; Schulz & Reynolds, 2013). Under normal physiological conditions, striatal ChIs are generally considered to correlate with TANs on the basis of particular anatomical and functional similarities between ChAT-ir neurons and *in vitro* and *in vivo* electrophysiologically recorded TANs in the dorsal striatum of rodents (Bishop *et al.*, 1982; Wilson *et al.*, 1990; Plenz & Aertsen, 1996; Reynolds *et al.*, 2004; Inokawa *et al.*, 2010; Goldberg & Reynolds, 2011; Schulz *et al.*, 2011; Schulz &

Reynolds, 2013). In regards to their morphology, a recent study that combined juxtacellular labeling and electrophysiological characterization of TANs in anesthetized rats showed that recorded striatal neurons with these electrophysiological features display the characteristic large-sized soma of ChIs (Bishop *et al.*, 1982; Wilson *et al.*, 1990; Kawaguchi *et al.*, 1995; Plenz & Aertsen, 1996; Pisani *et al.*, 2001; Reynolds *et al.*, 2004; Inokawa *et al.*, 2010; Goldberg & Reynolds, 2011; Schulz *et al.*, 2011), along with immunoreactivity for ChAT (Inokawa *et al.*, 2010). The descriptive nature of the morphology of TANs dendrites varies across studies, ranging from long, sparsely-branched dendritic trees (refer to ***Figure 1.2***A-C) (Plenz & Aertsen, 1996; Reynolds *et al.*, 2004; Schulz *et al.*, 2011) to thick, moderately-branched dendrites that taper into finer processes (refer to ***Figure 1.2***D, E) (Bishop *et al.*, 1982; Bennett & Wilson, 1998; 1999). Due to the necessary limitations and difficulties associated with primate studies, a direct relation between TANs and ChAT-labeled neurons remains to be established, although Aosaki et al. (1995) strongly suggested TANs recorded in vivo in the monkey striatum likely correspond to ChAT-labeled neurons based on their densities, large cell bodies and distribution across striatal regions (Aosaki *et al.*, 1995).

The firing properties of TANs in rodents and monkeys, which are quite different from those of the phasically active striatal projection neurons, are characterized by their more depolarized membrane potential (approximately -60 mV), tonic spike discharge around 2-10 spikes/sec, broad spike waveforms, and diverse spiking patterns (i.e., regular, irregular, and bursting) (Kimura *et al.*, 1984; Wilson *et al.*, 1990; Kawaguchi, 1992; Aosaki *et al.*, 1995; Raz *et al.*, 1996; Bennett & Wilson, 1999; Apicella, 2002; Wilson & Goldberg, 2006). Additionally, *in vitro* whole-cell and *in vivo* intracellularly recorded TANs display specific electrical responses to current injections, such that negative current pulses result in a prominent sag in the hyperpolarizing membrane potential of TANs regulated by an I_h current, whereas non-adaptive, regular spiking followed by a long-duration after-hyperpolarization occurs with depolarizing current pulses (Wilson *et al.*, 1990; Kawaguchi *et al.*, 1995; Reynolds *et al.*, 2004; Wilson &

Goldberg, 2006; Oswald *et al.*, 2009; Schulz *et al.*, 2011). On the other hand, TANs also fire spontaneously and maintain their diverse spiking patterns in the absence of current injections (Bennett & Wilson, 1999; Bennett *et al.*, 2000; Goldberg & Wilson, 2005; Wilson, 2005), suggesting that TANs patterns of spike output are regulated by both their intrinsic pacemaking mechanisms and synaptic afferents (Schulz & Reynolds, 2013).

In general, speculation is unavoidable when faced with the challenge of directly correlating TANs to ChIs, due to current technical limitations. However, the development of new techniques, such as optogenetics, may further advance our knowledge of the physiological properties of ChAT-expressing neurons and the chemical phenotype(s) of TANs. For example, recent findings from optogenetic studies in urethane-anesthetized rodents and extracellular recordings in awake rats reveal that other striatal interneurons, such as the parvalbumin-expressing interneurons, may also fire spontaneously in the dorsal striatum (Berke, 2008; Beatty *et al.*, 2012; Sharott *et al.*, 2012), demonstrating that TANs cannot be solely characterized on the basis of their tonic discharge patterns. Another challenge is to characterize the firing properties of TANs (and ChAT-expressing neurons) in the dorsal striatum, along with TANs in various functional and chemically-defined sub-compartments.

1.5 Cholinergic regulation of striatal activity

1.5.1 Cholinergic receptor expression in the dorsal striatum

1.5.1.1 Muscarinic receptor expression of striatal neurons

ChIs regulate the activity of striatal neurons through direct and indirect mechanisms via a wide range of pre- and post-synaptic cholinergic receptors, consisting of G-coupled muscarinic receptors (mAChRs) and ionotropic nicotinic receptors (nAChRs), located on the surface of striatal projection neurons, interneurons and synaptic afferents (for reviews, refer to (Zhou *et al.*, 2003; Exley & Cragg, 2008; Havekes *et al.*, 2011; Oldenburg & Ding, 2011; Wevers, 2011)). Five types of mAChRs have been genetically identified (M1-M5) and categorized into two

groups on the basis of their distinct pharmacological properties (Caulfield & Birdsall, 1998; Wess, 2003; Oldenburg & Ding, 2011). For instance, the $G_{q/11}$-coupled M1-like (M1, M3 and M5) receptors upon activation enhance internal calcium release through the stimulation of phospholipases, whereas activation of the $G_{i/o}$-coupled M2-like (M2 and M4) receptors reduces cyclic-AMP formation through the inhibition of adenyl cyclase, subsequently blocking calcium channel activity (Caulfield & Birdsall, 1998; Wess, 2003; Oldenburg & Ding, 2011).

The distributions of M1-M5 mRNA, protein, and binding sites in individual nuclei and in single cell populations demonstrate additional distinguishing features of these receptors. In regards to the macroscopic distribution of mAChRs, a highly heterogeneous expression has been revealed in the dorsal striatum of non-primates and primates by means of techniques that utilized radioligand binding, reverse transcription-polymerase chain reactions (RT-PCR), *in situ* hybridization, and immunohistochemistry (Nastuk & Graybiel, 1985; Cortés & Palacios, 1986; Cortés *et al.*, 1986; Cortés *et al.*, 1987; Brann *et al.*, 1988; Nastuk & Graybiel, 1988; Weiner *et al.*, 1990; Vilaró *et al.*, 1991; 1992; Flynn & Mash, 1993; Levey, 1993; Zubieta & Frey, 1993; Levey *et al.*, 1995; Aubert *et al.*, 1996; Rodríguez-Puertas *et al.*, 1997). M1 mAChRs represent the highest levels of mRNA and binding sites homogeneously distributed in the striatum, along with their particularly high concentrations in the striosomes of the rat and primate dorsal striatum (Nastuk & Graybiel, 1985; 1988; Flynn & Mash, 1993; Aubert *et al.*, 1996; Rodríguez-Puertas *et al.*, 1997). In rats and monkeys, M2 receptor mRNA is moderately expressed throughout the dorsal striatum (Vilaró *et al.*, 1992; Vilaró *et al.*, 1994; Aubert *et al.*, 1996). Contrastingly, mRNA for M3 receptors is mainly enriched in the mid-ventral and ventral caudate-putamen complex (Zubieta & Frey, 1993; Levey *et al.*, 1994). The expression of M4 receptor mRNA and protein occurs in a dense, patchy-like manner (not clear if correlated to striosomal boundaries) in the rat caudate-putamen complex,(Weiner *et al.*, 1990; Hersch *et al.*, 1994; Hersch & Levey, 1995). In cats, monkeys and humans, patches of minimal M1 and M2 binding sites were only observed in the AChE-rich regions of the ventral putamen (Nastuk & Graybiel, 1988),

demonstrating regional and species differences in the patterns of M1 and M2 expression. Although displacement radioligand binding experiments illustrate the likeliness of M3 and M4 receptor expression in the human caudate nucleus and putamen (Flynn & Mash, 1993; Rodríguez-Puertas *et al.*, 1997), a full characterization of these expression patterns using more specific markers remains to be established in the dorsal striatum of non-human and human primates.

At the cellular level, M1 mAChRs are highly expressed by direct (labeled with SP or D1 dopamine receptors) and indirect (labeled with Enk or D2 dopamine receptors) pathway MSNs, neuropeptide Y (NPY)/somatostatin-containing interneurons, and ChAT-positive neurons in the dorsal striatum (Bernard *et al.*, 1992; Sugaya *et al.*, 1997; Alcantara *et al.*, 2001; Santiago & Potter, 2001; Yan *et al.*, 2001; Ding *et al.*, 2006; Narushima *et al.*, 2007; Uchigashima *et al.*, 2007). A moderate number of Enk-containing projection neurons (Weiner *et al.*, 1990; Bernard *et al.*, 1992; Yan *et al.*, 2001) and ChAT-labeled neurons (Yan & Surmeier, 1996; Sugaya *et al.*, 1997; Ding *et al.*, 2006) also express M4 mAChRs. It is noteworthy that SP/D1 MSNs contain a five-fold increase in M4 mRNA expression in comparison to Enk-labeled neurons (Bernard *et al.*, 1992; Ince *et al.*, 1997; Yan *et al.*, 2001). At the light microscopic level, M2 mAChR expression highly correlates with the density and distribution of ChAT-labeled neurons in the rodent and monkey dorsal striatum (Bernard *et al.*, 1992; Hersch *et al.*, 1994; Vilaró *et al.*, 1994; Bernard *et al.*, 1998; Smiley *et al.*, 1999; Alcantara *et al.*, 2001), with the exception of the NPY/NADPH-containing interneurons that also exhibit M2 immunoreactivity (Bernard *et al.*, 1998; Smiley *et al.*, 1999). Single-cell RT-PCR findings suggest that mRNA for M3 receptors and the neuropeptide Enk likely coincide within around 10% of the same neurons in the striatum of rats (Yan *et al.*, 2001). Comparative data for M1, M3, and M4 expression in specific cell populations is currently unavailable in the primate caudate and putamen.

In regards to M5 mAChRs, immunostaining and *in situ* hybridization studies have had difficulties in providing evidence for their existence in the striatum, most likely resulting from relatively low levels of M5 expression and the lack of specific M5 receptor antibodies (Weiner *et*

al., 1990; Levey, 1993). However, by using purified fusion proteins to make polyclonal antisera to M5 receptors, the dorsal striatum and the midbrain were shown to exhibit M5 mAChR expression, although almost at undetectable levels (< 25 fmol/mg) (Yasuda *et al.*, 1993). In addition, Yan et al. (2001) used RT-PCR in single cells to demonstrate that M5 mRNA was expressed at very low levels in rat striatal slices, more specifically, in 9% of SP-, 5% of Enk-, and 18% of SP/Enk-containing neurons. However, no significant correlation was found between M5 expression and a particular peptide-containing cell group (Yan *et al.*, 2001). The expression of M5 mAChRs remains to be studied in the dorsal striatum of primates.

1.5.1.2 Nicotinic receptor expression of striatal neurons

Nicotinic receptors form pentameric ion (Na^+, K^+ and Ca^{2+}) channels that consist of either homomeric (α7 in the mammalian brain) or heteromeric combinations of α (α2-α10) and β (β2-β4) subunits, resulting in their highly diverse pharmacological and functional properties (for reviews, see (Dajas-Bailador & Wonnacott, 2004; Gotti & Clementi, 2004; Grady *et al.*, 2007; Exley & Cragg, 2008; Hurst *et al.*, 2013)). In regards to their general distribution in the dorsal striatum, α4 and β2 mRNA and protein is predominantly expressed, along with a moderate amount of α5, α6, and β3 subunits in non-primates and primates (reviewed in (Gotti & Clementi, 2004; Gotti *et al.*, 2009; Hurst *et al.*, 2013)). However, a difference in subunit mRNA and protein expression for nAChRs was found between the caudate nucleus and putamen of monkeys (Han *et al.*, 2000; Han *et al.*, 2003; Quik *et al.*, 2005; Quik & McIntosh, 2006) and humans (Rubboli *et al.*, 1994; Hellström-Lindahl *et al.*, 1998; Terzano *et al.*, 1998; Tohgi *et al.*, 1998; Hellström-Lindahl *et al.*, 1999; Martin-Ruiz *et al.*, 2000; Graham *et al.*, 2002; Guan *et al.*, 2002) in studies that utilized radioligand binding, immunolocalization of subunit-specific antibodies, and in situ hybridization. In particular, the α2, α3, α4, α7, and β2 subunits were similarly expressed in the caudate nucleus and putamen, whereas the putamen also contained α5, α6, and β3 subunits of nicotinic receptors.

At the cellular level, the co-expression of α7 and β2 nAChRs exists in ChIs and GABAergic axons of an unknown phenotype in the rat striatum (Lu *et al.*, 1998; Azam *et al.*, 2003). Nicotinic receptor expression by GABAergic striatal neurons of a specific chemical phenotype has not been directly demonstrated. However, it is generally thought that all striatal neurons (except MSNs) express nAChRs on the basis of their responsiveness to direct nicotinic activation (de Rover *et al.*, 2002; Koos & Tepper, 2002; Luo *et al.*, 2013).

1.5.1.3 Muscarinic and nicotinic receptor expression in striatal afferents

At this time, there is no detailed characterization of mAChR expression in extrinsic afferents to the striatum, although ultrastructural findings suggest that putative glutamatergic terminals (see section 1.5.2) from either the cortex or thalamus may contain M1, M2, M3 and/or M4 mAChRs (Hersch *et al.*, 1994; Hersch & Levey, 1995; Alcantara *et al.*, 2001). Additionally, functional findings in the striatum of M5 receptor knock-out mice suggest that striatal dopaminergic axons/terminals contain M5 muscarinic receptors co-localized with α4β2 nicotinic receptors (Zhang *et al.*, 2002b; Yamada *et al.*, 2003).

Attempts at characterizing nicotinic receptor expression in dopaminergic axons/terminals in the striatum have been on-going for decades, by means of *in situ* hybridization, autoradiography, and immunoprecipitation techniques in the SNc, VTA, and striatum (for examples, see (Klink *et al.*, 2001; Azam *et al.*, 2002; Zoli *et al.*, 2002)). Pharmacological and dopamine release studies in receptor subunit knock-out mice have also been achieved (for reviews, refer to (Luetje, 2004; Grady *et al.*, 2007; Exley & Cragg, 2008)). In addition, one study utilizing double-label electron microscopy revealed the co-expression of tyrosine hydroxylase and β2 nicotinic receptors on terminals in the dorsal striatum (Jones *et al.*, 2001). Currently, the general consensus is that α4, α5, α6, α3 (in primates), β2, and β3 subunits are present on dopaminergic axons/terminals in the striatum (Zoli *et al.*, 2002; Salminen *et al.*, 2004; Gotti *et al.*, 2005; Salminen *et al.*, 2007), where labeled nicotinic agonists revealed their formation of highly diverse and complex subunit compositions (Gotti & Clementi, 2004). Additionally, glutamatergic

terminals in the rodent striatum and non-dopaminergic axons in monkeys expressed nicotinic receptors with α7 subunits (Kaiser & Wonnacott, 2000; Quik *et al.*, 2005). Double-label confocal microscopy also revealed the co-expression of 5-hydroxytryptamine ($5HT_3$) serotonin receptors and α4 nAChRs on terminals of an unknown chemical phenotype in the dorsal striatum of rats (Nayak *et al.*, 2000).

1.5.1.4 Electron microscopic localization of muscarinic and nicotinic receptors

At the ultrastructural level, ChAT-labeled terminals form symmetric synapses with the cell bodies, dendritic shafts and spines of presumed striatal MSNs, along with direct inputs to PV- or NPY-expressing interneurons in the rodent dorsal striatum (Phelps *et al.*, 1985; Phelps & Vaughn, 1986; DiFiglia, 1987; Izzo & Bolam, 1988; Chang & Kita, 1992; Vuillet *et al.*, 1992; Ligorio *et al.*, 2009). To our knowledge, electron microscopic studies of cholinergic receptor localization have only been carried out in the caudate-putamen complex of rats and mice and the caudate nucleus of monkeys. In regards to pre-synaptic localization, M1 mAChRs were found in terminals forming asymmetric or symmetric synapses in the dorsal striatum of monkeys, while in rats, only boutons that formed asymmetric synapses were shown to express the M1 subtype (Hersch *et al.*, 1994; Hersch & Levey, 1995; Alcantara *et al.*, 2001). However, spines were found to be the main neuronal element that expressed M1 mAChRs in rats and monkeys (Hersch *et al.*, 1994; Hersch & Levey, 1995; Alcantara *et al.*, 2001). In double-labeling experiments at the light microscopic level, M1 receptor expression was found in calbindin-labeled, but not PV- or NADPH-labeled neurons, suggesting a preferential M1 expression in MSNs over GABAergic interneurons (Alcantara *et al.*, 2001). The expression of M2 mAChRs was found in soma, dendritic shafts, and in many terminals that typically formed symmetric synapses with dendritic shafts and spines in rats and monkeys (Hersch *et al.*, 1994; Hersch & Levey, 1995; Bernard *et al.*, 1998; Thomas *et al.*, 2000; Alcantara *et al.*, 2001). Interestingly, M2 expression was also seen at the sites of putative dendro-dendritic synaptic interactions (Hersch *et al.*, 1994). In the rodent dorsal striatum, M3 receptor expression occurred in the spines of small-sized spiny dendrites, and

in a similar manner as M4 receptors, in terminals that formed asymmetric axo-spinous synapses (Hersch *et al.*, 1994; Hersch & Levey, 1995). Electron microscopic observations with immunogold labeling was utilized by Bernard et al. (1998, 1999) to illustrate that M2 mAChRs are located at the plasma membranes of ChAT-labeled soma and dendrites, whereas M4 receptors are located intracellularly, especially in the endoplasmic reticulum, under normal conditions. However, after acute oxotremorine (i.e., a non-selective muscarinic agonist) treatment in rats, M2 receptors re-localized from the plasma membranes of ChAT-labeled neurons to internally-located endosomes, while M4 receptors expressed in ChIs did not re-locate (Bernard *et al.*, 1998; Bernard *et al.*, 1999). In addition to their intracellular localization, immunogold-labeled M4 receptors were found to be located extrasynaptically under the electron microscope (Bernard *et al.*, 1999), contrasting prior observations with M4 immunoperoxidase labeling that suggested these receptors were located at post-synaptic densities (Hersch *et al.*, 1994).

Through mainly pharmacological and electrophysiological approaches, nicotinic acetylcholine receptors were shown to be pre-synaptically located in neurons from various areas of the brain (Léna *et al.*, 1993; Brumwell *et al.*, 2002; Jones & Wonnacott, 2004; Poisik *et al.*, 2008), resulting in the assumption that nAChRs are similarly localized in the striatum. However, electron microscopic observations in the rat dorsal striatum demonstrated the extrasynaptic localization of β2 subunits in tyrosine hydroxylase-labeled nigrostriatal terminals that formed symmetric synapses with spines (Jones *et al.*, 2001). Additionally, β2 subunits were extrasynaptically located at non-labeled symmetric or asymmetric synapses with dendritic shafts (Hill *et al.*, 1993; Jones *et al.*, 2001). Electron microscopic studies are needed to characterize the location of the remaining subunits of nicotinic receptors in the dorsal striatum of rodents and primates.

1.5.2 Modulation of striatal activity by cholinergic receptors

1.5.2.1 Muscarinic and nicotinic regulation of cholinergic excitability in the striatum

Spike firing by ChIs sets a regulatory tone in the striatum through the tonic release of ACh, acting upon diverse muscarinic and nicotinic receptors located on striatal GABAergic neurons and their afferents (see sections 1.6.1.1 and 1.6.1.2; refer to reviews (Zhou *et al.*, 2003; Exley & Cragg, 2008; Havekes *et al.*, 2011; Oldenburg & Ding, 2011; Wevers, 2011)). Additionally, they can influence endogenous cholinergic activity, mainly through mAChRs located on ChIs themselves, as well as nAChRs on their non-cholinergic synaptic inputs. However, a discrepancy in the literature exists regarding the subtype of mAChR (M2 versus M4) that is responsible for ChI autoinhibition in the dorsal striatum of rodents (reviewed by (Zhou *et al.*, 2003)).

In striatal slices from rats, the application of oxotremorine (a non-selective muscarinic agonist) was shown to decrease potassium-evoked ACh release(Zhang *et al.*, 2002a) and calcium currents through N- and P-type channels located on the somatodendritic membranes of ChAT-expressing neurons, which was antagonized by a muscarinic or $G_{i/o}$ antagonist, suggesting an M2 and/or M4 modulation (Yan & Surmeier, 1996; Calabresi *et al.*, 1998c; Calabresi *et al.*, 2000). In a later study, decreased calcium currents were found to reduce the slow spike after-hyperpolarization of ChIs, thereby converting spontaneous, tonic firing to rhythmic bursting (Bennett *et al.*, 2000). In knock-out mice, the deletion of M4 but not M2 mAChRs abolished muscarinic pre-synaptic inhibition of striatal ACh release (Zhang *et al.*, 2002a), whereas a fairly selective M2 receptor antagonist (AF-DX 116) caused an increase in endogenous ACh release in rodent brain slices, likely through the hindrance of muscarinic autoinhibition (Galarraga *et al.*, 1999). Additionally, intracellular recordings from rat slices revealed an ACh-induced hyperpolarization of ChIs, which was blocked by the application of methoctramine (a partially-selective M2 antagonist) (Calabresi *et al.*, 1998c; Centonze *et al.*, 2003). A few factors may

contribute to these differing results found in the rodent dorsal striatum, such as differences in (1) variable mAChR expression between individual ChIs (Weiner *et al.*, 1990; Bernard *et al.*, 1992; Hersch *et al.*, 1994; Yan & Surmeier, 1996), (2) differential muscarinic modulation of ChIs between rodent species and strains (Van der Zee & Keijser, 2011), (3) differential M2 and M4 receptor trafficking properties after acute and chronic muscarinic receptor activation (see section 1.6.1.1) (Bloch *et al.*, 2003), and (4) variable selectivity of muscarinic ligands used in these studies (refer to section 1.6.3) (Digby *et al.*, 2010). With the development of more highly selective drugs, the muscarinic modulation of ChIs in the striatum can be characterized more rigorously, which may provide insight into this discrepancy in rodents and primates.

Recent data propose that nicotinic modulation of ChIs also takes place in the dorsal striatum of rodents. In slices from the caudate-putamen complex, ChIs were shown to inhibit their own activity through nicotinic receptor activation of their GABAergic afferents (i.e., neurogliaform/NPY-containing interneurons and other GABAergic striatal neurons), resulting in a synchronized pause in cholinergic activity (for a review, see (Schulz & Reynolds, 2013)) (Sullivan *et al.*, 2008; English *et al.*, 2012). However, the subunit composition of the nAChRs expressed by these presynaptic afferents is currently unknown.

1.5.2.2 Muscarinic and nicotinic modulation of GABAergic striatal neurons

ACh-induced activation of mAChRs can facilitate or suppress the activity of striatal neurons (for reviews, see (Zhou *et al.*, 2003; Bonsi *et al.*, 2011; Oldenburg & Ding, 2011)), because of their different G protein-coupling, multifarious neuronal and synaptic expression, and close proximity to nAChRs (Raiteri *et al.*, 1990; Calabresi *et al.*, 2000; de Rover *et al.*, 2002; Wang *et al.*, 2006; Grilli *et al.*, 2009; Witten *et al.*, 2010; Oldenburg & Ding, 2011). In particular, cholinergic receptors can influence GABA release in the striatum, likely through complex interactions between mAChRs and nAChRs co-expressed on the same GABAergic terminals (Raiteri *et al.*, 1990; Sugita *et al.*, 1991; de Rover *et al.*, 2002; Grilli *et al.*, 2009; Luo *et al.*, 2013). For instance, in synaptosomes from the dorsal striatum, oxotremorine inhibits,

whereas atropine and the presumed M4 antagonist MT3 facilitate, depolarization-evoked release of GABA, likely from presynaptic terminals (Raiteri *et al.*, 1990; Grilli *et al.*, 2009). Contrastingly, nicotine and a variety of nAChR agonists evoked GABA release in striatal synaptosomes (and in other experimental configurations), which was counteracted by muscarinic activation and/or antagonism of α4β2 subunits (Lu *et al.*, 1998; Grilli *et al.*, 2009). Therefore, co-expression of M4 mAChRs and α4β2 nAChRs is likely to exist on the same GABAergic terminals (of unknown phenotype) (Grilli *et al.*, 2009).

