Tyrosine Hydroxylase-Expressing Interneurons
in Intact and Dopamine-Depleted Striatum

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Abstract of the Dissertation

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The sets of experiments in this thesis have been driven by the hypothesis that striatal interneurons, in addition to spiny projection neurons, are important sites of action for striatal dopamine and respond to loss of striatal dopamine by discernible changes in their anatomy and physiology.

Tyrosine hydroxylase-expressing (TH+) interneurons of the striatum stand in a critical point for our understanding of the interaction between striatal dopamine and striatal interneurons. Recent investigations from our laboratory have established that TH+ neurons comprise electrophysiologically distinct group striatal GABAergic interneurons by using transgenic mice expressing enhanced green fluorescent protein under the control TH promoter (TH-eGFP). Interestingly, although the number of striatal TH-eGFP neurons was estimated to be around 3000 per striatum per hemisphere using unbiased stereological methodology, TH-eGFP neurons form four electrophysiologically distinct subtypes. Furthermore, experimentally induced- or Parkinson’s disease (PD)-related loss of striatal dopamine is repeatedly reported to increase the number of TH+ interneurons.
Therefore, in order to understand the significance of striatal TH+ neurons in the normal and dopamine-depleted striatum, determining anatomical distribution of TH+ neurons with regard to their electrophysiological diversity and striatal anatomical heterogeneity was imperative. In the first part of my thesis, I investigated striatal localization of TH-eGFP neurons with respect to anatomical organization of the striatum. In the second set of experiments, I investigated the changes in anatomical distribution, morphology, and electrophysiology of TH-eGFP neurons following striatal dopamine-depletion.

Data presented in this thesis indicates that the electrophysiological subtypes of TH-eGFP interneurons are equally prevalent throughout striatum. However, when patch/striosome-matrix organization is taken into account, a different pattern emerges in the dorso-ventral axis of the striatum: a significantly higher proportion of striatal TH-eGFP interneurons were located in MOR-enriched domains of the ventral striatum. Finally, TH-eGFP interneurons in both compartments extended their neurites into the neighboring compartment.

In the second set of experiments, I investigated the effect of striatal dopamine loss achieved by unilateral intranigral 6-OHDA infusion on TH-eGFP interneuron anatomy and physiology. The data presented here show that dopamine loss has a temporally and regionally different effect on the number of striatal TH-eGFP interneurons. Several electrophysiological parameters of TH-eGFP interneurons show profound changes in dopamine-depleted striatum, such as reduction in plateau
potentials and increase in spontaneous synaptic inputs, which were accompanied by a significant increase in spine density on these neurons.

Taken together, the findings in this thesis establish that TH-eGFP neurons are a distinct group of striatal interneurons that show differential anatomical patterning throughout the dorsal-ventral axis of the striatum and respond to striatal dopamine-loss in a qualitatively and quantitatively different manner than striatal projection neurons.
Preface

In his interview as the one of the first recipients of Kavli Prize, Pasko Rakic states: “I find it fascinating that our cortical neurons are as old, or even a little older, than we are, and during our prolonged life span we always use the same cells.” (Nature Reviews Neuroscience, 9, 893-897 (December 2008) doi: 10.1038/nrn2548). This proposition holds true for the vast majority of the mammalian central nervous system, although there are certain neuroproliferative domains present. In its essence, Rakic’s statement puts forth an overwhelming phenomenon that the central nervous system is endowed with a set number of core constituents and they are irreplaceable. However, the neural niche is anything but static: it changes throughout life as a result of the interaction between genetically determined programs and experience. Furthermore, due to certain neurodegenerative diseases, progressive loss of neural elements is inevitable. Parkinson's disease is one particular example of such a neurodegenerative disease, the hallmark of which being the loss of nigrostriatal dopaminergic neurons. However, clinical symptoms do not surface until more than 50% of the nigral dopaminergic cells are lost. The striatum receives the heaviest dopaminergic innervation within the basal ganglia and is the main point of entry for cortical and thalamic drive in the basal ganglia. Therefore, understanding the mechanism and timeline of the changes taking place in striatum deprived of dopamine is key to understanding what other sequel of alterations take place in the rest of the basal ganglia in the diseased state. Striatal TH+ neurons stand at the very intersection of striatal microcircuit dynamics and striatal dopamine owing to their identifying neurochemical marker. However, findings accrued over 30 years on striatal TH+
neurons represent a tumultuous picture and certain critical information were still missing. This situation motivated me to pursue my dissertation work to fill in these gaps in our knowledge of TH+ interneurons in intact and dopamine-depleted striatum. Using transgenic mice allowed immediate detection of this group of neurons in striatal slices for electrophysiology and anatomy on the basis of the sensitive reporter activity of enhanced green fluorescent protein under the control of the TH promoter. This gave us a significant advantage in characterizing striatal TH+ neurons since immunocytochemical detection of the TH protein in these neurons is variable unless somatic TH protein level is artificially augmented (i.e., using colchicine injections). In the rest of this document, the data presented will attempt at answering the following questions:

- Do any of the electrophysiological subtypes of TH-eGFP interneurons have preferential striatal localization?
- Are TH-eGFP neurons randomly distributed with respect to the patch-matrix compartmental organization of the striatum?
- Is there a differential distribution of any of the electrophysiological subtypes of TH-eGFP neurons with respect to the compartmental organization of the striatum?
- What type of changes do TH-eGFP neurons undergo in dopamine-depleted striatum?
Acknowledgements

Throughout my dissertation work at Dr. Tepper's laboratory, I not only learned a lot about the basal ganglia but also about myself. I will forever remain grateful for the mentorship of Dr. James Tepper and Dr. Elizabeth Abercrombie and their constant support for my research endeavors. Their transcending passion for basal ganglia has shaped my scientific persona and will continue to be a source of inspiration for my future work. I am deeply grateful to Dr. Laszlo Zaborszky, Dr. Miller Jonakait and Dr. Yoland Smith for their invaluable advice on my dissertation work.

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<th>Full Form</th>
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<tbody>
<tr>
<td>6-OHDA</td>
<td>6-hydroxy-dopamine</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>AP-5</td>
<td>DL-2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyl-transferase</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CR</td>
<td>Calretinin</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>D1R</td>
<td>D1 subtype of dopamine receptor</td>
</tr>
<tr>
<td>D2R</td>
<td>D2 subtype of dopamine receptor</td>
</tr>
<tr>
<td>dSTR</td>
<td>Dorsal striatum</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FSI</td>
<td>Fast-spiking interneuron</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma amino butyric acid</td>
</tr>
<tr>
<td>GIRK</td>
<td>G-protein coupled inwardly rectifying potassium channel</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus pallidus external segment</td>
</tr>
<tr>
<td>GPi</td>
<td>Globus pallidus internal segment</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>I</td>
<td>Current</td>
</tr>
<tr>
<td>IEI</td>
<td>Inter-event interval</td>
</tr>
<tr>
<td>ISI</td>
<td>Inter-spike interval</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>LTS</td>
<td>Low-threshold spiking</td>
</tr>
<tr>
<td>PLTS</td>
<td>Persistent and low-threshold spiking</td>
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<tr>
<td>MGE</td>
<td>Medial ganglionic eminence</td>
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<tr>
<td>MOR</td>
<td>µ-Opioid receptor</td>
</tr>
<tr>
<td>NGF</td>
<td>Neurogliaform</td>
</tr>
<tr>
<td>Ni-DAB</td>
<td>Nickel diaminobenzidine</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric-oxide synthase</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PV</td>
<td>Parvalbumin</td>
</tr>
<tr>
<td>QX-314</td>
<td>N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide</td>
</tr>
<tr>
<td>RRF</td>
<td>Retrorubral field</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>sEPSC</td>
<td>Spontaneous excitatory post-synaptic current</td>
</tr>
<tr>
<td>sIPSC</td>
<td>Spontaneous inhibitory post-synaptic current</td>
</tr>
<tr>
<td>SNC</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNR</td>
<td>Substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SOM</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>STR</td>
<td>Striatum</td>
</tr>
<tr>
<td>TEA-Cl</td>
<td>Tetraethylammonium chloride</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>vMAT</td>
<td>Vesicular monoamine transporter</td>
</tr>
<tr>
<td>vGlut1</td>
<td>Vesicular glutamate transporter 1</td>
</tr>
<tr>
<td>vGlut2</td>
<td>Vesicular glutamate transporter 2</td>
</tr>
<tr>
<td>vSTR</td>
<td>Ventral striatum</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>( \tau )</td>
<td>Time constant</td>
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Chapter 1