At the cellular level, oxotremorine application resulted in a decreased sIPSC frequency and amplitude in MSNs. In contrast, these were increased by nicotine most likely acting on presynaptic nAChRs in slices from the caudate-putamen complex of rodents (de Rover *et al.*, 2002; Liu *et al.*, 2007). M1 receptor activation directly contributes to MSN depolarization and dendritic excitability through the coordinated modulation of calcium and potassium channels, or indirectly by regulating the suppression of the endocannabinoid system in the rodent dorsal striatum, resulting in the shaping of the spiking activity of MSNs (Shen *et al.*, 2005; Wang *et al.*, 2006; Narushima *et al.*, 2007; Shen *et al.*, 2007; Oldenburg & Ding, 2011). On the other hand, MSN inhibition can occur indirectly through the M2-induced suppression of neurotransmitter release from glutamatergic and/or cholinergic terminals (Alcantara *et al.*, 2001; Ding *et al.*, 2010; Goldberg *et al.*, 2012). M4 receptor signaling is thought to shape the firing activity of MSNs and their up- and down-state transitions (Howe & Surmeier, 1995; Perez-Rosello *et al.*, 2005; Ding *et al.*, 2006). Recently, findings from genetically-modified mice demonstrate a possible difference in the muscarinic regulation of direct and indirect MSN excitability. For example, M1 receptors modulate the basal dendritic excitability of striatopallidal (but not striatonigral) neurons via downregulation of their Kir2 potassium channels (Shen *et al.*, 2007), along with the facilitation of their dopamine/DARP-32 signaling (Kuroiwa *et al.*, 2012).

In contrast, a lack of evidence subsists for nAChR expression by MSNs, leading to the proposal that MSN excitability undergoes indirect nicotinic regulation, such as di-synaptically

through intermediate GABAergic, glutamatergic, serotonergic and/or dopaminergic neurons, as well as through the co-localization of nicotinic and muscarinic receptors on these afferents (Kaiser & Wonnacott, 2000; Wonnacott *et al.*, 2000; de Rover *et al.*, 2002; Koos & Tepper, 2002; Marchi *et al.*, 2002; Oldenburg & Ding, 2011; Luo *et al.*, 2013). In patch-clamp recordings from caudate-putamen slices, nicotinic agonists were shown to directly activate GABAergic interneurons that express PV, TH, or NPY (neurogliaform and non-neurogliaform) but not D1- or D2-containing MSNs (Liu *et al.*, 2007; Luo *et al.*, 2013). However, only neurogliaform/NPY and TH-containing interneurons in this particular environment demonstrated the ability to induce nAChR-mediated (i.e., carbachol plus atropine) GABAergic responses in MSNs, along with changes in their firing patterns (Luo *et al.*, 2013). On the other hand, in whole-cell recordings from rat dorsal striatal slices, ACh and nicotine application resulted in the depolarization of fast-spiking (putative PV-containing) interneurons (FSIs), while mAChR-mediated presynaptic inhibition interrupted MSN inhibition FSIs (Koos & Tepper, 2002). Taken together, these findings suggest that *in vitro* muscarinic modulation may overpower nicotinic regulation of PV-containing interneurons in the dorsal striatum of rats, an effect that could be dependent on the basal ACh levels and firing activity of ChIs at the time of recordings (Koos & Tepper, 2002). The subtypes of receptors involved in muscarinic and nicotinic modulation of GABA release and MSN excitability remain to be established in the dorsal striatum of primates.

Cholinergic modulation of glutamatergic transmission also occurs in the dorsal striatum, mainly through the activation of M1 and M2/M3 mAChRS. Presynaptic M2/M3 mAChR activation decreased the probability of multi-vesicular release from glutamatergic terminals, along with glutamate release during the induction phase of long-term potentiation (LTP) in rodents, thereby reducing corticostriatal synaptic transmission (Calabresi *et al.*, 1998a; Higley *et al.*, 2009). However, muscarine and a putative M1 receptor agonist enhanced NMDA (but not AMPA) receptor-induced increases in MSN depolarization in rat slices from the dorsal striatum, which could be blocked by antagonism of post-synaptic M1 mAChRs on striatal projection

neurons (Calabresi *et al.*, 1998b; Barral *et al.*, 1999). In slice preparations from the caudate-putamen, cholinergic single spikes depressed cortically-evoked EPSCs in one-third of MSNs located within 100 μm of the spiking ChI (Pakhotin & Bracci, 2007), whereas the non-selective muscarinic agonist oxotremorine, as well as a burst in ChI firing induced by thalamic activation (thought to mimic salient stimuli), inhibited corticostriatal and thalamostriatal excitation of D1- and D2-expresssing MSNs (Ding *et al.*, 2010). When a pause in ChI firing follows this initial burst, post-synaptic M1 receptors activated during the initial burst combined with the re-activation of cortical and thalamic inputs led to the facilitation of dendritic responsiveness by striatopallidal neurons, providing a possible explanation for how salient stimuli transiently impede action outcomes/behaviors (Ding *et al.*, 2010). With the development of highly selective muscarinic drugs, the mAChRs underlying these effects in the dorsal striatum can be delineated in primates.

1.5.2.3 Muscarinic modulation of striatal dopamine activity

On the basis of anatomical observations, ChIs may directly and indirectly regulate dopaminergic activity in the striatum through presynaptic activation of cholinergic receptors located on dopaminergic, GABAergic, glutamatergic, and cholinergic axons (Hersch *et al.*, 1994; Hersch & Levey, 1995; Alcantara *et al.*, 2001; Rice *et al.*, 2011). For example, muscarinic and nicotinic regulation of dopamine release in the caudate-putamen complex requires M2/M4 mAChRs and nAChRs with α4β2 and α4α5β2 subunits. However, these rodent data are at odds with nonhuman primate studies showing a strong α6β2 and α3β2 nicotinic receptor-mediated modulation of dopamine release in the striatum (Quik *et al.*, 2005; Perez *et al.*, 2009; Quik *et al.*, 2011; Quik & Wonnacott, 2011).

Findings from muscarinic knock-out mouse slices demonstrate that ACh-mediated (i.e., oxotremorine-induced) dopamine release is enhanced primarily by the activation of M4 and less so by M5 mAChRs, but inhibited by the activation of M3 receptors (Zhang *et al.*, 2002b; Yamada *et al.*, 2003). Even though strong anatomical evidence for the expression of M5 mAChRs are

currently lacking in the dorsal striatum, it has been hypothesized that their regulation of dopamine release occurs through their (possible) expression on dopaminergic terminals, while M4 mAChRs are likely indirectly mediating dopamine release through the regulation of neurotransmitter release from intrastriatal GABAergic terminals (Zhang *et al.*, 2002b; Yamada *et al.*, 2003). However, since the expression of M3 mAChRs is mainly localized in terminals forming asymmetric synapses (Hersch *et al.*, 1994; Hersch & Levey, 1995), it is unclear how these receptors may inhibit dopamine release by regulating neurotransmitter release from glutamatergic (and possibly serotonergic) terminals in the dorsal striatum. Dopamine release in the caudate-putamen complex has also been shown to be indirectly regulated by activation of the pedunculopontine (PPN) and laterodorsal (LDT) tegmental nucleus, respectively, by means of ACh activation of mAChRs located on dopaminergic neurons in the SNc and VTA (Chapman *et al.*, 1997; Miller & Blaha, 2005; Lester *et al.*, 2008; Schmidt *et al.*, 2010; Steidl *et al.*, 2011). In regards to the mAChR subtype involved in this regulation, M5-mediated excitation of dopamine neurons in the SNc and VTA and/or M3-mediated inhibition of dopamine release from the SNc were suggested (Miller & Blaha, 2005; Steidl *et al.*, 2011).

1.5.2.4 Nicotinic modulation of striatal dopamine activity

Over the past forty years, the nicotinic regulation of mesostriatal and nigrostriatal dopaminergic systems has been of significant interest (reviewed by (Zhou *et al.*, 2002; Grady *et al.*, 2007; Exley & Cragg, 2008; Rice *et al.*, 2011; Threlfell & Cragg, 2011)), resulting from their diverse roles in a number of neurological and psychiatric disorders (for reviews, refer to (Zhou *et al.*, 2003; Calabresi *et al.*, 2006; Grady *et al.*, 2007; Janhunen & Ahtee, 2007; Livingstone & Wonnacott, 2009; Threlfell & Cragg, 2011; Quik *et al.*, 2012)) , as well as in encoding motivational value and/or salience that underlie action selection (Morris *et al.*, 2004; Ding *et al.*, 2010; Threlfell *et al.*, 2012). As described by Exley and Cragg (2008), nicotinic receptor activation generally facilitates striatal dopamine release by acting as "presynaptic filters," utilizing the spiking activity (i.e., frequency and pattern) of dopaminergic and non-dopaminergic

neurons to regulate the probability of neurotransmitter release from dopaminergic axons/terminals (Exley & Cragg, 2008).

Because of their ability to detect subsecond changes in extracellular concentrations of dopamine, voltammetric and amperometric techniques using carbon-fiber microelectrodes were utilized to study the nicotinic modulation of dopamine release between striatal regions (Wightman, 2006; Rice *et al.*, 2011). For instance, the frequency dependence of dopamine release after intrastriatal or dopaminergic stimulation varies between the rat caudate-putamen complex and nucleus accumbens (Zhou *et al.*, 2001; Rice & Cragg, 2004; Exley & Cragg, 2008; Zhang *et al.*, 2009a; Zhang *et al.*, 2009b), as well as between the monkey dorsal and ventral putamen (McCallum *et al.*, 2006; Quik & McIntosh, 2006; Perez *et al.*, 2009). Because of their higher probability of dopamine release in response to a single stimulus (Cragg, 2003; Rice & Cragg, 2004; Zhang & Sulzer, 2004), dopaminergic terminals in the dorsolateral striatum responded more robustly (i.e., released more neurotransmitter) to an arriving single action potential than those in the accumbens shell, which could be suppressed by nicotinic (partially, β2) receptor antagonism and/or desensitization of these terminals (Zhou *et al.*, 2001; Rice & Cragg, 2004; Zhang & Sulzer, 2004; Zhang *et al.*, 2009a; Zhang *et al.*, 2009b; Cachope *et al.*, 2012) via possible changes in their pre-synaptic nAChR-mediated calcium regulation (Rathouz & Berg, 1994; Grady *et al.*, 1997; Wonnacott *et al.*, 2000; Zhou *et al.*, 2001). Contrastingly, pulse-train stimulations alone or in combination with nicotine application induced a larger enhancement of dopamine release in the accumbens shell than in the dorsolateral striatum (Zhou *et al.*, 2001; Rice & Cragg, 2004; Exley & Cragg, 2008; Zhang *et al.*, 2009a; Zhang *et al.*, 2009b), signifying that dopaminergic afferents in the nucleus accumbens have a greater potential for the facilitation of dopamine release perhaps due to their initial, lower probability of release (Zhang *et al.*, 2009a; Zhang *et al.*, 2009b). Interestingly, dopamine transporter (DAT) density differences (i.e., lower DAT levels in the shell than in the dorsal striatum (Coulter *et al.*, 1996)) could not entirely

account for the variable dopaminergic signaling found between these striatal regions (Zhang *et al.*, 2009a; Zhang *et al.*, 2009b) .

In monkey slices, antagonism of α3α6β2 nAChRs, with a minor contribution by α4β2 nicotinic receptors, decreased single-pulse electrically-stimulated dopamine release in the ventral putamen (McCallum *et al.*, 2006; Quik & McIntosh, 2006; Perez *et al.*, 2009), while this regulation mostly underwent α3α6β2 modulation in the monkey dorsal putamen (Perez *et al.*, 2009). In the monkey dorsal striatum, the effects of nicotinic antagonism on dopamine release induced by single pulse or burst stimulations were similar, in that dopamine release was predominantly regulated by α3α6β2 nAChRs (Perez *et al.*, 2009). In contrast, nAChR antagonists under these same conditions did not affect electrically-evoked dopamine release in the monkey dorsal striatum (Perez *et al.*, 2009). Additionally, chronic nicotine treatment increased single-pulse- and burst-evoked dopamine release across the entire putamen.(Perez *et al.*, 2009) Altogether, these findings imply that the striatal sub-region and dopaminergic firing frequency influence nicotinic modulation of dopamine release in the primate striatum (Cragg, 2003; Perez *et al.*, 2009), as observed in the rodent dorsal striatum (Rice & Cragg, 2004; Zhang *et al.*, 2009b; Exley *et al.*, 2011).

Additionally, Threlfell et al. (2012) have recently demonstrated that frequency-dependent dopamine release is not ChI-driven in the caudate-putamen of ChAT-cre mice (Cachope *et al.*, 2012; Threlfell *et al.*, 2012). The authors discovered that *in vitro* optogenetic activation of ChIs induced frequency-independent dopamine release in the dorsal striatum, suggesting that individual ChIs do not regulate their own firing frequency in order to drive dopamine release (Cachope *et al.*, 2012; Threlfell *et al.*, 2012). The authors suggest that the frequency sensitivity of ACh-driven dopamine release can be restored instead by blockade of nAChRs in the caudate-putamen complex, dominating over ascending activity from dopaminergic soma and axons (Threlfell *et al.*, 2012).

1.5.2.5 Nicotinic modulation of striatal serotonin release

In the dorsal striatum of rats, confounding findings have been shown in regards to nicotinic receptor-mediated serotonin release. In general, co-localized α4 (but not α3 and α5) nAChRs and $5HT_3$ receptors have been shown to increase presynaptic intracellular calcium in rat striatal synaptosomes (Nayak *et al.*, 2000; Dougherty & Nichols, 2009), indicating possible modulation of neurotransmitter release and/or other presynaptic events by both nicotinic and serotonergic receptors located on the same terminals (Wonnacott, 1997; Barnes & Sharp, 1999; Vizi & Lendvai, 1999; Gotti & Clementi, 2004). In superfused rat slices and synaptosomes from the dorsal striatum, nicotine increased serotonin release in a dose-dependent manner (Yu & Wecker, 1994; Reuben & Clarke, 2000), whereas locally-applied ACh or nicotine (but not mAChR agonists) decreased *in vivo* serotonin release in the feline caudate nucleus (Becquet *et al.*, 1988). Interestingly, in the latter study, the nicotine receptor-mediated effects on serotonin release were blocked by application of a GABAergic antagonist, implying that these effects are perhaps indirectly mediated (with unknown mechanisms) by striatal GABAergic interneurons but not projection neurons (refer to Section 1.6.1.2) (Becquet *et al.*, 1988). Although a discrepancy exists between the results from *in vitro* versus *in vivo* studies, evidence demonstrates that serotonin release in the dorsal striatum is under modulation by the nicotinic cholinergic system, but whether this occurs in the primate dorsal striatum remains to be established.

1.6 Synaptic regulation of striatal cholinergic interneurons

1.6.1 General synaptic innervation of cholinergic interneurons

Numerous studies have provided insight into the underlying regulatory mechanisms of striatal cholinergic neurons' activity (Nicola *et al.*, 2000; Zhou *et al.*, 2002; Pisani *et al.*, 2007; Smith & Villalba, 2008; Williams & Adinoff, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Goldberg & Reynolds, 2011; Gutierrez *et al.*, 2011; Mark *et al.*, 2011; Threlfell & Cragg, 2011; Goldberg *et al.*, 2012; Schulz & Reynolds, 2013). Analyses of their

receptor expression pattern and intrinsic membrane properties have been discussed in detail in recent reviews (Nicola *et al.*, 2000; Zhou *et al.*, 2002; Pisani *et al.*, 2007; Smith & Villalba, 2008; Williams & Adinoff, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Goldberg & Reynolds, 2011; Gutierrez *et al.*, 2011; Mark *et al.*, 2011; Threlfell & Cragg, 2011; Goldberg *et al.*, 2012; Schulz & Reynolds, 2013). Although various studies have provided qualitative evidence for diverse synaptic inputs to ChIs, the functional significance of these findings towards our understanding of the overall synaptic regulation of ChIs remains limited due to the mere qualitative description of these observations.

Qualitative electron microscopic observations of either Golgi-impregnated "spidery" neurons or ChAT-ir cells in the primate striatum have revealed that ChIs receive symmetric and asymmetric synaptic inputs along the full length of their somatodendritic domain (Difiglia *et al.*, 1980; DiFiglia & Carey, 1986; DiFiglia, 1987). Although it is often assumed that terminals forming symmetric synaptic specializations have a GABAergic phenotype (Gerfen, 1988; Pasik *et al.*, 1988; Ribak & Roberts, 1990; Roberts *et al.*, 1996), such is not the case in the striatum (***Figure 1.4***). Because of its highly heterogeneous chemical composition, symmetric synapses on striatal neurons can involve GABAergic, dopaminergic, and cholinergic terminals (Haber, 1986; Semba *et al.*, 1987; Gerfen, 1988; Graybiel, 1990; Smith *et al.*, 1994b; Bolam *et al.*, 2000; Tepper *et al.*, 2010; Crittenden & Graybiel, 2011). In comparison, rodent ChIs mainly receive symmetric and asymmetric synaptic inputs onto their distal dendrites (Chang & Kitai, 1982; Bolam *et al.*, 1984b; Phelps *et al.*, 1985; Phelps & Vaughn, 1986; Meredith *et al.*, 1990; Lapper & Bolam, 1992; Dimova *et al.*, 1993; Ligorio *et al.*, 2009; Sizemore *et al.*, 2010), but due to the lack of quantification, these data remain mainly descriptive and hard to interpret in relation to the functional compartmentation of synaptic afferents along the somatodendritic domain of ChIs in primates. Another limiting factor is the lack of neurochemical markers to identify the source(s) of the different synaptic afferents to ChIs.

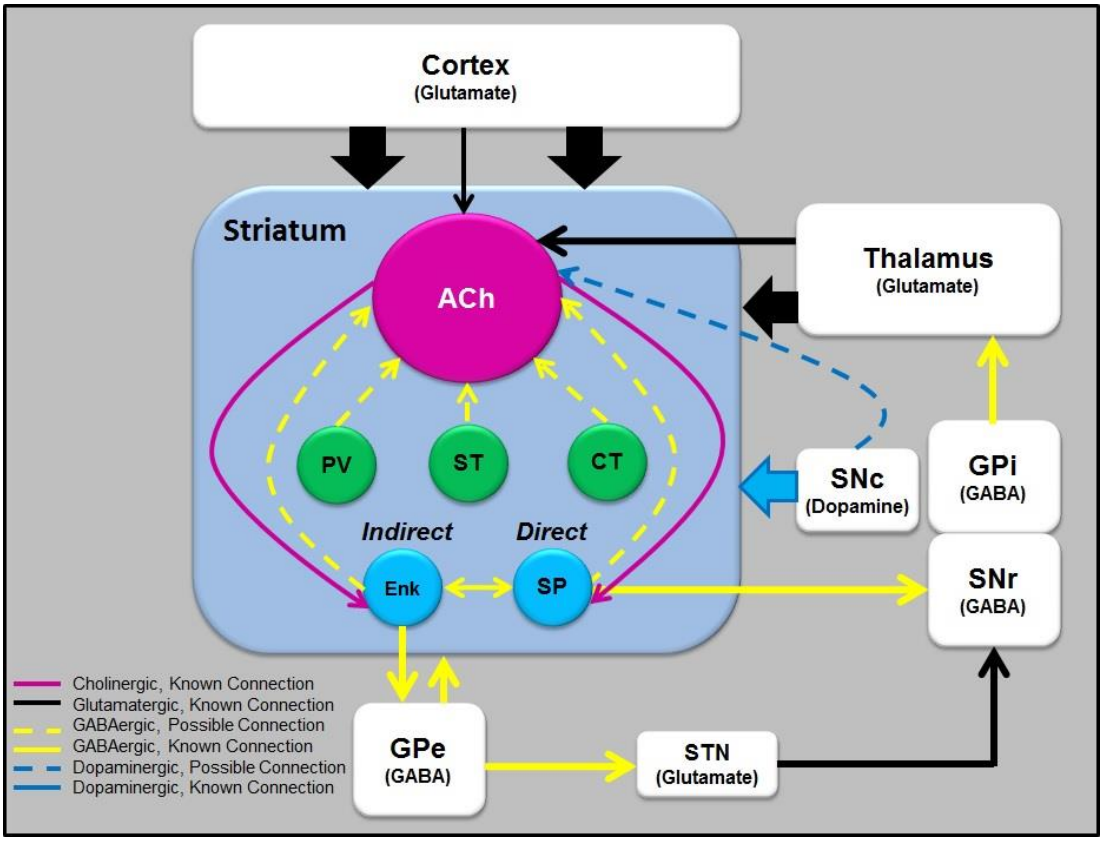

***Figure 1.4*. Schematic of the intrinsic microcircuitry of cholinergic interneurons within the dorsal striatum.**
The connections between striatal cholinergic interneurons and their afferents that have been previously reported (solid arrows) and those that are unknown (dotted arrows) are shown. The thickness of the arrows indicates that a large number of neurons from a particular nucleus form synapses in the target region.

1.6.2 Glutamatergic regulation of cholinergic interneurons

Although both the cerebral cortex and thalamus are the two main sources of glutamatergic inputs to the striatum, qualitative evidence has shown that the thalamostriatal system, particularly thalamic afferents from the caudal intralaminar nuclei (i.e., the CM/Pf), are a key source of glutamatergic synaptic inputs to striatal ChIs in rodents and primates (***Figure 1.4***) (Lapper & Bolam, 1992; Sidibe & Smith, 1999). In contrast, various studies aimed at assessing synaptic relationships between cortical terminals and ChIs have shown that corticostriatal afferents may provide a scarce and distal innervation of ChAT-labeled neurons in rodents and monkeys (***Figure 1.4***) (Lapper & Bolam, 1992; Dimova *et al.*, 1993; Thomas *et al.*, 2000). These

ultrastructural findings are hard to reconcile with some of the *in vitro* and *in vivo* electrophysiological and pharmacological studies in rats showing that cortical stimulation can elicit short-latency excitatory responses in TANs (Wilson *et al.*, 1990; Pisani *et al.*, 2000; Suzuki *et al.*, 2001; Nambu *et al.*, 2002; Reynolds & Wickens, 2004; Sharott *et al.*, 2012) and induce extracellular acetylcholine (ACh) release in the striatum (Taber & Fibiger, 1994; Baldi *et al.*, 1995; Consolo *et al.*, 1996a). On the other hand, despite anatomical evidence for a monosynaptic connection between thalamic inputs from the CM/Pf complex and striatal ChIs, our recent *in vivo* electrophysiological data have demonstrated that activation of the CM/Pf in monkeys results in complex multifaceted responses, consisting of combined increases and decreases in the firing of TANs and a reduction of striatal ACh levels (Nanda *et al.*, 2009). It remains to be determined whether these discrepancies rely on the fact that the correspondence between TANs and ChIs is not as close as originally thought (Beatty *et al.*, 2012), or result from the recruitment of complex intrastriatal GABAergic networks that impose strong inhibitory influences upon ChIs (see below) (Consolo *et al.*, 1996a; Matsumoto *et al.*, 2001; Suzuki *et al.*, 2001; Nanda *et al.*, 2009; Ding *et al.*, 2010).

Thus altogether, studies from the striatum have shown that the CM/Pf complex is a prominent source of thalamic inputs to ChIs in primates and non-primates (Lapper & Bolam, 1992; Sadikot *et al.*, 1992b). Nevertheless, the exact proportion of cortical and thalamic inputs at the level of single ChIs awaits further quantitative ultrastructural analyses in the dorsal striatum.

1.6.2.1 Glutamatergic receptors in cholinergic interneurons

Striatal ChIs express various subtypes of ionotropic and metabotropic glutamate receptor protein and mRNA (Landwehrmeyer *et al.*, 1995; Testa *et al.*, 1995; Chen *et al.*, 1996; Standaert *et al.*, 1999; Küppenbender *et al.*, 2000; Bell *et al.*, 2002; Bloomfield *et al.*, 2007; Deng *et al.*, 2007b; Deng *et al.*, 2010), which is consistent with a large number of pharmacological studies showing that direct or indirect activation of the glutamatergic system results in changes in ACh release and/or the depolarization of ChIs (Di Chiara *et al.*, 1994; Pisani *et al.*, 2002; Pisani *et al.*,

2003; Bonsi *et al.*, 2008; Goldberg & Reynolds, 2011). Additionally, thalamic inputs from Pf regulate ChIs activity predominantly through NMDA receptor activation, while the effects of cortical afferents are mainly mediated through AMPA receptor activation (Di Chiara *et al.*, 1994; Baldi *et al.*, 1995; Consolo *et al.*, 1996a; Consolo *et al.*, 1996b; Ellender *et al.*, 2013). Because of the limited information on the synaptic relationships between subtypes of glutamate receptors and their presynaptic afferent terminals from the cerebral cortex or thalamus, the underlying substrate for these specific glutamate receptor-mediated effects on ChIs is unknown in the dorsal striatum.

At the ultrastructural level, both group I metabotropic glutamate receptors (mGluRs), i.e.,mGluR1 and mGluR5, are expressed extrasynaptically, or at the edges of glutamatergic synapses, in dendrites of ChAT-labeled neurons in the rat dorsal striatum (Tallaksen-Greene *et al.*, 1998). Although direct evidence remains to be provided, the extrasynaptic glutamate spillover from cortical or thalamic terminals and/or the astrocytic release of glutamate are the most likely sources of activation of these receptors (Galvan *et al.*, 2006). Furthermore, because of the diverse chemical composition, receptor expression and afferentation between the functional subterritories of the striatum along with a heterogeneous population of ChIs, caution should be taken when translating observations made about the glutamatergic regulation of ChIs in a specific striatal region to other sectors of the dorsal striatum.

1.6.3 GABAergic regulation of cholinergic interneurons

Even if a detailed quantification of the synaptic innervation of ChIs has not yet been carried out in the primate striatum, GABAergic synapses could account for the largest contingent of afferent inputs to dorsal striatal ChIs, due to their high density of symmetric-like synapses (Difiglia *et al.*, 1980; DiFiglia & Carey, 1986; DiFiglia, 1987). GABAergic afferents from striatal projection neurons and various populations of striatal interneurons may contribute to this innervation in primates (***Figure 1.4***) (Chang & Kita, 1992; Vuillet *et al.*, 1992), thereby providing a solid substrate through which GABA may act as a key regulator of ChIs activity in the striatum.

In the rodent caudate-putamen complex, there is also qualitative evidence that GABAergic terminals are a source of inputs to ChIs (Bolam *et al.*, 1984b; Phelps *et al.*, 1985; Dimova *et al.*, 1993), in particular those that express substance P but not enkephalin (Bolam *et al.*, 1986; Martone *et al.*, 1992). In line with strong GABAergic influences, ChIs in the dorsal striatum are enriched in both GABA(A) and GABA(B) receptor subunits in rats and humans (Yan & Surmeier, 1997; Waldvogel *et al.*, 1999; Yung *et al.*, 1999; Waldvogel *et al.*, 2004), and electrophysiological evidence from *in vitro* and *in vivo* preparations demonstrate that activation of either receptor subtype elicits inhibitory synaptic responses in ChIs (Yan & Surmeier, 1997; Bennett & Wilson, 1998; Pisani *et al.*, 2000) and reduces *in vivo* ACh release (Stoof *et al.*, 1979; Anderson *et al.*, 1993; DeBoer & Westerink, 1994).

1.6.4 Peptidergic regulation of cholinergic interneurons

Another important observation made from studies discussed above is that the neuropeptides substance P and enkephalin, expressed in axon collaterals of striatal projection neurons, may also contribute to the regulation of ChIs. For instance, dorsal striatal ChIs display strong mRNA and protein expression for neurokinin 1 (NK1) receptors (Gerfen *et al.*, 1991; Kaneko *et al.*, 1993; Aubry *et al.*, 1994; Parent *et al.*, 1995; Richardson *et al.*, 2000) and 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). At the functional level, SP and Enk have opposite effects on the activity of ChIs in the rodent striatum. While ChIs become depolarized and increase their discharge of ACh after the striatal release of SP (or bath-applied SP) (Arenas *et al.*, 1991; Aosaki & Kawaguchi, 1996; Bell *et al.*, 1998; Perez *et al.*, 2007; Govindaiah *et al.*, 2010), opposite effects are elicited following local Enk-mediated activation of mu (and delta)-opioid receptors (Mulder *et al.*, 1984; De Vries *et al.*, 1989; Lapchak *et al.*, 1989; Izquierdo, 1990; Jiang & North, 1992; Lendvai *et al.*, 1993; Miura *et al.*, 2007; Ponterio *et al.*, 2013). In contrast, systemic administration of low doses of SP decreased extracellular ACh concentrations in the

striatum of freely moving rats (Boix *et al.*, 1994), most likely through activation of multi-synaptic pathways. The *in vitro* activation of mu- and delta-opioid receptors resulted in decreased ACh release in the caudate-putamen complex (Mulder *et al.*, 1984; De Vries *et al.*, 1989; Lapchak *et al.*, 1989; Izquierdo, 1990; Jiang & North, 1992; Lendvai *et al.*, 1993; Miura *et al.*, 2007).

Altogether, it appears that ChIs in the dorsal striatum are regulated by GABAergic afferents from unknown sources in primates, while in rodents, this relationship involves mainly GABAergic axon collaterals from direct pathway striatofugal neurons. In addition to GABA, the striatal neuropeptides SP and Enk also contribute to the regulation of ChIs. Nevertheless, further studies must be performed to determine if this peptidergic modulation occurs in concert or in parallel with that mediated by the GABAergic system.

1.6.5 Dopaminergic regulation of cholinergic interneurons

A substantial amount of pharmacological, electrophysiological and neurochemical data suggest close functional interactions between the nigrostriatal and mesostriatal dopaminergic systems and striatal ChIs in normal and diseased states (Nicola *et al.*, 2000; Zhou *et al.*, 2002; Pisani *et al.*, 2007; Smith & Villalba, 2008; Williams & Adinoff, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Crittenden & Graybiel, 2011; Goldberg & Reynolds, 2011; Gutierrez *et al.*, 2011; Mark *et al.*, 2011; Threlfell & Cragg, 2011; Goldberg *et al.*, 2012; Schulz & Reynolds, 2013). However, the exact synaptic mechanisms by which these interactions are mediated are complex and remain poorly understood (***Figure 1.4***). At the ultrastructural level, Kubota et al. (1987) demonstrated that ChAT-ir soma and proximal dendrites receive direct synaptic inputs from tyrosine hydroxylase (TH)-labeled terminals in the rat caudate-putamen, while other studies in rodents have shown close appositions, with only scarce direct synaptic connections, between putative dopaminergic terminals and ChIs (Freund *et al.*, 1984; Aoki & Pickel, 1988; Chang, 1988; Pickel & Chan, 1990; Smith *et al.*, 1994b).

Despite this paucity of synaptic contacts, it is clear that ChIs activity is highly sensitive to dopamine receptor modulation. In the dorsal striatum, D2 dopamine receptor mRNA is strongly expressed by ChIs (Le Moine *et al.*, 1990; Berendse & Richfield, 1993; Nicola *et al.,* 2000; Alcantara *et al.*, 2003), with the greatest densities being found in the dorsolateral caudate-putamen complex of rodents (Jongen-Rêlo *et al.*, 1995). The majority of striatal ChIs also express moderate to high levels of D5 dopamine receptors (Le Moine *et al.*, 1991; Bergson *et al.*, 1995; Jongen-Rêlo *et al.*, 1995; Yan & Surmeier, 1997; Nicola *et al.*, 2000; Rivera *et al.*, 2002; Berlanga *et al.*, 2005). Due to the high degree of D2 and D5 dopamine receptor expression by ChIs, it is likely that D2/D5 co-expression exists in the majority of dorsal striatal ChIs. However, D3 dopamine receptor expression is undetectable in ChIs of the rodent dorsal striatum (Yan & Surmeier, 1997).

In rodents and monkeys, *in vitro* and *in vivo* electrophysiological and pharmacological studies have revealed that cholinergic activity in the dorsal striatum is significantly altered by manipulation of the nigral dopaminergic systems, resulting in a wide range of receptor subtype-specific responses (i.e., no effect, increased or decreased cholinergic activity) (Damsma *et al.*, 1991; Henselmans & Stoof, 1991; Imperato *et al.*, 1992; Aosaki *et al.*, 1994a; Di Chiara *et al.*, 1994; DeBoer *et al.*, 1996; Abercrombie & DeBoer, 1997; Yan *et al.*, 1997; Yan & Surmeier, 1997; Consolo *et al.*, 1999; Pisani *et al.*, 2007; Aosaki *et al.*, 2010; Goldberg & Reynolds, 2011; Mark *et al.*, 2011; Threlfell & Cragg, 2011; Schulz & Reynolds, 2013). Additionally, dopamine can act as a modulator of cholinergic function in the caudate-putamen complex by regulating ChIs intrinsic cellular properties and synaptic inputs from their afferents, particularly those from the glutamatergic corticostriatal network (Yan *et al.*, 1997; Aosaki *et al.*, 1998; Bennett & Wilson, 1998; Maurice *et al.*, 2004; Reynolds *et al.*, 2004; Salgado *et al.*, 2005; Deng *et al.*, 2007a; Schulz & Reynolds, 2013). Functions of the intrastriatal cholinergic network in the dorsal striatum are significantly altered following acute or chronic chemical disruption of the nigrostriatal (Pisani *et al.*, 2007; Bonsi *et al.*, 2011; Goldberg *et al.*, 2012; Schulz & Reynolds,

2013) dopaminergic systems. Thus, it is reasonable to conclude that the ascending dopaminergic systems are major regulators of ChIs activity, but that such control is largely mediated by diffusion of non-synaptic dopamine release that can exert opposing and/or synergistic effects through the activation of specific dopamine receptors expressed by ChIs and by the manipulation of ChIs synaptic afferents and intrinsic conductances (Stoof & Kebabian, 1981; Bertorello *et al.*, 1990; Svenningsson *et al.*, 2000; Fetsko *et al.*, 2003).

1.7 Technical limitations of cholinergic receptor drugs, antibodies, and knock-out mice

The majority of drugs used to determine the autoradiographic distributions and functional activities of mAChRs in earlier studies were later found to target multiple mAChR subtypes (for reviews, see (Zhou *et al.*, 2003; Lebois *et al.*, 2010; Wevers, 2011; Melancon *et al.*, 2012b; Xiang *et al.*, 2012)), thereby significantly hampering the interpretation of data obtained with these drugs to characterize the localization and function of specific mAChR subtypes. This lack of specific muscarinic receptor compounds recently leads to a vigorous drive to develop drugs with higher mAChR selectivity for research and therapeutics (for examples, see (Bridges *et al.*, 2010a; Bridges *et al.*, 2010b; Digby *et al.*, 2010; Lebois *et al.*, 2011; Melancon *et al.*, 2012a; Salovich *et al.*, 2012)). Although specific receptor knock-out studies can avoid issues with drug selectivity, findings obtained in such animals should be interpreted with great caution. The normal endogenous activity of mAChRs is not represented in these animals, along with their ability to cross-regulate the activity of other cholinergic receptors, possibly leading to detrimental changes in mAChR levels and distribution that may not occur in the natural physiological state (Dhein *et al.*, 2013). In addition, recent findings have demonstrated that most antibodies directed against individual mAChRs (except those for M2) and α7 nAChRs may not be as specific as originally thought, based on the absence of detected differences in antibody immunolabeling in peripheral

and central tissue from wild-type and knock-out mice (Jones & Wonnacott, 2005; Jositsch *et al.*, 2009; Kirkpatrick, 2009; Michel *et al.*, 2009; Pradidarcheep *et al.*, 2009).

1.8 Experimental design, rationale, and significance

Striatal ChIs, which display unique morphological characteristics, a heterogeneous and widespread distribution throughout the striatum, and an extensively diverse microcircuitry, have long been recognized as significant regulators of striatal and basal ganglia functions in normal and diseased states (Nicola *et al.*, 2000; Zhou *et al.*, 2002; Pisani *et al.*, 2007; Smith & Villalba, 2008; Williams & Adinoff, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Goldberg & Reynolds, 2011; Gutierrez *et al.*, 2011; Mark *et al.*, 2011; Threlfell & Cragg, 2011; Goldberg *et al.*, 2012; Schulz & Reynolds, 2013). Anatomical, electrophysiological and pharmacological studies in rodents and primates have suggested that ChIs excitability is under strong GABAergic regulation through direct synaptic connectivity between cholinergic and GABAergic neurons (DiFiglia, 1987; Sullivan *et al.*, 2008; Tepper *et al.*, 2008; Ding *et al.*, 2010; Bonsi *et al.*, 2011; English *et al.*, 2012). While it is likely that most of these afferents originate from intrastriatal sources, including axon collaterals of striatal output neurons and GABAergic interneurons (Bolam *et al.*, 1986; Martone *et al.*, 1992; Bolam *et al.*, 2000; Tepper *et al.*, 2004; Wilson, 2007; Tepper *et al.*, 2008), the relative abundance and precise locations of these various GABAergic influences upon ChIs is unknown. To address this issue, I quantitatively assessed the prevalence and relative distribution of GABAergic inputs from direct (SP-expressing) and indirect (Enk-expressing) striatofugal neurons and PV-containing neurons onto ChIs in the monkey post-commissural putamen.

Striatal tissue from rhesus monkeys was processed at the electron microscopic level for the dual immunocytochemical localization of (1) GABA/ChAT; (2) SP/ChAT; (3) Enk/ChAT; and (4) PV/ChAT. Post-embedding immunogold localization of GABA combined with peroxidase immunostaining for ChAT was first carried out to determine the GABAergic and

glutamatergic innervation of ChIs in the primate dorsal striatum. In addition, pre-embedding immunogold localization of SP or Met-/Leu-Enk to label GABAergic terminals from collaterals of direct and indirect striatal projection neurons, respectively, or PV to label boutons from GABAergic PV-containing interneurons, in combination with ChAT immunoperoxidase was performed to delineate the subtype(s) of intrastriatal GABAergic inputs onto ChIs in the monkey putamen.

Altogether, findings from this thesis will provide the first detailed map of the synaptic microcircuitry of cholinergic interneurons in the sensorimotor striatum of non-human primates, thereby providing insight into the diverse nature of ChIs responses to afferent inputs, along with their highly complex downstream effects.

Chapter 2 :

GABAergic inputs from direct and indirect striatal projection neurons onto cholinergic interneurons in the primate putamen

Contains excerpts from:

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2.1 Introduction

The dorsal striatum receives glutamatergic inputs from all functional regions of the cerebral cortex and thalamus (Parent & Hazrati, 1995; Smith *et al.*, 2004; Smith *et al.*, 2009) and sends GABAergic projections to the GPe, GPi and 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 GPi and SNr) relay this information back to the cerebral cortex and striatum via the thalamus (Alexander *et al.*, 1990; Smith *et al.*, 2004; Smith *et al.*, 2009). Processing of information within the dorsal striatum involves dopaminergic inputs from the SNc, GABAergic afferents from the GPe, and intrastriatal GABAergic and cholinergic interneurons (Galvan & Wichmann, 2007; Wilson, 2007; Tepper *et al.*, 2008; Gerfen & Surmeier, 2011).

In rodents, the striatum comprises 95% projection neurons (i.e., medium spiny neurons; MSNs) and 5% interneurons (Kemp & Powell, 1971a; b; Wilson & Groves, 1980; Graveland & DiFiglia, 1985; Bolam *et al.*, 2000), while in primates, interneurons account for as much as 24% of the striatal neuron population (Pasik *et al.*, 1976; Graveland & DiFiglia, 1985; Graveland *et al.*, 1985; Roberts *et al.*, 1996; Wu & Parent, 2000). The large 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 & Kitai, 1982; DiFiglia & 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 & 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). While 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; Martone *et al.*, 1992; Bolam *et al.*, 2000; Tepper *et al.*, 2004; Wilson, 2007; Tepper *et al.*, 2008), the relative abundance of these various GABAergic influences upon ChIs is unknown. To address this issue, we quantitatively assessed the prevalence and relative distribution of GABAergic inputs onto ChIs in the monkey post-commissural 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 SP- or 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.

2.2 Materials and methods

2.2.1 Tissue processing

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).

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; 0.01M, 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 anti-freeze solution (1.4% NaH_2PO_4-H_20, 2.6% Na_2HPO_4-$7H_2O$, 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.

2.2.2 Primary antibodies

Sections from the post-commissural putamen were processed for light and electron microscopy immunoperoxidase localization of ChAT or immunogold localization of SP, Leucine [Leu^5]- and Methionine [Met^5]-Enk, or GABA using highly specific, well characterized monoclonal and polyclonal antibodies (refer to ***Table 2.1***). 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 *et al.*, 1993; Smith *et al.*, 1994a), and that preadsorption immunohistochemical assays resulted in the reduction of ChAT staining in these regions (Shiromani *et al.*, 1987; Shiromani *et al.*, 1990; Smith *et al.*, 1993; Holt *et al.*, 1997). Furthermore, Western blot analysis from human brain or placental tissue showed a distinct band at 68kDa 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 (see ***Table 2.1***). 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 & McGeer, 1984; Bolam & Izzo, 1988; Smith *et al.*, 1998; Reiner *et al.*, 1999a; Wolansky *et al.*, 2007). The lack of tissue labeling following incubation with non-immune rat serum or primary antibodies pre-absorbed 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 & 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 & 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 (Haber & Elde, 1981; DiFiglia *et al.*, 1982; Williams & Dockray, 1983; Beach & McGeer, 1984; Reiner *et al.*, 1999a).

Lastly, affinity purified rabbit polyclonal antibodies against GABA-bovine serum albumin (BSA) were used to localize GABA-containing terminals in the putamen (***Table 2.1***). Details about the production, characterization, and specificity of this antiserum have been extensively described in previous studies (Hodgson *et al.*, 1985; Somogyi & Hodgson, 1985; Smith *et al.*, 1987). In brief, biochemical specificity tests with nitrocellulose paper strips and pre-adsorption 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).

Table 2.1. Immunogens, sources, and dilutions of primary antibodies used in this study.

Antibody	Immunogen	Manufacturer Data	Dilution used
Choline acetyltransferase	Purified human placental enzyme	Chemicon (AB143), rabbit polyclonal	1:2000
Choline acetyltransferase	Purified human placental enzyme	Pro Sci (50-265), goat polyclonal	1:100
Substance P	Synthetic peptide corresponding to the full Substance P protein conjugated to BSA	Millipore (MAB356), rat monoclonal	1:200
Methionine-Enkephalin	Synthetic Met^5-ENK peptide (H-TGGPM-OH) conjugated through the N-terminal tyrosine to BSA	Millipore (AB5026), rabbit polyclonal	1:1000
Leucine-Enkephalin	Synthetic Leu^5-ENK peptide (H-TGGPL-OH) conjugated through the N-terminal tyrosine to BSA	Millipore (AB5024), rabbit polyclonal	1:1000
γ-aminobutyric acid	GABA-BSA	Sigma (A2052), rabbit polyclonal	1:1000

2.2.3 Single immunoperoxidase labeling for light microscopy

Sections from the post-commissural putamen were treated at room temperature (RT) with sodium borohydride for 20 minutes (min) followed by a pre-incubation for 1 hour (h) in a solution containing 1% normal horse or goat serum, 0.3% Triton-X-100, and 1% bovine serum

albumin (BSA) in PBS. Sections were then incubated for 24 h at RT in a solution containing goat anti-ChAT (1:100), rat anti-SP (1:100), or rabbit anti-Met/Leu-Enk (1:1000) 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 min at RT. Sections were exposed to an avidin-biotin-peroxidase complex (ABC; 1:100, Vector for 90 min followed by rinses in PBS and Tris buffer (50mM; pH 7.6). Sections were then incubated within a solution containing 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma), 10 mM imidazole, and 0.005% hydrogen peroxide in Tris buffer for 10 min at RT, rinsed with PBS, placed onto gelatin-coated slides, and coverslipped with Permount. Lastly, the sections were analyzed using a Leica DMLB light microscope (Vienna, Austria) and photographed using a ScanScope light microscope (Aperio Technologies; Vista, CA).

2.2.4 Preparation for electron microscopic observations

2.2.4.1 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), and frozen at -80°C for 20 min each, before being returned to PBS-based solutions with decreasing gradients of cryoprotectant, and lastly, washed thoroughly in PBS. The tissue was then pre-incubated for 1 h at RT in a solution containing PBS, 1% normal goat serum (NGS), and 1% BSA, followed by a 48 h 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, Burlingame, CA), for 90 min 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 min 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 min in the dark. After alcohol dehydration, sections were placed in propylene oxide and embedded in epoxy resin (Durcupan ACM, Fluka, Buchs, Switzerland) for at least 12 h, mounted onto slides, and placed in a 60°C oven for 48 h.(Smith & Bolam, 1990) Tissue samples from the post-commissural 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 min, 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).

2.2.4.2 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 (1:1000; 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 min) and incubated with 15 nm gold-conjugated

goat anti-rabbit IgG (1:50 in TBS 0.05 M, pH 8.2; British Biocell, Cardiff, United Kingdom, 90 min). The sections were then rinsed in TBS (0.05 M, pH 8.2) for 20 min, washed in distilled water for 5 min, and stained with 1% uranyl acetate (in distilled water, 90 min). Lastly, the grids were washed in distilled water and stained with lead citrate for 5 min before being examined with the electron microscope.

2.2.4.3 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 min 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 h 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 h 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 min to increase gold particle sizes to 30-50nm 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 min, and the tissue was stained with 1% uranyl acetate for 10 min in the dark.

2.2.5 Analysis of material

2.2.5.1 GABAergic 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 post-commissural putamen of 4 animals were randomly examined to localize ChAT-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 & 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 (Rasband, 1997-2013). 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 cut-off were categorized as "symmetric/unknown". Because of inter-individual variability in the absolute number of gold particles associated with GABA-positive and GABA-negative terminals across animals and post-embedding reactions, these measurements were collected for each run of post-embedding reaction in the striatal tissue from each monkey (***Fig. 2.4***E). 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 ANOVA with a post-hoc Tukey's test (significance level set at $p < 0.05$; ***Fig. 2.5***B).

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

Sections from the post-commissural putamen double-labeled with antibodies for rat anti-SP (gold-labeled) and goat anti-ChAT (peroxidase-labeled; 5 animals) or rabbit anti-Met-/Leu-Enk (gold-labeled) and goat anti-ChAT (peroxidase-labeled; 4 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. 2.6***F, G). The average proportion of SP- or Met/Leu-Enk-positive terminals forming synapses onto ChAT-labeled elements was compared using a Student's t-tests (significance level set at $p < 0.05$; ***Fig. 2.6***F) and between the postsynaptic targets within each group using a Student's t-test (significance level set at $p < 0.05$; ***Fig, 2.6***G).

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 25,000x. 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

using Image J (Rasband, 1997-2013), 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 & 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 2.6***E).

2.2.5.3 Total synaptic innervation of ChAT-positive neurons from SP- or Enk-positive terminals

In other measurements on the same double-immunostained striatal tissue in 3 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. 2.7***B, 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 25,000x. 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.

2.3 Results

2.3.1 Light microscopic observations

2.3.1.1 ChAT immunolabeling in the dorsal striatum

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 (***Figure 2.1***). These morphological characteristics are consistent with those described for ChAT-positive neurons in previous primate studies (DiFiglia, 1987; Bernacer *et al.*, 2007).