Introduction
1.1 General Organization of the Basal Ganglia

The basal ganglia are the subcortical system comprising a group of nuclei that span from the anterior telencephalon to the posterior mesencephalon. On the basis of the highly evolutionary conserved scheme across the vertebrate phylogeny (Marin et al., 1998; Diederich and Parent, 2012), the basal ganglia have been divided into two streams as dorsal striatopallidal and ventral striatopallidal systems (Marin et al., 1998; Murer et al., 2002). The striatum, the globus pallidus internal segment (or entopeduncular nucleus in rodents), the globus pallidus external segment, the subthalamic nucleus, the substantia nigra pars reticulata and the substantia nigra pars compacta are the core components of the dorsal striatopallidal system. There is substantiating evidence for reciprocal functional and structural connections between the dorsal basal ganglia system and the pedunculopontine nucleus (Hammond et al., 1983; Mena-Segovia et al., 2004). The dorsal striatopallidal stream is mainly involved in acquisition, initiation of motor behaviors, and successful switching between motor programs (Graybiel, 2005; Redgrave et al., 1999).

The ventral striatopallidal system, on the other hand, mediates limbic functioning of the basal ganglia and involves the flow of information among core and shell segments of the nucleus accumbens, ventral pallidum and ventral tegmental area. The ventral system receives glutamatergic cortical and thalamic innervation from topographically distinct areas in comparison to the dorsal system, and additional excitatory afferents to ventral system also arise from amygdala and hippocampus (Zaborszky et al., 1985; Groenewegen et al., 1999; Heimer et al., 1997; Voorn et al., 2004).
Basal ganglia structures are predominantly made up of GABAergic neurons, which show uncorrelated firing patterns under normal conditions (Bergman et al., 1998; Rivlin-Etzion et al., 2010). Excitatory glutamatergic inputs from the cerebral cortex and thalamus drives the striatal spiny projection neurons (SPNs) to synchronous up-states, during which SPNs show intermittent phasic firing (Stern et al., 1997; 1998; Wilson, 2004), whereby tonically active output structures of the basal ganglia get phasically silenced, while the thalamic or tectal targets are momentarily disinhibited. This scheme enables movement initiation through the corticospinal and tectospinal tracts, respectively. Exceptions to the GABAergic dominance of basal ganglia projections are the dense dopaminergic innervation arising from the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) as well as the glutamatergic subthalamic nucleus, a key relay of the hyperdirect pathway that carries excitatory drive from the cerebral cortex on to the GPe, GPi and SNr (Mathai and Smith, 2011; Inoue et al., 2012)

1.2 Striatal organization and information flow

The striatum is the largest structure and the major input domain within the basal ganglia. Excitatory afferents to the striatum originate from all areas of the cerebral cortex (Alexander et al., 1986) as well as from the caudal intralaminar centromedian-parafascicular thalamic complex and to a lesser degree from midline and specific relay nuclei of the thalamus (Smith et al., 2004). Excitatory projections from the cortex form synapses most exclusively on the heads of dendritic spines of the SPNs, while the thalamic inputs target both spine heads and dendritic shafts of striatal neurons (Alexander et al., 1986; McGeorge and Faull, 1989; Moss and Bolam, 2010;
O’Donnell, 2010; Smith and Bolam, 1990; Smith et al., 2004; Smith et al., 2009; Smith et al., 2010).