2.3.1.2 SP and Met-/Leu-Enk immunolabeling in the striatopallidal system

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 & Emson, 1980; Cuello *et al.*, 1981; Del Fiacco *et al.*, 1982; Beach & McGeer, 1984; Reiner *et al.*, 1999b). In agreement with previous reports (Haber & Elde, 1981; DiFiglia *et al.*, 1982; Williams & Dockray, 1983; Beach & McGeer, 1984; Reiner *et al.*, 1999b), the striatum and GPi showed strong positive immunostaining for SP (***Figure 2.3***A), while the antibodies for Met-/Leu-Enk positively labeled the striatum and GPe (***Figure 2.2***B-C).

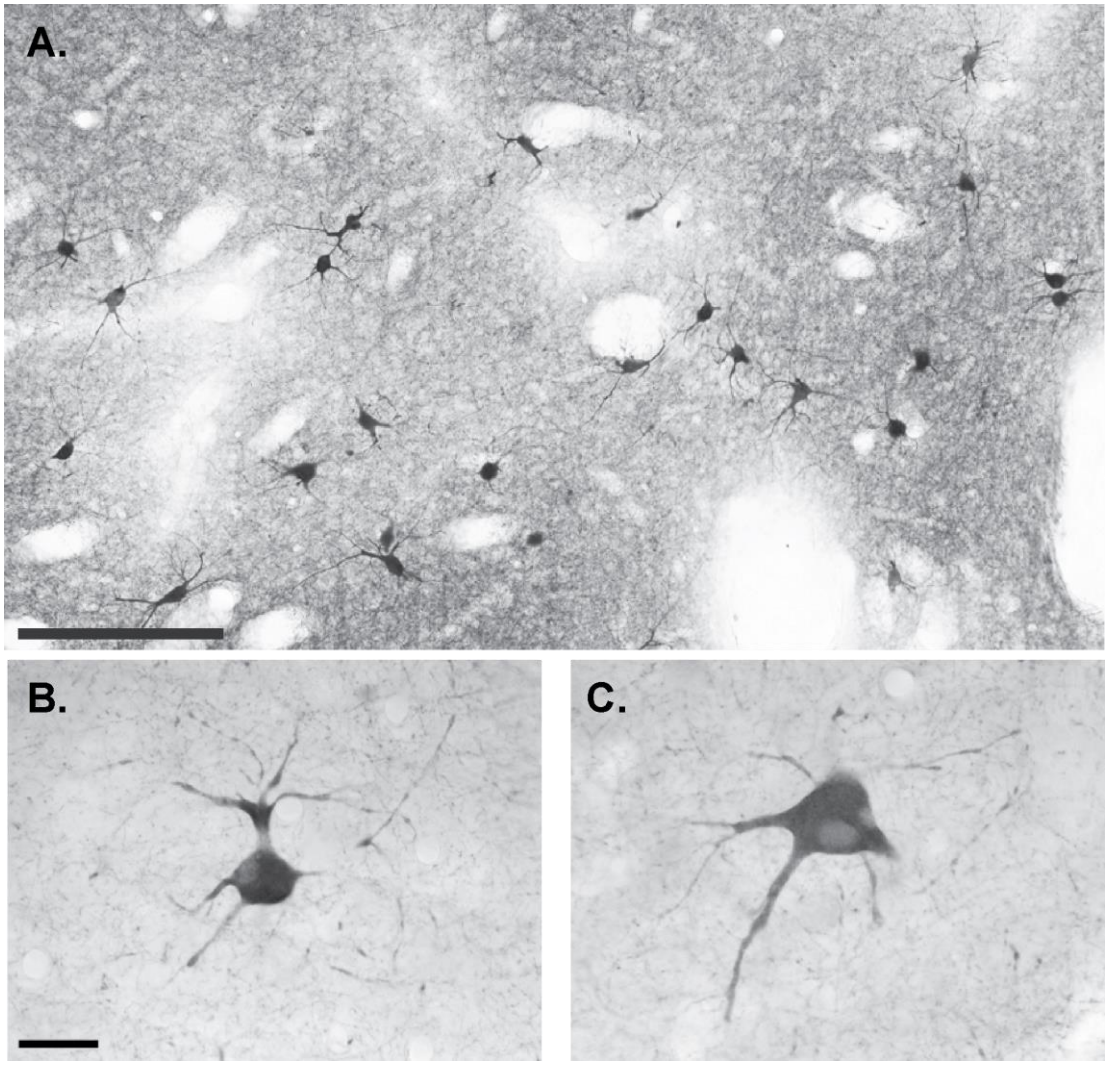

Figure 2.1. **General pattern of distribution and morphology of cholinergic interneurons in the monkey putamen at the light microscopic level.**
A: Low power view of ChAT-positive neurons and neuropil in the post-commissural putamen. **B,C:** High power views of typical ChIs from the monkey sensorimotor putamen. Notice 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 bars = 100 μm in (A); 25 μm in (B-C).

2.3.2 Electron microscopic observations

In double-label experiments, the pre-embedding immunogold method was used to localize SP or Enk immunoreactivity, while ChAT-positive neurons were labeled with the immunoperoxidase method (***Figure 2.6***A-D). Consistent with previous reports on the ultrastructural localization of SP and Enk immunoreactivity in the primate striatum (DiFiglia *et*

al., 1982; Hutcherson & Roberts, 2005), most immunoreactivity was found in terminals forming symmetric synapses. Less labeling was observed in dendrites, cell bodies and dendritic spines (***Figure 2.6***A-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 dense-core vesicles, a known vesicular storage site of neuropeptides in CNS neurons (Merighi *et al.*, 2011) (***Figure 2.6***A-D). The pattern of ChAT immunostaining in the double labeled tissue was the same as described below for the ChAT/GABA-immunostained material (***Figure 2.6***E). 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 co-localization (***Figure 2.6***A-D).

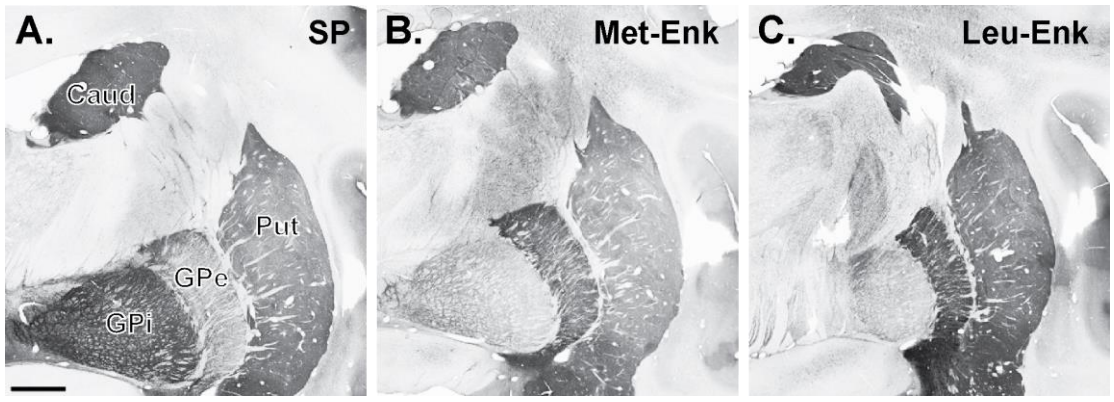

Figure 2.2. **Light microscopic distribution of immunoreactivity for substance P and enkephalin in the monkey striatopallidal complex.**
The distribution of SP (A), Met-Enk (B), and Leu-Enk (C) immunolabeling in the striatopallidal complex is shown in monkey tissue. SP labeling is intense in the caudate nucleus (Caud), putamen (Put), and internal globus pallidus (GPi), whereas strong Met- and Leu-Enk immunostaining is found in the Caud, Put, and external globus pallidus (GPe). Scale bar = 2 mm in A (applies to B and C).

2.3.2.1 GABAergic innervation of ChAT-labeled neurons

The GABAergic innervation of cholinergic cells was characterized from post-commissural putamen tissue that was double immunostained for ChAT (pre-embedding immunoperoxidase) and GABA (post-embedding immunogold). At the electron microscopic level, ChAT-immunostained cell bodies, dendrites, and axon terminals displayed the electron

dense amorphous DAB reaction product (***Figure 2.3***), while GABA-ir terminals were recognized by their large density of gold particles (***Figure 2.3***A, 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 (***Figure 2.3***). We performed this analysis separately for each of the 4 animals used in this part of the study (animals 1-4 in ***Figure 2.4***), because the absolute number of gold particles over labeled and unlabeled elements

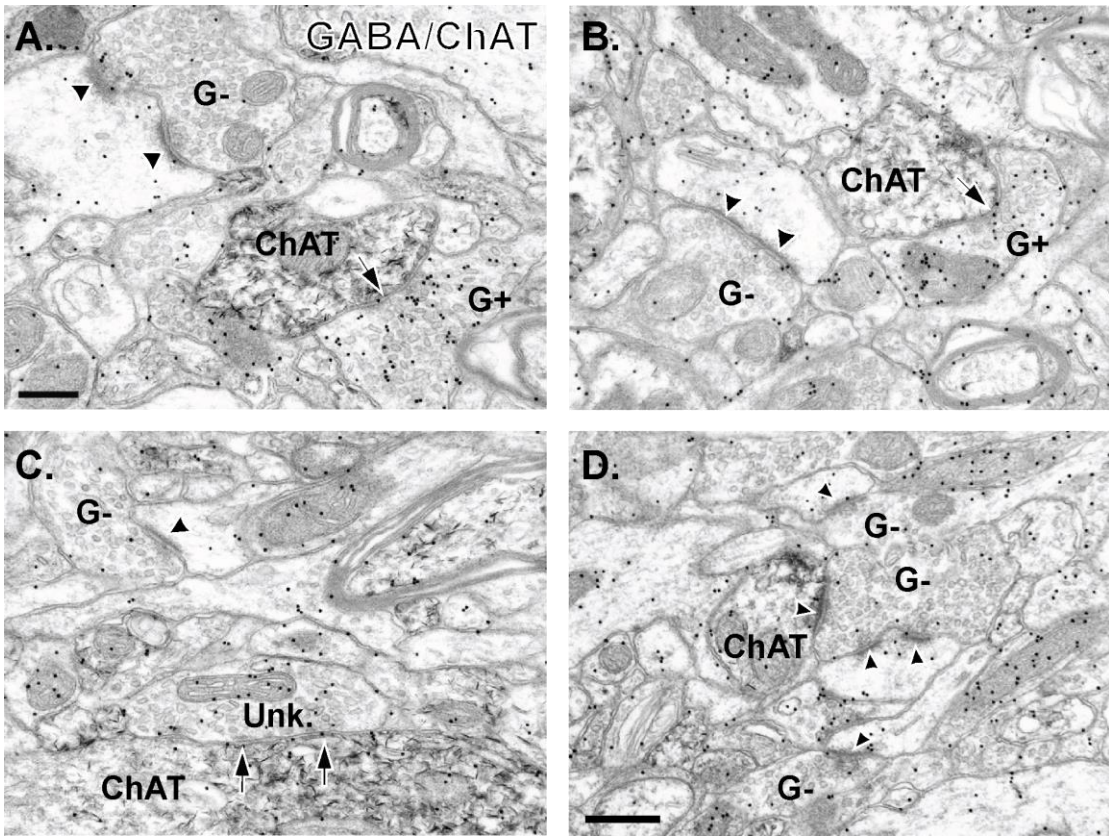

Figure 2.3. **Electron micrographs of tissue double immunostained for GABA (immunogold) and ChAT (immunoperoxidase) in the monkey putamen.**
A-B: Photomicrographs demonstrating GABA-positive (G+) terminals forming symmetric synapses (arrows) with medium-sized ChAT-labeled dendrites. Notice that a GABA-negative (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 unlabeled spine (arrowheads). Other GABA-negative (G-) terminals are also visible in this tissue. Scale bar = 0.2 µm in A-B; 0.5 µm in D.

across animals may vary when localized with the post-embedding immunogold method. In each animal, the average density of gold particles associated with terminals forming symmetric synapses outnumbered that in terminals forming asymmetric synapses (***Figure 2.3*** and ***Figure 2.4***) by a factor of 4:1 (***Figure 2.4***E). As described in the Methods, we used these data to categorize the neurochemical phenotype of terminals forming synapses onto ChAT- positive elements. As shown in ***Figure 2.5***A, 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 post-synaptic 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; ***Figure 2.5***B). 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; ***Figure 2.5***B).

2.3.2.2 SP-labeled inputs onto ChAT-immunoreactive neurons

In the first series of double-label experiments carried out using post-commissural putamen tissue from 5 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

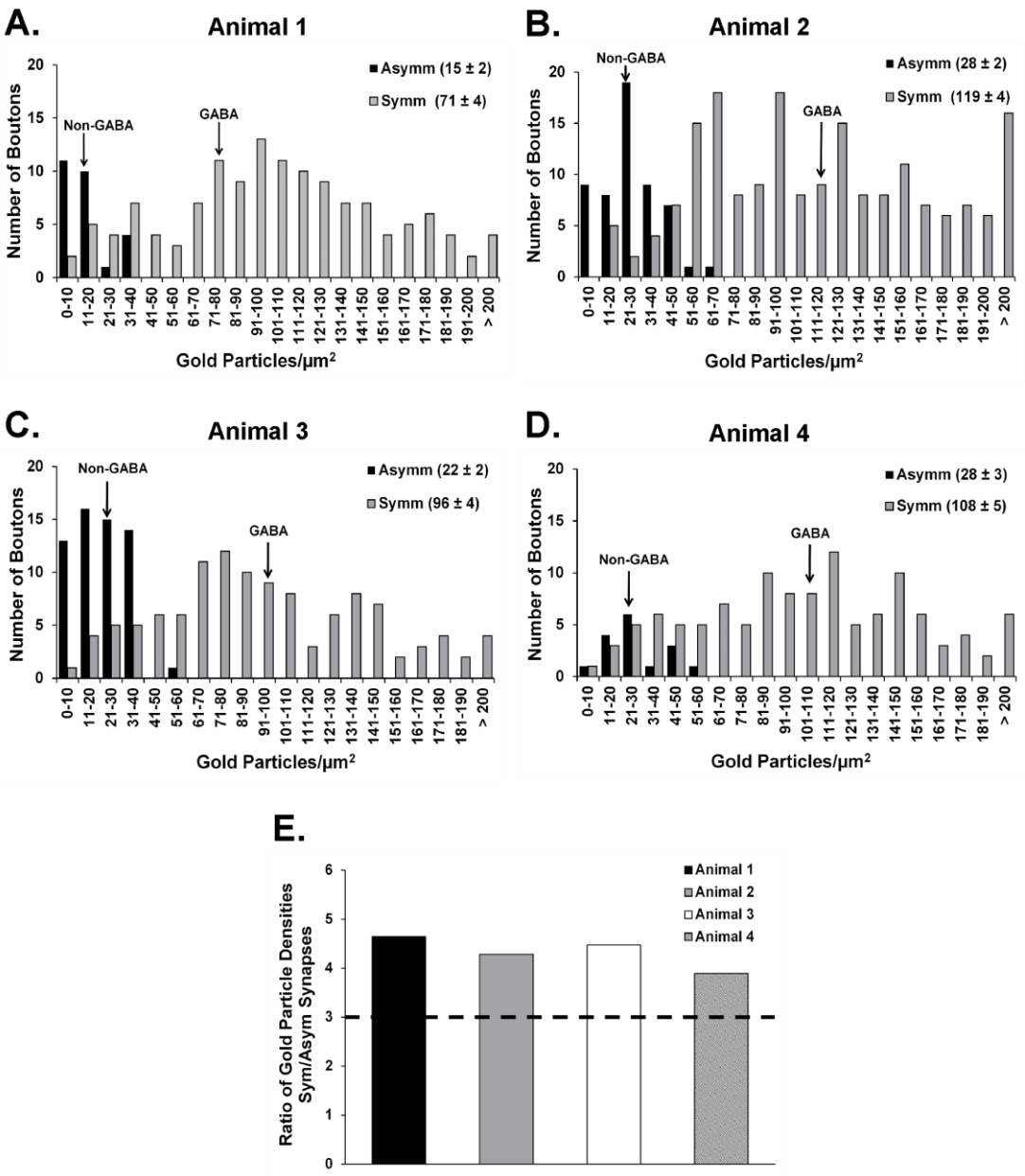

***Figure 2.4*. Gold particle densities in putative GABAergic striatal terminals.**

The frequency distribution histograms of the relative gold particle density in terminals forming synapses with ChAT-labeled elements are shown in the 4 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 ChIs in the post-commissural 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 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) cut-off to separate confirmed GABAergic from "unknown" symmetric terminals.

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 (***Figure 2.6***A-B), which were preferentially of the medium (52 ± 6%) or small (29 ± 3%) size diameter (***Figure 2.6***G).

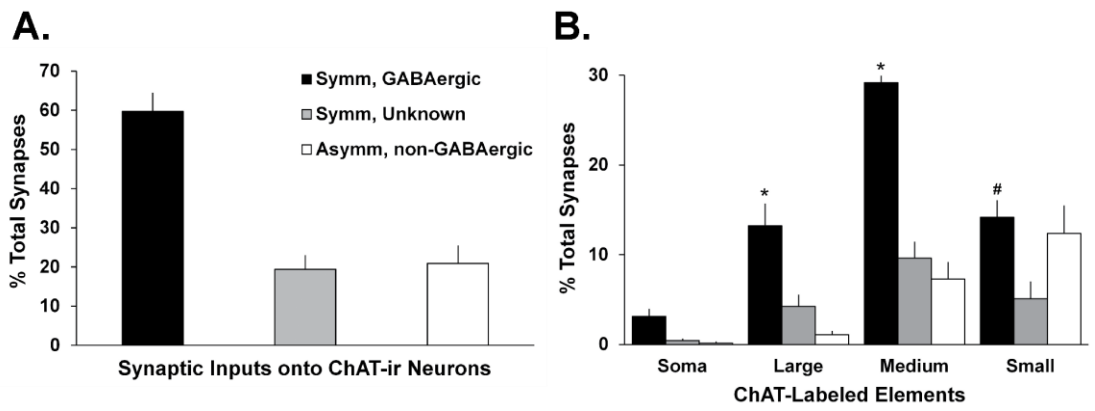

***Figure 2.5*. Proportion of GABAergic and non-GABAergic terminals in contact with ChAT-labeled neurons in the putamen of 4 monkeys.**
A: Mean percentage (± SEM) of terminals categorized as "symmetric/GABAergic", "symmetric/unknown" or "asymmetric/non-GABAergic." These categories were based on the relative 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 vs Symm, Unknown and Asymm, non-GABAergic; #, $p < 0.001$ Symm, GABAergic vs Symm, Unknown.

We also determined the proportion of the total synaptic innervation of ChAT-positive neurons that was accounted for by SP-ir terminals in 3 out of the 5 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, while 68 ± 3% originated from SP-negative boutons forming symmetric synapses (***Figure 2.7***B), and 16 ± 2% came from putatively glutamatergic terminals forming asymmetric synapses (***Figure 2.7***B). The proportion of SP-negative terminals forming symmetric synapses (i.e., Symm/SP(-)) with

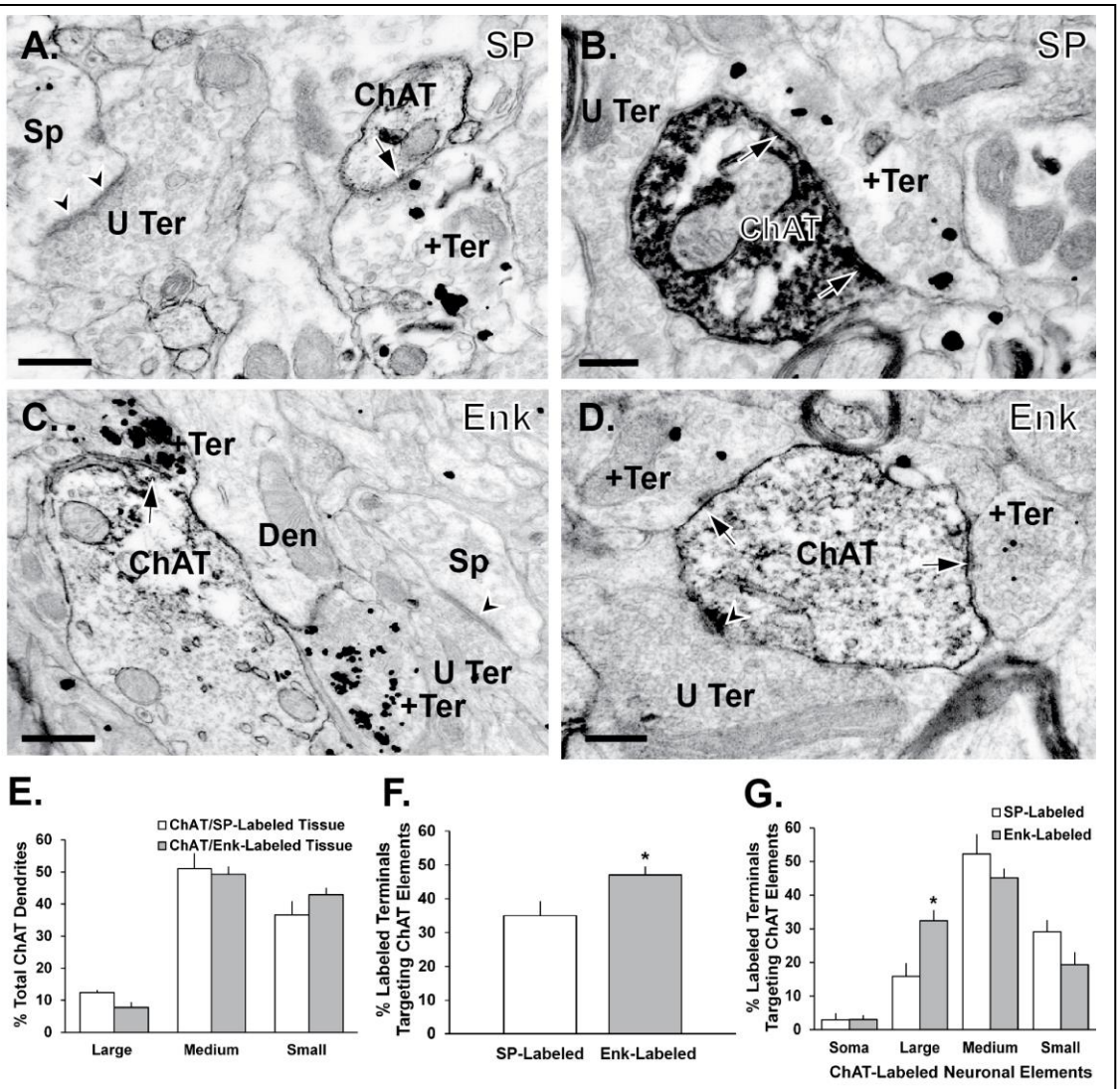

***Figure 2.6*. Synaptic relationships between terminals labeled with SP or Met-/Leu-Enk and ChAT-labeled dendrites in the monkey putamen.**

A,B: Photomicrographs showing SP-ir boutons (+Ter; immunogold) forming symmetric synapses (black arrows) with a small-sized and a medium-sized ChAT-labeled dendrite (immunoperoxidase) (A and B, respectively). **C,D:** Examples of Enk-ir terminals (+Ter; immunogold) forming symmetric synapses (arrows) with a large-sized (C) and a medium-sized (D) ChAT-labeled dendrite. Notice 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 vs SP-labeled on large dendrites. Abbreviations: Den, dendrite. Scale bar = 0.5 µm for A, C and 0.2 µm or B, D.

medium-sized ChAT-labeled dendrites was significantly larger than the two other populations of terminals in contact with these ChAT-positive elements (***Figure 2.7***B). 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 under-estimated 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; ***Figure 2.7***A). 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.

2.3.2.3 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 post-commissural putamen of 4 out of the 5 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 (***Figure 2.6***F), a proportion significantly greater ($p < 0.05$, Student's t-test) than that found for SP-positive terminals in contact with ChAT-positive cells (***Figure 2.6***F). This difference is accounted for by a significantly larger proportion of Enk-ir terminals in contact with large ChAT-positive dendrites (***Figure 2.6***G; $p = 0.012$; 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, while 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 (***Figure 2.7***C). 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; ***Figure 2.7***C). On the other hand, small-sized ChAT-ir dendrites also received a significantly greater synaptic innervation from the symmetric Enk-negative terminals than the

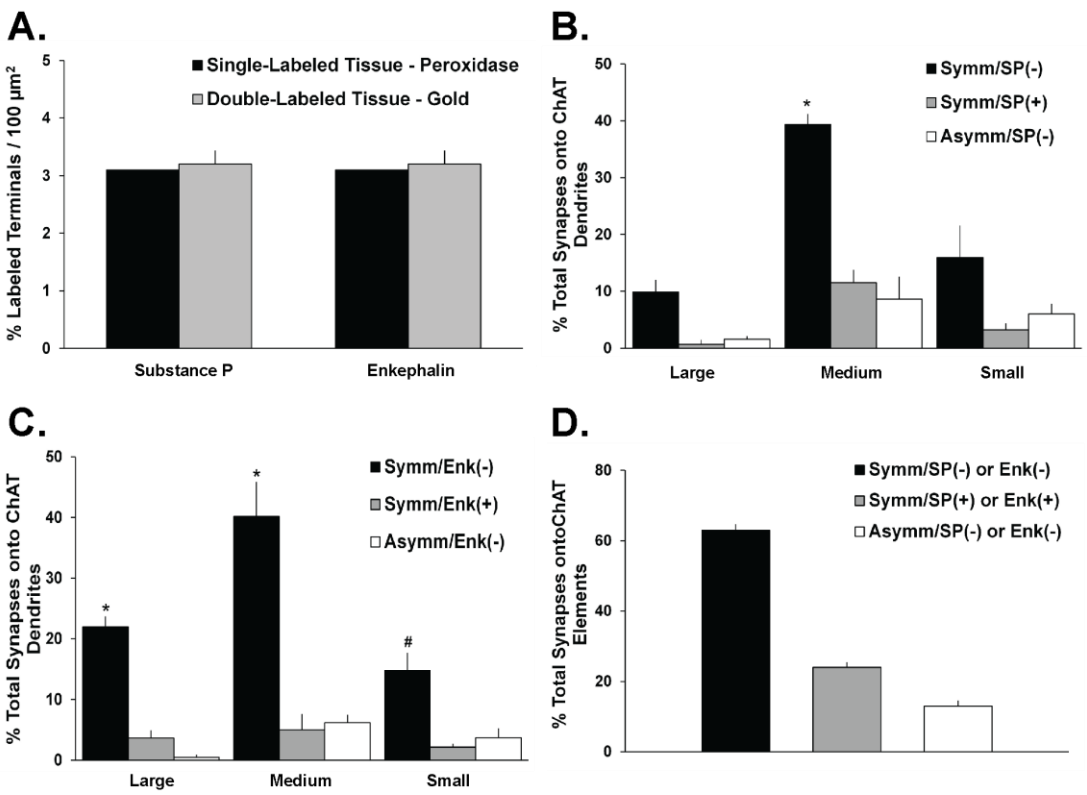

Figure 2.7. **Proportion of total synaptic inputs onto ChIs from SP- and Enk-ir terminals.**
A: Comparison of the mean densities of SP- or Enk-positive terminals as revealed with the pre-embedding immunogold method in double immunostained striatal tissue of 3 monkeys (SP/ChAT-left gray bar; Enk/ChAT-right 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(-) vs Symm/SP(+) and Asymm/SP(-) on medium dendrites. In (C), *, $p < 0.001$, Symm/Enk(-) vs Symm/Enk(+) and Asymm/Enk(-) on large or medium dendrites; #, Symm/Enk(-) vs Symm/Enk(+) on small dendrites. **D:** Mean percentages of synaptic inputs onto ChIs 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-).

symmetric Enk-positive terminals ($p < 0.001$, One-way ANOVA with a post-hoc Tukey's test; ***Figure 2.7***C), 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 using the immunoperoxidase method (***Figure 2.7***A).

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 post-commissural putamen originate from axon collaterals of GABAergic direct and indirect striatal projection neurons, while 63 ± 2% arise from other subpopulations of terminals that form symmetric synapses, and 13 ± 2% from putatively glutamatergic boutons (***Figure 2.7***D).

2.4 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 post-commissural putamen. Through these anatomical connections, the intrinsic axon collaterals of GABAergic striatal projection neurons may exert a powerful control of ChI activity in primates.

2.4.1 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 & Westerink, 1994; Yan & Surmeier, 1997; Fujiyama *et al.*, 2000; Waldvogel *et al.*, 2004), readily evoked IPSPs in TANs (Yan & Surmeier, 1997; Bennett & Wilson, 1998; Pisani *et al.*, 2000) and decreased striatal ACh release (Anderson *et al.*, 1993; DeBoer & 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 & Kitai, 1982; Bolam *et al.*, 1984b; 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 & 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 *et al.*, 1994b; Bolam *et al.*, 2000; Tepper *et al.*, 2010; Crittenden & 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; Sadikot *et al.*, 1992b; Bolam *et al.*, 2000; Kubota & 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 & Kawaguchi, 2000; Tepper *et al.*, 2010), or extrinsic GABAergic inputs from the GPe (Beckstead, 1983; Oertel *et al.*, 1984; Smith *et al.*, 1987; Kita & 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 NPY/somatostatin-containing interneurons, known to express a low level of GABA (Aoki & Pickel, 1989; Chesselet & Robbins, 1989; Vuillet *et al.*, 1992; Kubota *et al.*, 1993; Catania *et al.*, 1995; Kubota & Kawaguchi, 2000). However, low levels of non-specific gold labeling in dopaminergic SNc inputs (Kubota *et al.*, 1987; Pickel & Chan, 1990; Dimova *et al.*, 1993) or cholinergic terminals from striatal ChIs themselves (Calabresi *et al.*, 1998c; 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 (Dubé *et al.*, 1988; Lapper & Bolam, 1992; Sadikot *et al.*, 1992a; Dimova *et al.*, 1993; Bennett & Wilson, 1998; Sidibe & Smith, 1999; Thomas *et al.*, 2000), although most of them are likely to arise from projections of the centromedian/parafascicular thalamic nuclei (Meredith & Wouterlood, 1990; Lapper & Bolam, 1992; Bennett & Wilson, 1998; Sidibe & 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 & 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 & Wickens, 2004; Nanda *et al.*, 2009; Ding *et al.*, 2010). It is noteworthy that additional phenomena not studied here, such as spike back-propagation, dendritic calcium entry, or the type and density of post-synaptic 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 & Plenz, 2002; Plenz, 2003; Carter & Sabatini, 2004; Kerr & Plenz, 2004; Tepper & Bolam, 2004; Wilson, 2007; Day *et al.*, 2008; English *et al.*, 2012).

2.4.2 Axon collaterals of striatal projection neurons are a main source of GABAergic inputs onto 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 8 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 co-exist 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 & 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 & Emson, 1980; Del Fiacco *et al.*, 1982; DiFiglia *et al.*, 1982; Beach & McGeer, 1984; Smith *et al.*, 1998; Reiner *et al.*, 1999b). 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 & 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 post-commissural 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 *et al.*, 1985; Roberts *et al.*, 1996; Wu & Parent, 2000), and that striatal ChIs in primates have a denser and larger dendritic tree than rodents (Difiglia *et al.*, 1980; Bolam *et al.*, 1984b; Phelps *et al.*, 1985; DiFiglia & Carey, 1986; Yelnik *et al.*, 1991; Yelnik *et al.*, 1993; Bernacer *et al.*, 2007). On the other hand, technical differences cannot be ruled out, especially the increased sensitivity of the ultrastructural double immunocytochemical technique used in our study. In regards 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 are 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 & Plenz, 2002; Tunstall *et al.*, 2002; Tecuapetla *et al.*, 2005; Wilson, 2007; Blomeley & Bracci, 2008; Taverna *et al.*, 2008; Tepper *et al.*, 2008; Blomeley *et al.*, 2009; Blomeley & Bracci, 2011). 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.

2.4.3 Peptidergic modulation of cholinergic interneurons by striatal projection neurons?

In addition to their GABAergic influences, MSN collaterals could also modulate the activity of ChIs through the release of their co-expressed neuropeptides, SP and Enk. It has, indeed, been shown that these neuropeptides participate in the synaptic modulation of striatal neuronal activity (Aosaki & Kawaguchi, 1996; Miura *et al.*, 2007; Miura *et al.*, 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; Lindefors *et al.*, 1989; Llorens-Cortes *et al.*, 1990; Ruzicka & Jhamandas, 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 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 rodent 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 FSIs or other MSNs (Aosaki & Kawaguchi, 1996; Kawaguchi *et al.*, 1997; Bell *et al.*, 1998; Govindaiah *et al.*, 2010), whereas Enk activation of opioid receptors results in the hyperpolarization and decreased spontaneous firing activity of ChIs (Jiang & North, 1992; Miura *et al.*, 2007; Ponterio *et al.*, 2013). 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.

Chapter 3 :

Synaptic regulation of cholinergic interneurons: Inputs from parvalbumin-containing neurons in the primate putamen

3.1 Introduction

Striatal intrinsic circuits and their external afferents form a multi-level, highly complex network that unitarily facilitates information outflow from the striatum to other basal ganglia nuclei about behaviorally-significant events (Nicola *et al.*, 2000; Voorn *et al.*, 2004; Smith & Villalba, 2008; Aosaki *et al.*, 2010; Bonsi *et al.*, 2011; Crittenden & Graybiel, 2011; Smith *et al.*, 2011). Within the striatum, ChIs contribute to this network through their various roles in reward-related behaviors, conditioned learning, and action selection, as well as through their modulation of striatal synaptic plasticity (Williams & Adinoff, 2008; Ragozzino *et al.*, 2009; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Deiana *et al.*, 2011; Gutierrez *et al.*, 2011; Havekes *et al.*, 2011; Mark *et al.*, 2011; Threlfell & Cragg, 2011; Goldberg *et al.*, 2012; Schulz & Reynolds, 2013). ChIs may be capable of this functional diversity because of their unique morphological characteristics, highly specific but widespread distribution, distinctive spiking characteristics, and feedforward and feedback connections with intrastriatal and extrinsic afferents (Zhou *et al.*, 2002; Bonsi *et al.*, 2011; Goldberg & Reynolds, 2011; Threlfell & Cragg, 2011; Gonzales *et al.*, 2013; Schulz & Reynolds, 2013).

In regards to this connectivity, we recently established that ChIs receive inputs from a substantial number of GABAergic, putative glutamatergic, and other types of neurons in the monkey post-commissural putamen, which includes a significant innervation from collaterals of direct (SP-containing) and indirect (Enk-containing) striatal projection neurons (Gonzales *et al.*, 2013). However, we also demonstrated that the source(s) of a considerable proportion (approximately 36%) of GABAergic inputs to ChIs still remains to be uncovered in the monkey putamen (Gonzales *et al.*, 2013). The potential sources of this GABAergic innervation in primates include the heterogeneous group of striatal GABAergic interneurons that co-express PV, NPY/somatostatin, or calretinin (Hussain *et al.*, 1996; Kawaguchi *et al.*, 1997; Meredith, 1999; Tepper *et al.*, 2010), and the PV-containing pallidostriatal afferents (Kita *et al.*, 1999).

The populations of striatal interneurons that express GABA/PV (i.e., FSIs) or ACh (i.e., ChIs/TANs) are of particular interest because of their well-recognized roles as regulators of striatal and basal ganglia function in normal and diseased states (Zhou *et al.*, 2002; Pisani *et al.*, 2007; Smith & Villalba, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Gittis & Kreitzer, 2012; Goldberg *et al.*, 2012). Data about the functional connectivity between these two groups of striatal interneurons are absent in primates. However, recent electrophysiological findings from mouse brain slices demonstrated that putative ChIs do not show any synaptic events in FSI-TAN paired recordings or in response to optogenetic activation of FSIs (Gittis *et al.*, 2010; Szydlowski *et al.*, 2013). In agreement, electron microscopic analysis of double-label adult rat striatal tissue revealed that PV-containing terminals could not be found forming synapses with ChIs (Chang & Kita, 1992). Although these data are indicative of a potential lack of synaptic inputs from PV-containing interneurons onto ChIs in rodents, such analysis might be more difficult to achieve in primates, because electron microscopic observations demonstrated that PV-containing striatal afferents and intrastriatal synaptic microcircuits are considerably different between rats and monkeys (Kita *et al.*, 1990; Bennett & Bolam, 1994a; b; Kita *et al.*, 1999; Sidibe & Smith, 1999; Jones, 2007). For example, PV-labeled terminals in the primate striatum mainly form asymmetric synapses, indicative of extrinsic glutamatergic afferents (Bennett & Bolam, 1994a) most likely from the thalamus (Sidibe & Smith, 1999; Jones, 2007), while PV-containing terminals in the rodent form only symmetric synapses (Kita *et al.*, 1990; Bennett & Bolam, 1994b). In addition, the PV-containing terminals that form symmetric synapses in the monkey striatum originate from two sources: striatal GABAergic interneurons (Bennett & Bolam, 1994a) and pallidostriatal afferents (Kita *et al.*, 1999), contrasting those in rodents that originate only from PV GABAergic interneurons (Kita *et al.*, 1990; Bennett & Bolam, 1994b; Kita & Kita, 2001; Mallet *et al.*, 2012).

Given the striking differences in the potential sources of PV-positive terminals in the primate versus non-primate striatum, we undertook a detailed quantitative analysis of the

prevalence and relative distribution of PV-labeled inputs onto ChIs in the post-commissural putamen of monkeys. Our findings reveal that ChIs receive inputs from PV-positive terminals that form symmetric or asymmetric synapses that account for a substantial proportion of the total GABAergic and glutamatergic innervation of ChIs in the monkey putamen.

3.2 Materials and methods

3.2.1 Animals

In these studies, brain tissue was used from five adult (3-9 years old; 5-8 kg) female rhesus monkeys (*Macaca mulatta*) originating from the Yerkes National Primate Research Center colony. At the time of sacrifice, deep anesthesia was first induced in these monkeys with a pentobarbital overdose (100 mg/kg, i.v.) followed by transcardial perfusion with a cold oxygenated Ringer's solution and a fixative containing 0.1% glutaraldehyde and 4% paraformaldehyde in PB (0.1 M, pH 7.4). All procedures were approved by the Institutional Animal Care and Use Committee at Emory University and conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (Garber *et al.*, 2010).

3.2.2 Tissue Processing

After perfusion, the removed brains were cut into 10-mm-thick blocks in the coronal plane and placed into cold PBS (0.01M, pH 7.4). Shortly afterward, these blocks were sectioned into serial 60-µm-thick coronal slices using a vibrating microtome, then placed into an anti-freeze solution (1.4% NaH_2PO_4-H_20, 2.6% Na_2HPO_4-$7H_2O$, 30% ethylene glycol, 30% glycerol dissolved in distilled water) for long-term storage in a -20^oC freezer. Prior to all immunostaining, sections were treated with a 1% sodium borohydride/PBS solution for 20 minutes, followed by thorough washes with PBS.

3.2.3 Primary antibodies

Sections from the post-commissural putamen were processed for double-label electron microscopy with immunogold localization of PV and immunoperoxidase localization of ChAT using highly specific, well characterized monoclonal and polyclonal antibodies (Table 3.1), respectively. Findings from experiments that utilized immunofluorescence and immunoperoxidase labeling, immunoenzymatic labeling of immunoblots, and radioimmunoassays have demonstrated the specificity of monoclonal anti-PV antibodies, along with a common epitope to the 1st binding site of PV in carp, mouse, rat, monkey, and human tissue (Celio & Heizmann, 1981; 1982; Celio, 1986; Celio *et al.*, 1988; Celio, 1989). Overall, the pattern of immunostaining obtained with these antibodies, i.e., strong labeling in the cortex, hippocampus, striatum, hypothalamus, olfactory bulb, and cerebellum, is consistent with areas known to express detectable levels of PV (Celio & Heizmann, 1981; Celio, 1986; 1989; Prensa *et al.*, 1998). Additionally, these antibodies did not cross-react with other calcium-binding proteins (i.e.,calmodulin and calbindin D-28k) (Celio *et al.*, 1988). The specificity of the goat anti-ChAT antibodies has been verified through Western blot analysis in rat and human brain tissue (Karson *et al.*, 1993), along with abolished striatal cell body and fiber immunostaining in control incubations from which the primary antibodies were omitted (Holt *et al.*, 1999). The pattern of ChAT immunostaining observed in our study was similar to that described in previous non-human primate studies using different ChAT antibodies (refer to section 1.3) (Graybiel *et al.*, 1986; DiFiglia, 1987; Bernacer *et al.*, 2007).

Table 3.1. Immunogens, sources, and dilutions of primary antibodies used in this study.

Antibody	Immunogen	Manufacturer Data	Dilution used
Parvalbumin	Purified Carp-II parvalbumin from muscle; directed against an epitope at the 1st calcium binding site	SWant (325), mouse monoclonal	1:10,000
Choline acetyltranserase	Purified human placental enzyme	Pro Sci (50-265), goat polyclonal	1:100

3.2.4 Preparation for electron microscopic observations

3.2.4.1 Single pre-embedding immunoperoxidase labeling for electron microscopy

Sections from the post-commissural putamen were processed for electron microscopic observations of PV and ChAT as described previously in sections 2.2.4.1 and 2.2.4.3 and in our prior publication (Gonzales *et al.*, 2013). Thus, we will only briefly discuss the details of the tissue preparation in this section. Striatal sections were first processed according to our cryoprotectant protocol, followed by pre-incubation in a solution containing 1% normal horse serum and 1% BSA in PBS for 1 h at RT, and then, a 48 h incubation in the primary antibody solution containing mouse anti-PV and goat anti-ChAT antibodies. On the following day, sections were incubated for 90 min at RT in either (secondary) biotinylated horse anti-goat IgGs or horse anti-mouse IgGs (1:200; Vector, Burlingame, CA), exposed for 90 min to an ABC complex (1:100, Vector), and then, processed for DAB (Sigma) for 10 min. After PB (0.1 M, pH 7.4) rinses, the tissue underwent treatment with 1% osmium for 20 min and 1% uranyl acetate in 70% ethanol for 35 min, followed by dehydration with decreasing ethanol concentrations. Sections were placed in propylene oxide, embedded in epoxy resin (Durcupan ACM, Fluka, Buchs, Switzerland) for at least 12 h, and then baked in a 60°C oven for 48 h.

Prior to being cut into 60-nm ultrathin sections (Leica Ultracut T2), post-commissural putamen samples from the resin-embedded sections were cut and glued onto resin blocks. The ultrathin sections were stained for 5 min with lead citrate and therefore, ready to be examined with an electron microscope (EM; model 1011, Jeol, Peabody, MA). Immunoreactive elements were digitally collected with a Gatan CCD camera (Model 785; Warrendale, PA) controlled by Digital Micrograph software (version 3.11.1).

3.2.4.2 Double pre-embedding immunogold for PV and immunoperoxidase for ChAT

After being processed with the cryoprotectant protocol, sections from the post-commissural putamen were pre-incubated in a PBS solution containing 5% milk for 30 min, followed by an overnight incubation at RT in a primary antibody solution consisting of mouse anti-PV and goat anti-ChAT antibodies and 1% dry milk in TBS-gelatin buffer (0.02 M, 0.1% gelatin, pH 7.6). In regards to the processing of control sections, each primary antibody was omitted from the solution to verify the specificity of the immunogold and immunoperoxidase labeling. On the next day, sections were first incubated for 90 min with secondary goat anti-mouse Fab' fragments conjugated to 1.4-nm gold particles (1:100; Nanoprobes, Yaphank, NY) and 1% dry milk in TBS-gelatin to limit cross-reactivity of the secondary antibodies. Sections underwent incubation for approximately 10 min in the dark with a HQ Silver Kit (Nanoprobes) to increase gold particle sizes to 30-50-nm through silver intensification, in order to optimize PV visualization. Sections were incubated with the next secondary antibody solution containing biotinylated horse anti-goat IGg (1:200; Vector) and 1% milk in PBS buffer for 90 min at RT, followed by ABC and DAB procedures to localize ChAT. Following washes in PB (0.1 M, pH 7.4), sections were treated for osmification, dehydration, embedding, and tissue selection as described above, with the exception that these sections were kept in 0.5% OsO4 and 1% uranyl acetate for 10 min instead of 20 and 35 min, respectively.

3.2.5 Analysis of material

3.2.5.1 PV-positive terminals targeting ChAT-positive interneurons

In order to quantify the proportion of PV-ir terminals in contact with ChAT-labeled neurons, sections from the post-commissural putamen of five monkeys were double-labeled with mouse anti-PV (gold-labeled) and goat anti-ChAT (peroxidase-labeled) antibodies. This tissue was randomly scanned for gold-labeled PV terminals to determine their post-synaptic targets (i.e., ChAT-labeled or unlabeled), regardless of their association with ChAT-positive or ChAT-

negative elements. If PV-ir terminals formed clear synapses (symmetric or asymmetric; refer to section 3.3.1) and contained synaptic vesicles at their pre-synaptic membranes, identification of their post-synaptic target(s) was carried out, resulting in our analysis of approximately 100 PV-immunostained terminals per animal (***Figure 3.5*** and ***Figure 3.6***). However, it should be noted that the differentiation between some PV-labeled terminals and dendrites was not always possible, because these elements had the irregular shape and size of dendrites but contained synaptic vesicles like terminals. Therefore, they were excluded from our analysis.

3.2.5.2 Total synaptic innervation of ChAT-positive neurons from PV-positive terminals

In the same double-immunostained tissue from the post-commissural putamen, the proportion of synaptic inputs onto ChIs represented by PV-positive terminals was determined as well. However, in this set of analyses, the tissue was randomly scanned for ChAT-labeled dendrites to determine their synaptic innervation, irrespective of their association with PV-positive or PV-negative boutons. The characterization of approximately 50 ChAT-labeled dendrites and their synaptic afferents (n=295 terminals) were identified and categorized as follows: (1) Symmetric/PV-positive, (2) Symmetric/PV-negative, (3) Asymmetric/PV-positive, and (4) Asymmetric/PV-negative (see ***Figure 3.7*** and ***Figure 3.8***). In addition, the total innervation of ChIs by sub-types of PV-labeled terminals (Type 1-Symmetric and Type 2-Symmetric) was also determined in the double immunostained sections (see ***Figure 3.8***). The results were statistically analyzed with a Student's t-test (significance set at $P < 0.05$).

Additional analyses were performed, however, before this quantification was carried out to ensure that the quantitative analysis of the double-labeled tissue was not affected by poor antibody or gold particle penetration (refer to our previous publication, (Gonzales *et al.*, 2013). Therefore, the density of PV-positive terminals was compared in single-labeled (immunoperoxidase; one monkey) and double-labeled (immunogold; five monkeys) sections from the post-commissural putamen. At 25,000x, digital micrographs were randomly taken of PV-ir boutons in every visible field of ultrathin sections stained with PV or PV/ChAT, resulting

in the analysis of 464 PV-labeled boutons (***Figure 3.7***A). The terminal density index was calculated by dividing the number of labeled boutons in the micrographs by the total surface area of these examined micrographs. The tissue was not further analyzed if the density of labeled boutons in the double immunostained sections was 10% less than that quantified in singly labeled tissue.

3.2.5.3 Dendritic densities in single- and double-labeled tissue

In order to determine whether the overall pattern of ChAT labeling was similar across double-label immunoreactions, we quantified the relative distribution and abundance of ChAT-ir dendrites from each block of tissue used in the double-label studies to ensure an adequate representation of dendritic labeling. At 30,000x, approximately 30 digital micrographs of ChAT-labeled dendrites were randomly taken from sections cut on the surface of tissue blocks from five animals, followed by the measurement of the cross-sectional diameter of each dendrite using the Image J software (Rasband, 1997-2013). Dendritic profiles were then categorized as large-sized (diameter > 0.99 µm, presumably representing proximal dendrites), medium-sized (diameter 0.5-

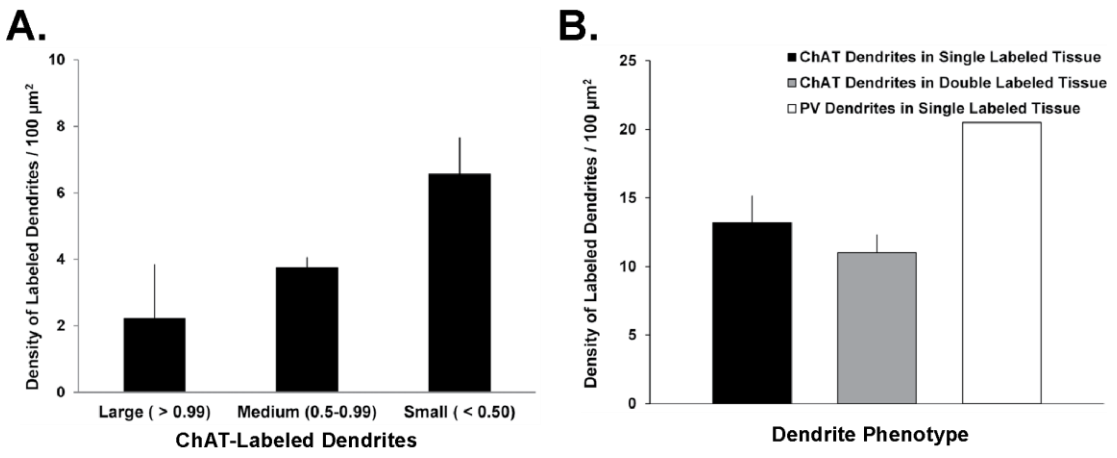

***Figure 3.1*. Density histograms for PV- and ChAT-immunoreactive dendrites in the putamen.** **A:** Mean percentages (± SEM) of ChAT-labeled dendrites of different sizes in double-labeled tissue (n=5). **B:** Comparison of the mean densities (± SEM) of ChAT- or PV-labeled dendrites as revealed with pre-embedding immunoperoxidase in single- (n=1, dendrites=170 for PV; n=2, dendrites=237 for ChAT) and double- (n=5, dendrites=424 for PV/ChAT) immunostained tissue. The density of ChAT-labeled dendrites was not significantly different ($p < 0.05$) regardless of single or double immunolabeling reactions, suggesting the dendrites labeled with immunoperoxidase in the double-labeled tissue most likely originate from ChAT-containing neurons.

0.99 µm), and small-sized (diameter < 0.5 µm, presumably representing distal dendrites) (DiFiglia & Carey, 1986; DiFiglia, 1987; Peters *et al.*, 1991; Yelnik *et al.*, 1991). Further quantification was only performed if the proportion of ChAT-positive proximal and distal dendrites in our double-labeled tissue was comparable to that in single-labeled with ChAT antibodies (***Figure 3.1***A; refer to (Gonzales *et al.*, 2013).

In a separate series of analyses, we wanted to determine if cross-reactivity occurred between the secondary antibodies used in our double-label immunostaining. As mentioned previously, our secondary antibodies were separately processed to avoid their cross-reactivity with each other (i.e., sections were first incubated with the goat anti-mouse immunogold secondary, followed by the biotinylated horse anti-goat secondary). To confirm that a cross-reaction did not occur in our tissue, we quantified the relative densities of ChAT-ir dendrites (i.e., percentage of labeled dendrites per 100 μm^2) in material immunostained with PV alone (immunoperoxidase in one monkey), ChAT alone (immunoperoxidase in two monkeys), and PV/ChAT together (immunogold and immunoperoxidase in five monkeys). In single- and double-labeled tissue, ultrathin sections from the surface of blocks were randomly scanned for dendrites labeled with immunoperoxidase, and electron micrographs were taken of these elements at 30,000x. To calculate the dendrite density index, the number of immunoreactive dendrites in these images was counted and divided by the total surface area of the examined micrographs. The results were then statistically analyzed with a Student's t-test (significance set at $P < 0.05$). Data in Figure 3.1B demonstrate that the density of dendrites labeled with immunoperoxidase in our double-labeling experiments was not significantly different from that found in single ChAT-immunolabeled tissue, providing evidence for the specificity and sensitivity of ChAT immunostaining in single and double immunoreactions. Thus, we confirmed that cross-reactivity of the secondary antibodies did not occur in these reactions (***Figure 3.1***B). If this were the case, we would have observed an increase in the density of ChAT-labeled dendrites beyond that for PV-labeled dendrites.

3.3 Results

3.3.1 General ultrastructural characterization of PV immunoreactivity in the striatum

In double-label immunoreactions, the pre-embedding immunogold method was employed to localize PV immunolabeling, along with immunoperoxidase detection of ChAT-positive neurons in the post-commissural putamen of monkeys (***Figure 3.2***, ***Figure 3.3***, and ***Figure 3.4***). A detailed description of striatal ChAT immunostaining at the light and electron microscopic levels is provided in sections 1.4, 2.3.1.1, and 2.3.2.1 (Gonzales *et al.*, 2013). In regards to PV labeling, immunoreactivity in single-and double-labeled sections was observed in axons, terminals, and dendritic shafts, a pattern reminiscent of that previously described in the monkey caudate nucleus and putamen (Bennett & Bolam, 1994a).

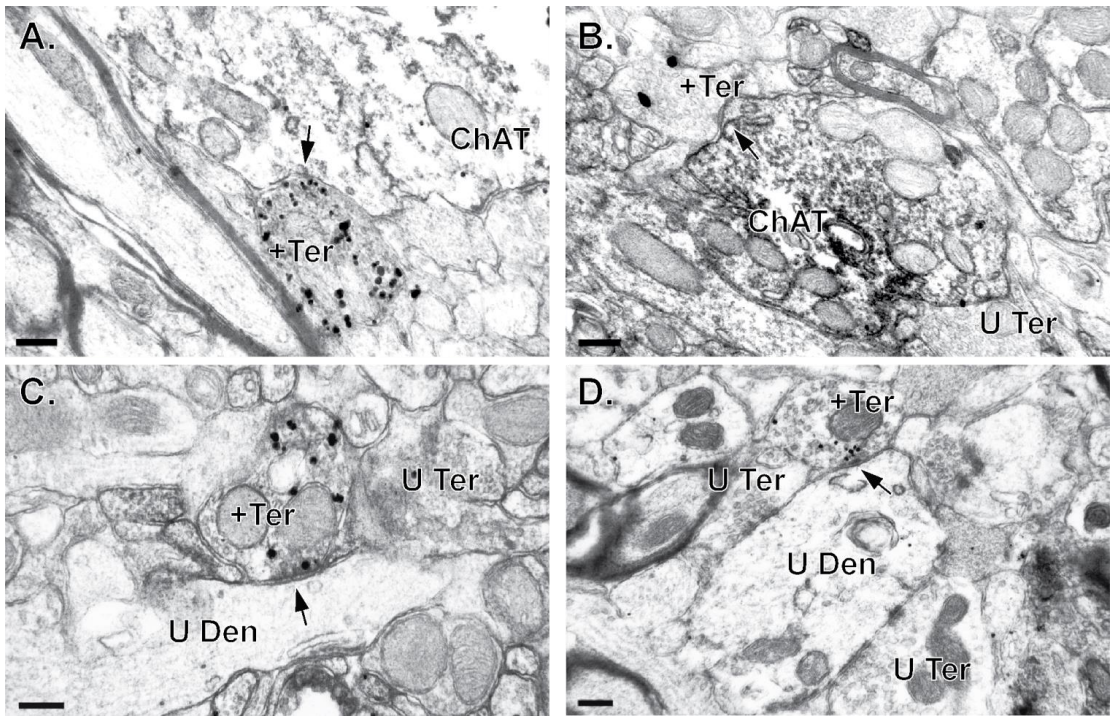

***Figure 3.2*. "Type 1-Symmetric" PV-labeled terminals in contact with ChAT-ir neurons in the monkey striatum.**
A-D: Photomicrographs of "Type1-Symmetric" PV-immunoreactive terminals (+ Ter; immunogold) and their post-synaptic targets in the monkey putamen. (A-B) PV-positive terminals form symmetric synapses (arrows) with large-sized ChAT-labeled dendrites (immunoperoxidase). (C-D) PV-positive terminals in contact with medium-sized unlabeled dendrites (U Den). Unlabeled PV-negative terminals (U Ter) are also shown in these micrographs. Scale bar = 0.2 µm in A-D.

3.3.2 Characterization of PV-labeled terminals in single and double immunoreactions

Based on their heterogeneous ultrastructural characteristics and synaptic specializations, PV-positive terminals (i.e., contained two or more gold particles) in the monkey putamen were divided into three distinct subtypes: Type1-Symmetric (T1-PV; ***Figure 3.2***), Type 2-Symmetric (T2-PV; ***Figure 3.3***), and Type 3-Asymmetric (T3-PV; ***Figure 3.4***).

3.3.2.1 PV-positive terminals that form symmetric synapses

The analysis of 746 PV-ir terminals in the post-commissural putamen revealed that 35 ± 4% of these boutons formed symmetric synapses (***Figure 3.2***, ***Figure 3.3***, and ***Figure 3.5***A). Of these, 42 ± 7 % were in contact with ChAT-labeled dendrites and 58 ± 3% with unlabeled elements (***Figure 3.5***). ChAT-labeled and unlabeled medium- and small-sized dendrites were the preferential targets of PV-positive terminals that formed symmetric synapses (***Figure 3.5***C,D). Unlabeled spines also received significant inputs from these subtypes of PV-ir boutons (***Figure 3.5***D). In contrast, ChAT-labeled cell bodies were not contacted by PV-positive terminals.

Within this group of PV-positive boutons forming symmetric synapses, two major subtypes of terminals were identified based on their ultrastructural features (compare ***Figure 3.2*** and ***Figure 3.3***). The T1-PV terminals (representative of 13 ± 3 % of total PV-ir boutons; ***Figure 3.6***A) had a round or slightly irregular shape, a medium to large size, 1-2 mitochondria, and a moderate amount of small-sized synaptic vesicles (***Figure 3.2***). Contrastingly, the T2-PV terminals (representative of 22 ± 2% of total PV-labeled boutons displayed the appearance of "en passant" type vesicle-filled axonal processes (***Figure 3.6***A) (Peters *et al.*, 1991; Peters & Palay, 1996) that had an elongated shape and contained a few large, loosely packed synaptic vesicles, with rare mitochondria (***Figure 3.3***). Although both types of terminals formed symmetric synapses, the active zones of synaptic junctions formed by T2-PV boutons were shorter than those associated with T1-PV terminals, as seen in single ultrathin sections (***Figure 3.2*** and ***Figure 3.3***). A total of 13 ± 3 % of T1-PV terminals and 29 ± 5% of T2-PV terminals targeted ChAT-

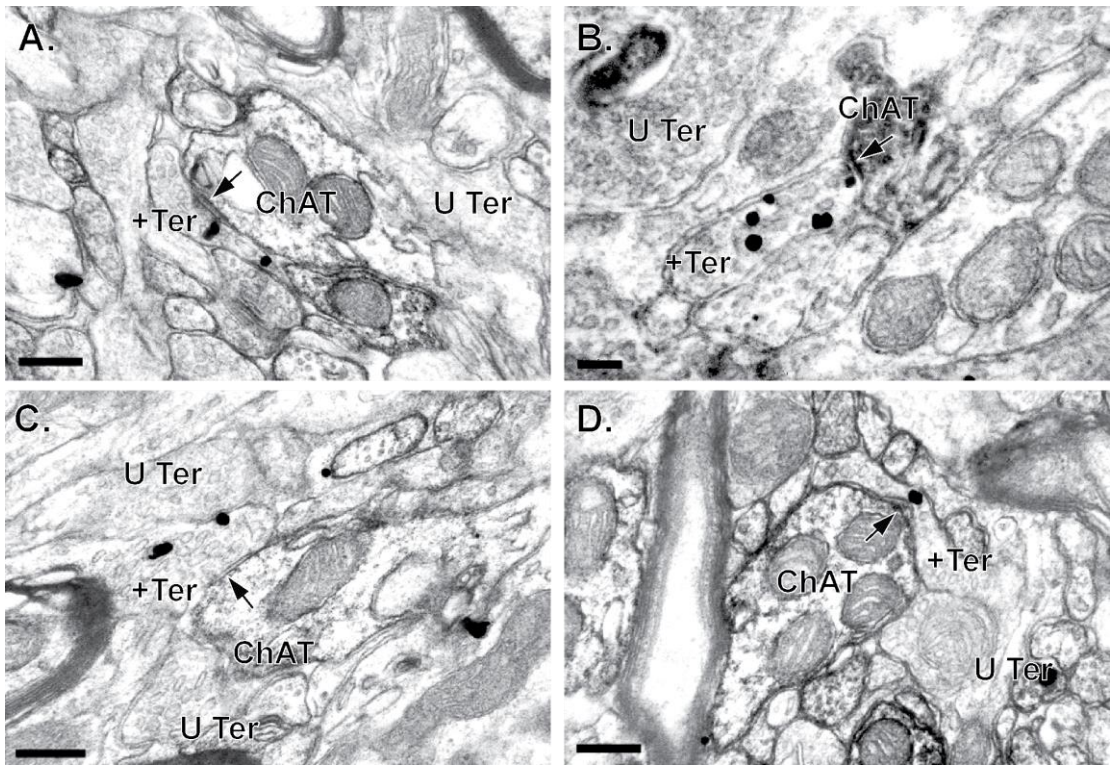

***Figure 3.3*. "Type 2-Symmetric" PV-labeled terminals in contact with ChAT-ir neurons in the monkey striatum.**

A-D: Photomicrographs of "Type 2-Symmetric" PV-positive terminals (+ Ter; immunogold) forming symmetric synaptic contacts (arrows) with small- (**A, B,** and **D**) and medium- (**C**) sized ChAT-labeled (immunoperoxidase) dendrites in the monkey putamen. Note the thin, elongated shape, and the content in fairly large-sized, flattened and loosely-packed synaptic vesicles of these terminals. PV-negative Terminals (U Ter) are also shown in these micrographs. Scale bar = 0.2 μm in A-D.

positive neurons (***Figure 3.6***B). There was no significant difference in the distribution of the two types of labeled boutons along the somatodendritic domain of cholinergic neurons, both being found predominantly on medium- and small-sized dendrites without any innervation of ChAT-positive cell bodies (***Figure 3.6***C).

3.3.2.2 PV-labeled boutons that form asymmetric synapses

The remaining (64.8 ± 3.98%) PV-ir terminals (referred to as T3-PV boutons) formed asymmetric synapses with ChAT-labeled (17 ± 3%) and unlabeled (83 ± 3%) elements in the post-commissural putamen (***Figure 3.4***; ***Figure 3.5***A, B; ***Figure 3.6***A, B). These terminals exhibited ultrastructural features distinct from PV-labeled boutons that formed symmetric

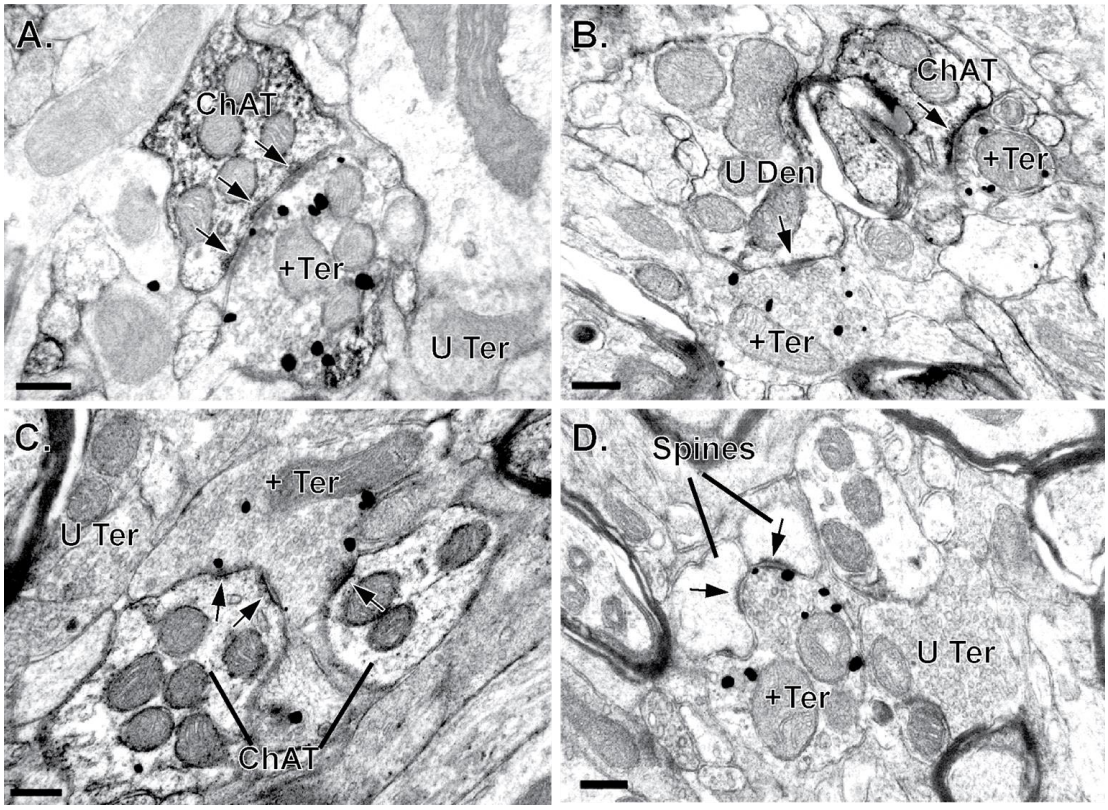

***Figure 3.4*. "Type 3-Asymmetric" PV-labeled terminals in contact with ChAT-ir neurons in the monkey striatum.**

A-D: Photo micrographs of "Type 3-Asymmetric" PV-labeled terminals (+ Ter; immunogold) forming synaptic contacts (arrows) with small- (**B** and **C**) and medium- (**A** and **C**) sized ChAT-positive (ChAT; immunoperoxidase) dendrites in the monkey putamen, along with two unlabeled spines (Spines) (D). These terminals have a fairly large size in diameter and contain many round-shaped synaptic vesicles, as well as the ability to form multiple synapses on the same element (**A**) or on two different elements (**C** and **D**). Scale bar = 0.2 µm in A-D.

synapses (***Figure 3.2*** and ***Figure 3.3***). They were more irregular in shape, had a larger size (), contained more than 1 mitochondria (most often), and were densely packed with a large number of small synaptic vesicles (***Figure 3.4***). Additionally, they formed clear asymmetric synapses with a thick post-synaptic density and were often tightly associated with peri-synaptic glia and non-synaptic puncta adherens (for more details, refer to (Peters *et al.*, 1991)) (***Figure 3.4***A, C, D). The main synaptic targets of these terminals were unlabeled spines (37 ± 4%; ***Figure 3.5***C, D). As seen for T1-PV and T2-PV symmetric terminals, the T3-PV terminals were mostly found on medium- and small-sized ChAT-labeled dendrites (***Figure 3.5***C; ***Figure 3.6***C). The T3-PV

terminals also often formed more than one synaptic contact with single (***Figure 3.4***A, C) or multiple elements (***Figure 3.4***C, D).

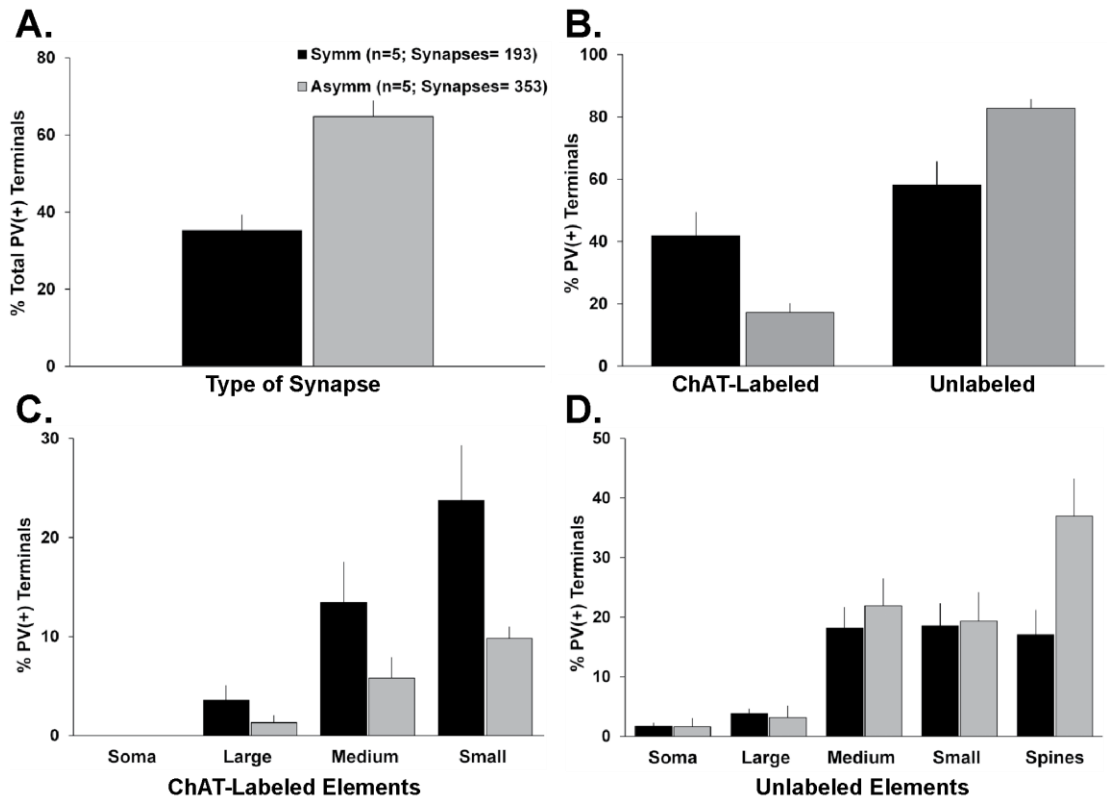

***Figure 3.5*. Proportion of ChAT-labeled and unlabeled elements targeted by PV-immunolabeled terminals in the monkey putamen.**
A: Mean percentages (± SEM) of the types of synapses (Symmetric-black bars; Asymmetric-gray bars) formed by PV-positive terminals in double-labeled tissue (N=5; 546 synapses). **B:** Mean percentages (± SEM) of PV-labeled terminals in contact with ChAT-immunoreactive and unlabeled elements. **C,D:** Mean percentages (± SEM) of PV-positive terminals in contact with ChAT-labeled (C) and unlabeled (D) dendrites of various sizes. Legend in (A) corresponds to (B-D).

3.3.2.3 Total synaptic innervation of ChIs represented by PV-labeled terminals

Additional analyses were carried out in the same double-labeled tissue to determine the proportion of total synaptic innervation of ChAT-labeled neurons that was accounted for by PV-positive terminals. Under the electron microscope, regions that contained both gold- and peroxidase-labeled elements were randomly scanned for ChAT-ir dendrites. The analysis of 225 ChAT-positive dendrites (large-sized = 20; medium-sized = 94; small-sized = 111) and 287 of their synaptic inputs (Symm/PV(-) = 142; Symm/PV(+) = 23; Asymm/PV(-) = 92; Asymm/PV(+)

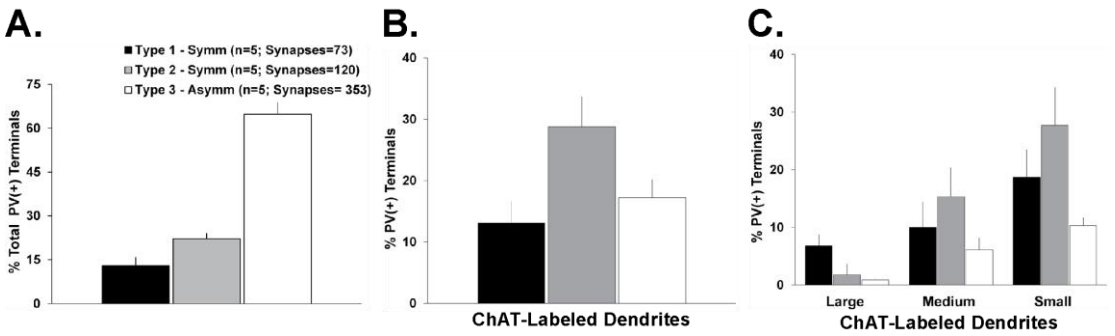

***Figure 3.6*. Proportions of specific subtypes of PV-immunolabeled terminals in contact with ChIs**.

A: : Mean percentages (± SEM) of PV-positive terminal subtypes in double immunostained tissue from the monkey putamen (N=5 monkeys; 546 synapses). **B,C:** Mean percentages (± SEM) of Type 1, Type 2 and Type 3 PV-positive terminals that formed symmetric (T1, T2) or asymmetric (T3) synapses with ChAT-labeled dendrites (B) of various sizes (C). Legend in (B) corresponds to (C).

= 30) revealed that 19 ± 1% of their total synaptic innervation originates from PV-ir terminals in the monkey post-commissural putamen (***Figure 3.7***B). More specifically, 9 ± 1% and 10 ± 1% of this innervation were from PV-positive terminals that formed symmetric or asymmetric synapses, respectively (***Figure 3.7***B). Interestingly, all three sizes of ChAT-ir dendrites received comparable innervation from PV-labeled terminals (***Figure 3.7***C), even though small-sized ChAT-positive dendrites were more abundant in our tissue (***Figure 3.1***A).

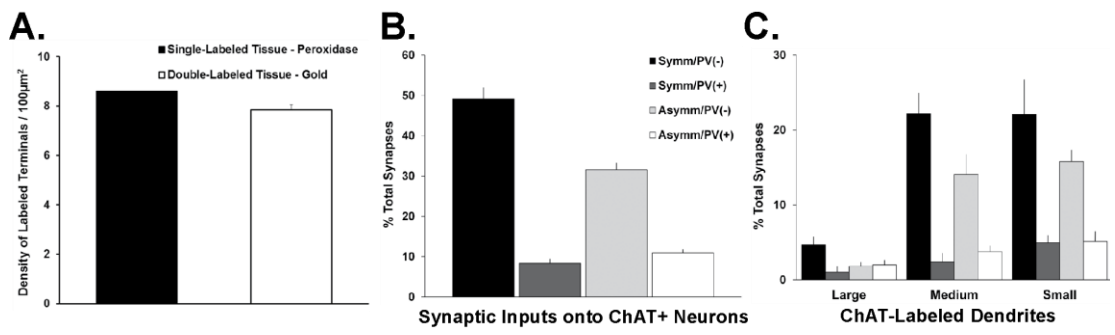

***Figure 3.7*. Proportion of total synaptic inputs onto ChIs from PV-immunoreactive terminals in the monkey putamen.**

A: Comparison of the mean densities (± SEM) of PV-labeled terminals in single-labeled tissue (N=1 monkey; 79 terminals) and PV/ChAT double-labeled tissue (N=5 monkeys; 385 terminals), revealing that the densities are comparable regardless of single or double immunoreactions. **B,C:** Mean percentages (± SEM) of total PV-positive terminals in contact with ChAT-labeled dendrites (B) of various sizes (C) (N=5; 287 terminals; 225 dendrites). The characterization of terminals is based on their synaptic specializations (symmetric vs. asymmetric) and PV expression (PV+ vs. PV-). Legend in (B) corresponds to (C).

When PV-ir terminals were divided into their sub-types, ChAT-labeled dendrites were found to receive a significantly greater innervation (57 ± 5%; $p < 0.05$) from T3-PV inputs (in comparison with those that formed T1-PV (11 ± 2%) and T2-PV(32 ± 5%)) and T2-PV inputs (in comparison with those that formed T1-PV) (***Figure 3.8***A). In regards to the PV innervation of specific sizes of ChAT-positive dendrites, the small-sized ChAT-containing dendrites were the recipients of a significantly greater (56 ± 4%; $p < 0.05$) PV innervation than large- (14 ± 2%) or medium- (32 ± 3%) sized dendrites (***Figure 3.8***B). Additionally, T3-PV terminals contacted more frequently ($p < 0.05$) large- and medium-sized ChAT-labeled dendrites than T1-PV and T2-PV terminal sub-types (***Figure 3.8***B). In the case of small-sized dendrites, they received the same proportion of synaptic inputs from T3-PV and T2-PV terminals, which were significantly higher than the proportion of T1-PV terminals in contact with these dendrites (***Figure 3.8***B).

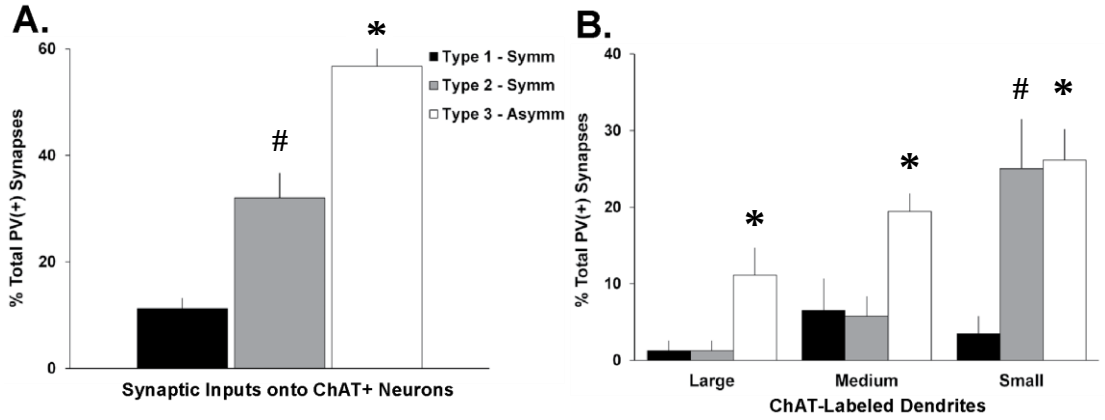

***Figure 3.8*. Proportion of total synaptic inputs onto ChIs from subtypes of PV-positive terminals.** **A, B:** Mean percentages (± SEM) of the subtypes of PV-positive terminals in contact with ChAT-labeled dendrites (A) of various sizes (B). The characterization of terminals is based on their synaptic specializations (Type1-symmetric vs. Type-2 symmetric vs. Type 3-asymmetric) and expression of PV (PV+ vs. PV-). Legend in (A) corresponds to (B). *, $p < 0.05$ Type 1 vs Type 3, Type 2 vs Type 3; #, $p < 0.05$ Type 1 vs Type 2.

3.4 Discussion

Our findings demonstrate that the synaptic innervation of ChIs by PV-containing neurons in the monkey post-commissural putamen is highly complex and involves various sources of intrinsic and extrinsic GABAergic and glutamatergic inputs. We found that a greater proportion

of PV-containing terminals formed asymmetric than symmetric synapses, suggesting that PV innervation of the putamen largely originates from the thalamus but less so from the GABAergic systems of the striatum and pallidum. In agreement with a diverse PV striatal innervation, PV-labeled boutons in the monkey putamen could be categorized into three morphologically-distinct subtypes based on their synaptic specialization and ultrastructural features. The PV-T1 inputs displayed "typical" morphological characteristics of terminals that form symmetric synapses, whereas "en passant" synapses were characteristic of PV-T2 inputs. In contrast, PV-T3 inputs formed asymmetric synapses with multiple sites of contact and peri-synaptic glia. Significant targets of PV-positive boutons, especially the "en passant" and asymmetric subtypes, were ChI dendrites with a medium- and small-sized diameter (but not the cell bodies), providing one-fifth of their total innervation. Through this complex anatomical connectivity, a highly heterogeneous PV network consisting of striatal GABAergic interneurons and axons of extrinsic glutamatergic and GABAergic afferents may tightly modulate the activity of ChI dendrites in the monkey putamen.

3.4.1 Symmetric versus asymmetric synapses onto ChIs

Glutamatergic projections from the cortex and thalamus, along with nigral dopaminergic and GABAergic pallidal afferents, provide substantial, topographic innervation of individual cell populations within the dorsal striatum (Alexander *et al.*, 1986; Alexander *et al.*, 1990; Hoover & Strick, 1993; Groenewegen & Berendse, 1994; Parent & Hazrati, 1995; Bevan *et al.*, 1998; Kita *et al.*, 1999; Mallet *et al.*, 2012). In addition, multiple feedforward and feedback connections are thought to exist between striatal neuron populations (Sullivan *et al.*, 2008; Tepper *et al.*, 2008; Gittis *et al.*, 2010; Planert *et al.*, 2010; Oldenburg & Ding, 2011; English *et al.*, 2012; Szydlowski *et al.*, 2013). We have previously shown that the GABAergic and glutamatergic innervation of striatal ChIs is highly organized and diverse in the monkey post-commissural putamen (Gonzales *et al.*, 2013). For example, ChIs receive synaptic inputs from various populations of striatal

GABAergic neurons on their entire somatodendritic domain or from extrinsic glutamatergic afferents mainly on their distal dendrites (see ***Figure 4*** in (Gonzales *et al.*, 2013). Our present finding that almost 60% of inputs onto striatal ChIs are putatively GABAergic (i.e., form symmetric synapses) is consistent with results of our recent study (Gonzales *et al.*, 2013). However, in regards to asymmetric synaptic inputs onto ChIs, findings from these two studies differ (i.e., current study, 42 ± 1% versus 21 ± 4% in (Gonzales *et al.*, 2013), most likely due to the more extensive immunolabeling of distal dendrites, the main targets of asymmetric synaptic inputs, by the ChAT antibodies used in the present study (refer to ***Figure 6***E in (Gonzales *et al.*, 2013). However, the overall pattern of innervation of ChIs by terminals forming symmetric or asymmetric synapses was similar between the two studies (see ***Figure 4***B in (Gonzales *et al.*, 2013).

3.4.2 Intrastriatal and extrinsic PV-containing afferents of ChIs

In regards to PV synaptology in the primate striatum, our data confirm and extend the previous report by Bennett and Bolam (1994) that PV-containing terminals form either symmetric or asymmetric synapses with striatal neurons in monkeys (Bennett & Bolam, 1994a), although this group reported a much larger proportion of PV-positive terminals forming asymmetric synapses than in our study. This discrepancy could result from the type of immunohistochemical technique used to label PV terminals (i.e., immunoperoxidase versus immunogold) and/or the particular striatal area analyzed. Altogether, our findings indicate that intrinsic and extrinsic putative GABAergic and glutamatergic afferents express PV in the monkey striatum. These findings are in striking contrast with rodent studies suggesting that PV-containing striatal interneurons are the sole source of PV terminals in the rat striatum (Gerfen *et al.*, 1985; Cowan *et al.*, 1990; Kita *et al.*, 1990; Bennett & Bolam, 1994b).

3.4.2.1 PV-positive terminals forming symmetric synapses

In the present study, we found that 42% of PV-labeled terminals forming symmetric synapses contacted ChAT-positive dendrites, accounting for about 9% of the total synaptic innervation of ChIs in the monkey putamen. Striatal GABAergic PV interneurons and/or pallidostriatal afferents are the most likely source(s) of these inputs onto ChIs, because no other sources of striatal afferents that contain PV and give rise to terminals forming symmetric synapses have been shown in the monkey striatum (Gerfen *et al.*, 1985; Côté *et al.*, 1991; Lapper *et al.*, 1992; Bennett & Bolam, 1994a; Kita *et al.*, 1999). Two distinct sub-types of PV-containing terminals forming symmetric synapses were observed in the monkey putamen. The PV-T1 and PV-T2 inputs displayed clear ultrastructural differences in their pattern of innervation of ChI dendrites (see ***Figure 3.2*** and ***Figure 3.3***). Although a similar proportion of both types of terminals were found in the monkey post-commissural putamen, ChIs received a significantly larger proportion of symmetric synaptic inputs from Type 2 than Type 1PV-containing terminals, suggesting heterogeneous sources of inhibitory regulation of ChIs by PV-positive striatal boutons. However, without further axonal tracing and immunohistochemical studies, we cannot make a firm conclusion about the exact origin(s) of these two sub-types of putative GABAergic PV-containing terminals.

Interestingly, our monkey data are different from those in the rodent caudate-putamen complex. In double-label electron microscopic studies from adult rats, synaptic connections between PV terminals and cholinergic interneurons were not found (Chang & Kita, 1992). In line with these anatomical observations, the Silberberg group recently found that putative ChIs (characterized as TANs) do not respond in FSI-TAN paired recordings or after optogenetic activation of FSIs in recent *in vitro* electrophysiological studies in mice (Planert *et al.*, 2010; Szydlowski *et al.*, 2013). However, the authors were cautious in interpreting these data as evidence for the absolute lack of connections between PV terminals and ChIs. They proposed that the effects of potential PV-expressing inputs from intrinsic striatal neurons onto ChI distal

dendrites may have been attenuated in brain slices used in their studies, due to the longer electrotonic distances causing an "apparent depression" of the synaptic driving forces at distally-located synaptic inputs (Banitt *et al.*, 2005; Planert *et al.*, 2010; Szydlowski *et al.*, 2013). Additionally, they suggested that extrinsic afferents from the pallidum were not activated by the local light stimulation in the rat striatum (Planert *et al.*, 2010; Szydlowski *et al.*, 2013), because the pallidostriatal projection does not originate from PV-positive neurons in the rodent globus pallidus (Kita & Kita, 2001; Mallet *et al.*, 2012). In contrast, pallidostriatal neurons in the monkey GPe are strongly immunoreactive for PV (Kita *et al.*, 1999), thereby indicating that the pallidostriatal system is a major source of PV-positive GABAergic terminals in the monkey putamen, but not in the rodent striatum. The functional significance of this differential expression of PV along the pallidostriatal projection between primates and rodents is discussed below.

3.4.2.2 PV-positive terminals forming asymmetric synapses

In the monkey post-commissural putamen, we found that 18% of PV-labeled boutons that form asymmetric synapses contacted ChIs and that 10% of the total innervation of ChIs originates from these putative "glutamatergic" terminals. Thalamostriatal projections are the most likely source of these terminals in the monkey striatum (Jones & Hendry, 1989; Bennett & Bolam, 1994a; Sidibe & Smith, 1999; Jones, 2007). More specifically, the CM/Pf thalamic complex is a likely candidate for the following reasons (reviewed by (Galvan & Smith, 2011): (1) in contrast to most thalamic nuclei, both cell bodies and axonal projections of CM/Pf neurons express PV (Jones & Hendry, 1989; Jones, 2007); (2) PV-expressing terminals from the CM/Pf form asymmetric synapses onto ChIs in the monkey striatum (Sidibe & Smith, 1999); and (3) CM/Pf-striatal projections mainly innervate the matrix compartment of the striatum, where PV-containing terminals form mainly asymmetric synapses compared with the striosomes that contain approximately an even proportion of asymmetric and putative "inhibitory" PV-positive terminals forming symmetric synapses (Sadikot *et al.*, 1992a; Sadikot *et al.*, 1992b; Bennett & Bolam, 1994a). Besides other thalamic nuclei and the amygdala (Celio, 1990; Pitkänen &

Amaral, 1993; Jones, 2007), a small proportion of PV-labeled boutons forming asymmetric synapses in the striatum could also originate from the somatosensory cortex, although such PV-containing corticostriatal projections have only been found in the caudal striatum in mice (Jinno & Kosaka, 2004). Therefore, if the thalamus is the main source of PV-positive terminals forming asymmetric synapses in the monkey striatum, our findings and others (Bennett & Bolam, 1994a) demonstrate a striking species difference between primates and non-primates about PV expression along the thalamostriatal systems. In contrast to monkeys, PV-positive terminals in the rat striatum only form symmetric synapses (Kita *et al.*, 1990; Bennett & Bolam, 1994b; Kubota & Kawaguchi, 2000), and Pf neurons do not express PV immunoreactivity in rodents (Celio, 1990; Jones, 2007). The functional significance of this species difference in PV expression by the thalamostriatal system between rodents and primates remains to be determined.

3.4.3 PV modulation of cholinergic interneurons by GABAergic and glutamatergic projections

Our anatomical findings provide a substrate through which the activity of striatal ChIs can be regulated by both intrastriatal and extrinsic GABAergic and glutamatergic PV-containing afferents in monkeys. As mentioned above, this possible regulatory mechanism is at odds with functional and anatomical data in rodents suggesting that ChIs do not receive inputs from striatal PV interneurons and pallidostriatal afferents in this species (Chang & Kita, 1992; Bevan *et al.*, 1998; Kita & Kita, 2001; Planert *et al.*, 2010; Szydlowski *et al.*, 2013). Because the electrophysiological characterization of the synaptic interactions between GABAergic PV interneurons and ChIs has not been performed in the monkey striatum, we can only speculate about the functional significance of the anatomical data presented in our study.

Striatal GABAergic interneurons and/or pallidal afferents that express the calcium-binding protein PV may have a unique role in regulating the dendritic excitability of ChIs because of the diversity of their symmetric synaptic specializations (i.e., “typical” and “en passant-like”)

with small-sized dendrites of cholinergic neurons. In support of this role, the calcium-binding protein PV is known to act as a slow-onset, mobile calcium buffer in order to maintain calcium homeostasis within neurons (Lee *et al.*, 2000; Schwaller *et al.*, 2002; Schwaller, 2010), especially in regions enriched with PV such as the striatum (Caillard *et al.*, 2000). Additionally, recent studies demonstrated that PV may act as a fast calcium buffer at high concentrations and under particular physiological conditions (Franconville *et al.*, 2011; Bischop *et al.*, 2012). For example, following rises in intracellular calcium levels in response to depolarization, PV utilizes mitochondria and endoplasmic reticulum to precisely modulate levels of calcium transients, thereby precisely mediating synaptic transmission over time (Fierro *et al.*, 1998; Chen *et al.*, 2006). An example of such modulation on the activity of FSIs and MSNs has recently been shown in the rat striatum (Orduz *et al.*, 2013). However, it remains to be determined whether a similar modulatory relationship (i.e., pre-synaptic short-term plasticity) exists between PV-containing FSIs and/or pallidal afferents and ChIs in the striatum of monkeys.

PV-containing inputs (i.e., PV-T3) from the thalamus (likely the CM/Pf) largely innervate ChI dendrites as well and therefore, may serve as another unique regulator of ChI activity in the primate striatum. For example, electrophysiological studies in monkeys demonstrated that thalamic inputs likely recruit intrastriatal GABAergic interneurons (or projection neurons) to mediate their functional connectivity with ChIs (Matsumoto *et al.*, 2001; Nanda *et al.*, 2009). The subtype(s) of GABAergic interneurons that mediate these connections are currently unknown, but our findings suggest that GABAergic PV interneurons may be a participant in this thalamic-ChI communication in monkeys. Additionally, the fact that PV-containing thalamic inputs from CM/Pf contact PV striatal interneurons in the monkey dorsolateral putamen (Sidibe & Smith, 1999) provide further support for this possibility. In rodents, similar electrophysiological findings have been shown (Suzuki *et al.*, 2001; Ding *et al.*, 2010), along with those demonstrating that GABAergic interneurons mediate the functional connectivity between pairs of ChIs, providing evidence that GABAergic interneurons can serve

roles as intermediary regulators of ChI activity (Sullivan *et al.*, 2008; English *et al.*, 2012). However, because recent rodent studies show a lack of FSI-ChI functional interactions (Planert *et al.*, 2010; Szydlowski *et al.*, 2013), these intermediary GABAergic neurons in the rat striatum may not be FSIs but rather NPY-containing interneurons or SP-expressing MSNs (Bolam *et al.*, 1986; Martone *et al.*, 1992; Vuillet *et al.*, 1992).

PV-T3 terminals in the monkey putamen also have specialized ultrastructural characteristics indicative of synaptic plasticity and efficacy (Lushnikova *et al.*, 2009; Bourne & Harris, 2011; Lushnikova *et al.*, 2011; Bourne & Harris, 2012; Tewari & Majumdar, 2012). Previous electron microscopic observations revealed that highly plastic hippocampal terminals are associated with puncta adhaerentia (or puncta adherens), perforated synapses, multiple sites of vesicular release, and perisynaptic astroglial processes (Perea & Araque, 2007; Lushnikova *et al.*, 2009; Bourne & Harris, 2011; Lushnikova *et al.*, 2011; Bourne & Harris, 2012; Navarrete *et al.*, 2012; Tewari & Majumdar, 2012; Navarrete *et al.*, 2013), as was observed in our studies of PV-T3 inputs to striatal ChIs (***Figure 3.4***). Interestingly, putative thalamic, i.e., vesicular glutamate transporter 2 (vGluT2)-labeled, terminals in the monkey striatum were previously reported to have similar structural characteristics as hippocampal boutons (Villalba & Smith, 2010; 2011a; b), suggesting that the asymmetric PV-containing terminals encountered in our tissue may, indeed, originate from the thalamus and participate in synaptic plasticity in the primate striatum.

To our knowledge, the effects of PV on GABA and glutamate release from pallidal and thalamic terminals and/or on the activity of ChIs have not been analyzed in the monkey striatum. Because PV is not expressed by the rodent pallido- and Pf-striatal systems, the expression of this calcium binding protein in primate pallidal and thalamic inputs to ChIs may provide a unique and different means of synaptic modulation, plasticity, and learning. Altogether, the prominent synaptic interactions between PV-containing terminals and cholinergic neurons revealed in our study open up numerous opportunities to learn more about the interactions between these systems within the striatum.

Chapter 4 :

Conclusions & Implications

4.1 Summary of main findings

Although ChIs are nearly as well-studied as striatal projection neurons, numerous discrepancies exist in the literature about their morphological characterization, distribution, synaptic connectivity, and receptor expression. However, after my in-depth analyses of their synaptic connectivity in the monkey post-commissural putamen (refer to ***Figure 4.1*** and ***Figure 4.2***), the following conclusions can be proposed:

1) ChIs receive a diverse synaptic innervation in the monkey putamen that largely originates from GABAergic inputs, while their remaining afferents come from glutamatergic neurons and other chemically uncharacterized sources.
2) ChIs receive GABAergic inputs from various sources in the monkey putamen. The collaterals of SP- or Enk-containing striatal projection neurons represent one-quarter of these inputs, while the PV-containing terminals from either local striatal interneurons or extrinsic pallidal projection neurons contribute to one-tenth of this innervation. The source(s) of the remaining GABAergic inputs to ChIs are currently unknown.
3) ChIs receive glutamatergic inputs from various sources in the monkey putamen. The PV-expressing afferents, most likely from the thalamus, represent one-tenth of these inputs. The source(s) of the remaining glutamatergic inputs to ChIs are currently unknown.
4) ChIs receive diverse patterns of GABAergic and glutamatergic synaptic innervation. The collaterals of GABAergic direct and indirect striatofugal neurons evenly innervate the somatodendritic domain of ChIs. Contrastingly, the more distal portions of ChI dendrites receive a larger innervation from PV-containing GABAergic and glutamatergic neurons.

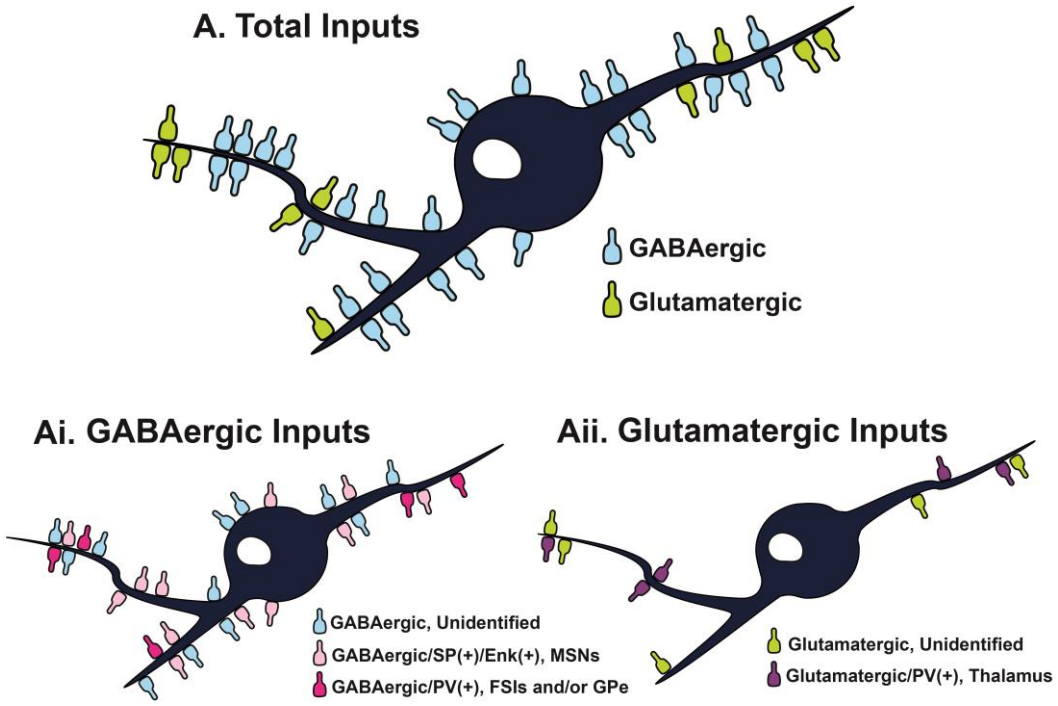

***Figure 4.1*. Summary schematic of the various chemically characterized synaptic inputs onto ChIs in the monkey putamen.**
The relative location and proportion of the different terminal sub-types along the somatodendritic domain of ChIs are represented (**A**, **Ai**, and **Aii**). The total GABAergic and glutamatergic inputs onto ChIs are shown in (A). In addition, the chemical phenotypes of these GABAergic (Ai) and glutamatergic (Aii) inputs onto ChIs are revealed. Abbreviations: substance P (SP); enkephalin (Enk); parvalbumin (PV); fast-spiking interneurons (FSIs); globus pallidus, external segment (GPe).

4.2 Implications

4.2.1 New views on striatal circuitry of ChIs

4.2.1.1 GABA, neuropeptides, and the striatal cholinergic systems

The strong association between MSNs and ChIs shown in this thesis (refer to ***Figure 4.2***) 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 (for reviews, see (Zhou *et al.*, 2003; Bonsi *et al.*, 2011; Oldenburg & Ding, 2011), 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, while their dendrites extend beyond the boundaries of each striatal compartment (Mesulam *et al.*, 1984; Hirsch *et al.*, 1989; Lehéricy *et al.*, 1989; Kubota & Kawaguchi, 1993; Aosaki *et al.*, 1995; Holt *et al.*, 1996; Bernacer *et al.*, 2007; Crittenden & Graybiel, 2011).

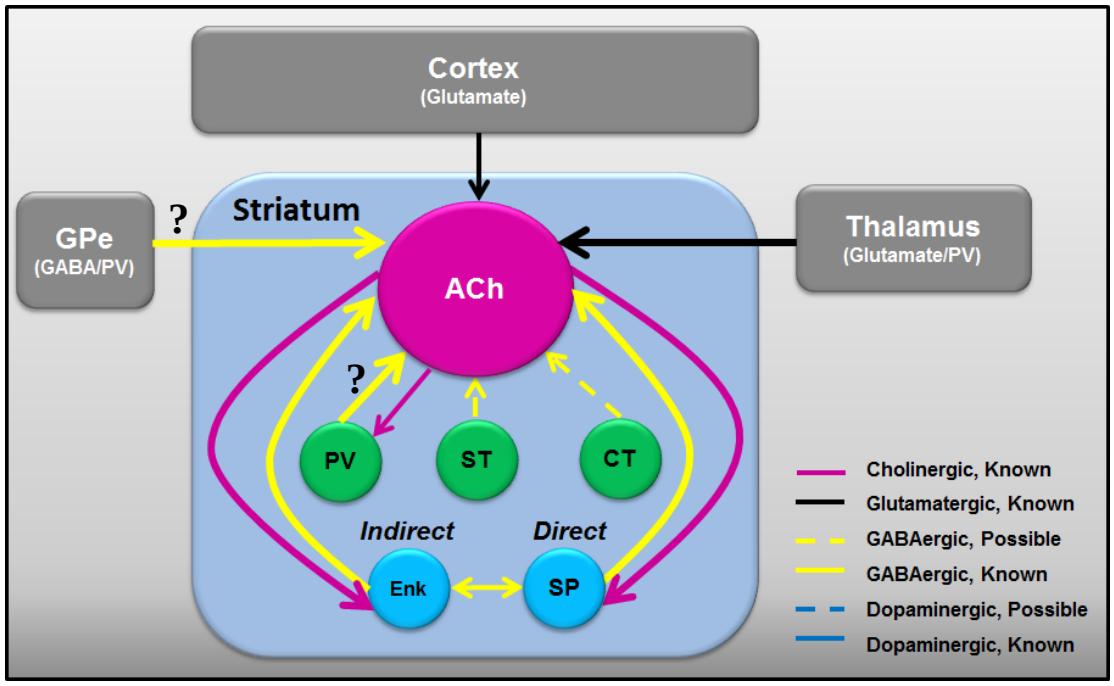

***Figure 4.2*. Current connectivity of ChIs in the monkey putamen.**
Based on the findings from the double-label immunoreactions performed in this thesis, new views of the striatal circuitry in primates can be put forth. The "?" indicates that further studies are needed to assess the exact proportion of PV GABAergic inputs that originate from the GPe versus striatal PV interneurons in monkeys.

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 (Solis *et al.*, 2007; Raju *et al.*, 2008; Villalba & Smith, 2010; 2011a; b). The overall firing frequency of striatal ChIs does not change in parkinsonism (Aosaki *et al.*, 1994a; Raz *et al.*, 1996; Raz *et al.*, 2001). 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 DA from the system (MacKenzie *et al.*, 1989; Joyce, 1991; 1993; Ding *et al.*, 2006). Thus, in light of our data that show 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.

4.2.1.2 ChIs in synaptic plasticity: roles of PV, calcium, and glia

ChIs undergo plastic activity changes in response to sensory stimuli associated with behavioral events such as acquiring rewards during associative learning or reacting to aversive signals, and to cortical, thalamic, nigral, and intrastriatal stimulation and/or pharmacological lesion (Kimura *et al.*, 1984; Aosaki *et al.*, 1994a; Aosaki *et al.*, 1994b; Pisani *et al.*, 2000; Suzuki *et al.*, 2001; Apicella, 2002; Pisani *et al.*, 2002; Bonsi *et al.*, 2003; Bonsi *et al.*, 2004; Nanda *et al.*, 2009; Ding *et al.*, 2010; Bradfield *et al.*, 2013). ChI intrinsic properties and synaptic afferentation have been shown to greatly contribute to these changes, in particular, the induction of long-term changes in their synaptic efficacy. For example, in rat slices, ChIs demonstrated typical characteristics of plastic responses to high frequency intrastriatal stimulation (3 trains at 100 Hz for 3 sec, 20-sec interval), consisting of a sustained potentiation in their EPSP amplitude for 30-60 minutes and a significant increase in their intracellular calcium levels (Bonsi *et al.*, 2004). Interestingly, the induction of long term potentiation of ChIs was mediated by L-type calcium channels, GABA-A receptors, and D1 dopamine receptors, but not by NMDA and AMPA receptors, suggesting a key role for GABAergic transmission in the plasticity occurring at

ChI synapses after high frequency stimulation (Bonsi *et al.*, 2004). On a number of levels, my anatomical findings (see ***Figure 4.2***) may support this concept: (1) ChIs are largely innervated by GABAergic terminals; (2) some of these boutons co-express neurochemicals known to be involved in synaptic plasticity such as SP and PV (Aosaki & Kawaguchi, 1996; Caillard *et al.*, 2000; Schwaller, 2010); and (3) the dendrites of ChIs contain subcellular machinery, such as the subsurface cisternae of endoplasmic reticulum (Broadwell & Cataldo, 1983), that allows for the regulation of extracellular calcium entry and membrane excitability directly at the post-synaptic site (McGraw *et al.*, 1980; Pozzo-Miller *et al.*, 2000; Kawamoto *et al.*, 2012). However, the most supportive evidence may exist between ChIs and their PV-containing GABAergic and glutamatergic inputs, as described in section 3.4.3.

4.2.1.3 Functional impact of the morphology and synaptic afferentation of ChI dendrites

In comparison to other neuron populations in the primate striatum (Difiglia *et al.*, 1980; Yelnik *et al.*, 1991; Yelnik *et al.*, 1993), the morphology of ChI dendritic trees is the most complex, consisting of thick primary dendrites that profusely branch with successive tapered or thin dendritic arborizations (***Figure 1.1*** and ***Figure 2.1***). Additionally, the general distribution of ChI synaptic inputs follows two well-defined patterns in the monkey post-commissural putamen: (1) symmetric synapses that express GABA, SP, or Enk are found on ChI dendrites of all sizes (***Figure 2.5*** and ***Figure 2.7***), whereas (2) the medium- and small-sized dendrites of ChIs are the main recipients of PV-containing symmetric and asymmetric synapses (***Figure 3.7***). In contrast, the striatal principal neurons in primates mainly receive basket-like innervation from striatal GABAergic interneurons onto their somata, as well as glutamatergic asymmetric synapses onto their spine heads and necks (Difiglia *et al.*, 1980; DiFiglia *et al.*, 1982). However, MSN proximal and distal dendrites in primates are practically synapse-free, except for a few symmetric and asymmetric synaptic inputs. Based on their intrinsic membrane properties (Bennett *et al.*, 2000; Goldberg & Reynolds, 2011; Schulz & Reynolds, 2013), complex dendritic morphology, and diverse synaptic afferentation and distribution, ChIs may uniquely partake in a highly variable

synaptic integration and coupling of incoming signals in the primate striatum, possibly contributing to the regulation of their excitability and participation in synaptic plasticity (Suzuki *et al.*, 2001; Bonsi *et al.*, 2003; Bonsi *et al.*, 2004).

Traditionally, signals transmitted from the soma to the dendrites of neurons, and vice versa, were thought to simply attenuate as they propagate along a neuron, as suggested by the cable theory (Rall *et al.*, 1967; Spruston *et al.*, 1993). In addition, an individual neuron was believed to only be capable of a linear summation of its synaptic inputs, whereas groups of neurons were required for more complex computational processing of information (reviewed by (Silver, 2010). However, electrophysiological and computational evidence now suggests that individual neurons can integrate and compartmentalize incoming signals at the level of their dendritic trees (for examples, see (Rall, 1967; Rall *et al.*, 1967; Blomfield, 1974; Koch *et al.*, 1983; Braitenberg, 2001; Silver, 2010; Ferrante *et al.*, 2013). The processing of these signals into dendritic (or back-propagating) spikes is dependent upon their intrinsic firing properties (Losonczy *et al.*, 2008) and branch point morphology (i.e., dendrite/trunk diameter ratio, number of branch points, length of the tapering dendrite, and diameter of the tapering region at the trunk) (Gulledge *et al.*, 2005; Migliore *et al.*, 2005; Migliore & Shepherd, 2005; Ferrante *et al.*, 2013), along with the chemical phenotype, density and distribution of their synaptic inputs (Gulledge *et al.*, 2005; Li & Ascoli, 2006). Thus, dendrites are capable of regulating the rate and precise timing of action potential initiation at the soma/axon hillock and axonal output (Gulledge *et al.*, 2005; Ferrante *et al.*, 2013). Taken together, it is highly plausible that striatal ChIs participate in this type of dendritic signaling, because substantial examples of neurons with similar morphologies as ChIs (i.e., highly branching dendrites with different sized diameters), as well as other striatal neurons, participate in this type of dendritic signaling in the cortex and hippocampus (for recent instances, refer to (Gulledge *et al.*, 2005; Day *et al.*, 2008; Gertler *et al.*, 2008; Stokes & Isaacson, 2010; Evstratova *et al.*, 2011; Xu *et al.*, 2012; Fortier & Bray, 2013; Harnett *et al.*, 2013).

4.3 Cholinergic interneuron dysfunction

4.3.1 Structural changes in ChI innervation in Parkinson's disease

Morphological and ultrastructural changes of spines and glutamatergic synapses at MSNs are well-established in parkinsonian animal models or PD patients (reviewed by (Smith & Villalba, 2008) (Solis *et al.*, 2007; Raju *et al.*, 2008; Villalba & Smith, 2010; 2011a; b). Contrastingly, only a few studies have examined similar plastic changes in ChIs or GABAergic interneurons in response to nigrostriatal dopamine depletion (Smith & Villalba, 2008; Taverna *et al.*, 2008). Despite the well-established evidence for a striatal dopamine-acetylcholine imbalance in Parkinson's disease (for reviews, refer to (Pisani *et al.*, 2007; Smith & Villalba, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011; Crittenden & Graybiel, 2011; Goldberg *et al.*, 2012), the limited knowledge about the complex functional interactions between cholinergic and dopaminergic systems at the striatal level (see sections 1.2.4, 1.6.2.3, and 1.6.2.4) have significantly slowed down advances in this field.

The changes in dopamine-acetylcholine relations that occur in various animal models of Parkinson's disease have been challenging to interpret because of the complex interplay between nigrostriatal dopamine and ACh. For instance, although increased *in vitro* extracellular levels of striatal ACh (Ding *et al.*, 2006; Fino *et al.*, 2007; Sanchez *et al.*, 2011) or decreased AChE activity (Sirviö *et al.*, 1989; Zang & Misra, 1993; Hadjiconstantinou *et al.*, 1994; Zang & Misra, 1996; 2003) were shown in rodent and monkey PD models, respectively neither the spontaneous firing rate or the level of synchronization of TANs (i.e., putative ChIs) activity appeared to be significantly affected in MPTP-treated monkeys, contrary to accepted belief (correspondence with Dr. Hagai Bergman) (Aosaki *et al.*, 1994a; Raz *et al.*, 1996; Raz *et al.*, 2001). However, TANs demonstrated an increase in oscillatory activity (Raz *et al.*, 1996; Raz *et al.*, 2001), along with a decrease in sensory responsiveness to rewards (Aosaki *et al.*, 1994a), in the dopamine-denervated monkey striatum. In addition, TANs displayed an apparent decreased after-

hyperpolarization current in dopamine-depleted rodents, possibly contributing to their reduced spike frequency adaptation under these conditions (Sanchez *et al.*, 2011). However, it is not fully understood how these changes in cholinergic activity transpire and then, influence overall striatal function and output in parkinsonism.

As described above, the existence of dopaminergic inputs onto ChIs is somewhat controversial. The DA-ChI connectivity has only been analyzed primarily in the rodent striatum, where ChIs were identified on the basis of their ultrastructural characteristics (Freund *et al.*, 1984; Aoki & Pickel, 1988; Chang, 1988; Pickel & Chan, 1990; Smith *et al.*, 1994b). Therefore, this leaves us questioning whether or not these connections exist in the primate striatum. However, because ChIs densely express D1-like and D2-like dopamine receptors and respond to dopaminergic activity changes in the monkey striatum (refer to sections 1.2.4, 1.6.2.3, and 1.6.2.4), the existence of connections between dopaminergic terminals/axons and ChIs seems highly plausible. In parkinsonian rodent models, dopamine receptors in the striatum become hypersensitive to dopamine, often resulting in enhanced cholinergic responsiveness to their activation in the dopamine-depleted state (for reviews, refer to (Pisani *et al.*, 2007; Smith & Villalba, 2008; Aosaki *et al.*, 2010; Lester *et al.*, 2010; Bonsi *et al.*, 2011). Thus, the inhibitory and excitatory actions of dopamine on ChI activity may be significantly altered in parkinsonism, resulting in the previously observed changes in striatal cholinergic activity (Aosaki *et al.*, 1994a; Raz *et al.*, 1996; Raz *et al.*, 2001; Sanchez *et al.*, 2011).

In addition, the connectivity of ChI inputs may be transformed in the dopamine-depleted striatum. For instance, both ChIs and FSIs/PV interneurons were found to increase their connectivity with striatopallidal (D2-expressing) projection neurons in a rat and mouse model of PD, respectively (Salin *et al.*, 2009; Gittis *et al.*, 2011). However, only cholinergic innervation of striatonigral (D1-expressing) neurons was decreased after dopamine depletion, whereas FSIs connections with these MSNs remained unchanged (Salin *et al.*, 2009; Gittis *et al.*, 2011). In contrast to the majority of thalamic nuclei, substantial cell loss occurs in the CM/Pf nuclei of

MPTP-treated monkeys (Villalba *et al.*, 2013) and parkinsonian humans (Henderson *et al.*, 2000a; b; Halliday *et al.*, 2005; Halliday, 2009), suggesting that the glutamatergic innervation of ChIs may also undergo changes in parkinsonism.

Altogether, these findings demonstrate complex changes in striatal cholinergic activity after dopamine depletion, while many uncertainties still exist. Thus, targeting the striatal cholinergic system in PD is reasonable, but a more detailed characterization of the localization and function of specific cholinergic receptor subtypes in the striatum and other basal ganglia nuclei are warranted to identify therapeutically relevant targets devoid of debilitating side effects (refer to section 4.2.1.1).

4.3.2 Cholinergic therapies for Parkinson's disease

Anti-cholinergic drugs, used alone or in conjunction with dopaminergic therapies, were utilized as one of the first treatments for PD patients (Barbeau, 1962; Duvoisin, 1967; Bartholini *et al.*, 1987), although their use was limited because of significant cognitive and autonomic side effects (reviewed by (Katzenschlager *et al.*, 2003). More than a century later, the use of anti-cholinergic agents as PD therapeutics still remains a major challenge.

At this point, the most commonly used anti-parkinsonian treatments are dopaminergic agonists that focus on targeting the motor symptoms (akinesia/bradykinesia and muscular rigidity) of PD, whereas anticholinergics are utilized as secondary treatments for PD, alleviating non-motor and motor symptoms, such as bladder dysfunction and tremor, respectively (Duvoisin, 1967; Katzenschlager *et al.*, 2003; Smith & Villalba, 2008; Lester *et al.*, 2010). However, long-term use of dopaminergic therapies, involving the progressive ramping up of drug dose, leads to abnormal, involuntary movements (dyskinesias) and psychiatric complications in PD patients, while aversive psychiatric and cognitive side effects are the consequence of the non-selectivity of current anti-cholinergic therapies (Lester *et al.*, 2010; Xiang *et al.*, 2012). As a result, decades of research have been directed towards the search for alternative monotherapies or those in

conjunction with dopaminergic agonists, especially those that can selectively target striatal cholinergic function, due to its tight relationship with the nigrostriatal dopaminergic system.

In regards to cholinergic therapy for PD, drugs targeting M1 muscarinic receptors have been developed in order to attenuate increased M1-signaling at striatal projection neurons (Goldberg *et al.*, 2012; Xiang *et al.*, 2012). In rodents, genetically deleting M1 muscarinic receptors partially prevented the loss of glutamatergic innervation of MSN dendritic spines in parkinsonian mice (Shen *et al.*, 2007). However, the current M1 antagonists also inhibit M4 muscarinic receptors, resulting in additional effects on MSN activity, likely due to the decrease of autoregulation of cholinergic activity (Xiang *et al.*, 2012). In contrast, it may be more practical to enhance M2/M4 muscarinic autoreceptors, largely expressed by ChIs, in order to inhibit ACh release, but promote dopaminergic neurotransmission (Pisani *et al.*, 2007; Threlfell *et al.*, 2010; Threlfell & Cragg, 2011). In support of this concept, the mRNA expression of M4 mAChRs was significantly decreased in the dorsal striatum of dopamine-depleted rodents (Kayadjanian *et al.*, 1999), as well as M4 autoreceptor signaling that normally regulates ChIs spiking and ACh release (Ding *et al.*, 2006).

Another potential target is nAChRs expressed by striatal neurons (data inconclusive for ChIs) and the nigrostriatal system, the α4β2 and α6β2 nicotinic receptors, which show potential neuroprotective and anti-parkinsonian roles (Zhou *et al.*, 2003; Lester *et al.*, 2010; Perez *et al.*, 2010; Quik *et al.*, 2011; Quik & Wonnacott, 2011; Quik *et al.*, 2012). Numerous studies have demonstrated a drastic loss of α6β2 nicotinic receptors (primarily responsible for ACh-induced dopamine release in primates) in parkinsonian animal models and patients, along with a less severe reduction in α4β2 subtypes (Zhou *et al.*, 2003; Bordia *et al.*, 2007; Perez *et al.*, 2010; Quik *et al.*, 2011; Quik & Wonnacott, 2011). More specifically, with minimal to moderate nigrostriatal damage, α4α6β2-expressing dopaminergic terminals are significantly reduced, whereas dopaminergic terminals containing α4β2, α6β2, and α4α6β2 subtypes are all lost with severe nigrostriatal damage (Quik & Wonnacott, 2011), leaving only α4β2 and α6β2-expressing non-

dopaminergic neurons to regulate ACh-induced dopamine release in the striatum of late stage parkinsonism. Additionally, in partially-depleted parkinsonian animals, nicotine acting on α4β2 and α6β2 nicotinic receptors reduces abnormal, involuntary movements induced by commonly used dopaminergic therapies (Quik *et al.*, 2011). Therefore, a promising role for nicotinic acetylcholine receptors lies in the early intervention of parkinsonism with the use of nicotinic agonists acting at the α6β2 (and possibly α4β2) subtype to induce dopamine release from the intact dopaminergic axons, whereas in later stages of PD, it is unclear what the effects on striatal and dopaminergic activity will be with activation of the remaining α4β2 and α6β2 nicotinic receptors located on non-dopaminergic neurons in the striatum. Further electrophysiological and pharmacological studies could help delineate these uncertainties.

4.4 Concluding remarks

Within the striatum, ChIs are involved in a highly complex network formed by intrastriatal circuits and their external afferents that systematically regulate information outflow from the striatum to other basal ganglia nuclei about behaviorally-significant events. The precise mechanisms in which striatal ChIs contribute to this network under normal physiological conditions and in wide range of neurological and psychiatric disorders are difficult to delineate because of their diverse responses to afferent inputs and highly complicated downstream effects in the striatum. One approach to begin delineating this puzzle is to define the precise synaptic microcircuitry of striatal ChIs, in order to identify the density, location, and chemical phenotype of their synaptic afferents. The work presented here has significantly contributed to this understanding through the demonstration that two key striatal neuron populations, the direct and indirect GABAergic projection neurons, largely contribute to the total synaptic innervation of the somatodendritic domain of ChIs in the post-commissural putamen of monkeys. Importantly, this work also provided evidence for a significant connection between striatal ChIs and PV-containing GABAergic afferents, likely originating from GABAergic parvalbumin interneurons and/or

pallidostriatal projections, and glutamatergic afferents, likely originating from the CM/Pf thalamic nuclei, which are not found in the rodent species. These data suggest that ChIs are under a strong but diverse regulation by intrastriatal GABAergic and extrinsic glutamatergic neurons in the monkey putamen, but functional data demonstrating this scenario in the primate striatum does not currently exist. However, recent advances in the development of optogenetic tools combined with the introduction of more specific cholinergic receptor antibodies and drugs pave the way for exciting progress in the understanding the striatal cholinergic system in normal and diseased states, and potential therapeutic advances using ChIs and cholinergic receptors as therapeutic targets in brain disorders such as Parkinson's disease.

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