In the rat, the striatal neural population size is in the order of millions, whereas in other basal ganglia structures, the sizes of the neural populations are two to three orders of magnitude smaller (Oorschot, 1996; Oorschot, 2010; Wilson, 2004). The arrangement of neural populations in this way across basal ganglia gives rise to what is called “divergence-reconvergence” scheme (Alexander et al., 1986; Graybiel et al., 1994). Excitatory projections from diverse areas of the cerebral cortex innervate striatal neurons in an axo-cruciform manner and, as a result, each striatal SPN samples excitatory input coming from thousands of different corticostriatal projection neurons from functionally related areas of the cortex (Smith and Bolam, 1990; Moss and Bolam, 2010; Wilson, 2004). SPN projections carrying the striatal output, on the other hand, converge onto neurons of the GPe and SNr in a ratio of 1:100 to 1:1000, respectively (Oorschot, 1996; Oorschot, 2010). Arrangement of neural populations in this manner highlights the role of striatum in extracting synchronous excitatory drive from cortex/thalamus origin and condensing it to shape ongoing activity of its projection targets.

Anatomically and functionally, the striatum can be divided into two major domains. First is the dorsal striatum, which comprises the caudate nucleus and putamen (Kemp and Powell, 1971). The second division is the ventral striatum, which comprises the shell and core domains of nucleus accumbens and the olfactory tubercle (Zaborszky et al., 1985).
In addition to the dorsal and ventral division, there is superimposition of patch/striosome-matrix compartmental organization increasing the striatal anatomical and functional complexity. Each compartment also expresses different neurochemical markers. The matrix shows strong immunoreactivity for acetylcholinesterase and calbindin whereas patch/striosome compartments are characterized by strong(er) expression of substance P, enkephalin and limbic associated membrane protein (Bolam et al., 1988; Graybiel et al., 1981; Holt et al., 1997; Voorn et al., 1989). Additional striosome/matrix differences exist in terms of enhanced expression of other signaling molecules in the striosomes such as µ-opioid receptor (MOR), AMPA receptor subunit 1 (GluR1), voltage-gated potassium channel interacting protein 1 (Mikula et al., 2009), dopamine receptor subtypes (Rivera et al., 2002b), and olfactory-type G-protein alpha subunit expression (Sako et al., 2010).

As will be mentioned in subsequent sections, the dopaminergic afferents in patch and matrix compartments arise from developmentally and topographically distinct midbrain loci (Gerfen et al., 1987a; 1987b; Langer and Graybiel, 1989). There is also evidence that this compartmental organization reflects a complex molecular patterning requiring the orchestrated action of multiple molecules including retinoic acid (Liao et al., 2008), notch signaling (Mason et al., 2005), certain transcription factors (Arlotta et al., 2008) cell adhesion (Redies et al., 2002) and guidance molecules (Hamasaki et al., 2001, Janis et al., 1999). Moreover, patch and matrix compartments are recipients of differing cortical and thalamic inputs. On the one hand, the patch/striosome compartment receives afferents from limbic regions and deeper segment of layer 3 and 5 of the cortex and the paraventricular nucleus of the thalamus (Berendse et al., 1988; Gerfen, 1989). Conversely, the matrix compartment
receives cortical innervations heavily from somatosensory and motor cortices arising from superficial segment of cortical layer 5 and parafascicular and centromedian complex of the thalamus (Berendse et al., 1988; Gerfen, 1989).

The output of striatum in both domains is mediated by gamma amino butyric acid (GABA) releasing SPNs that give rise to direct and indirect pathways following distinct routes towards the output nuclei of the basal ganglia (Albin et al., 1989; Gerfen et al., 1990; Kawaguchi et al., 1990). Furthermore, the SPNs that constitute different striatofugal pathways express different dopamine receptors and neuropeptides (Gerfen et al., 1990; Gertler et al., 2008; Wu et al., 2000). Direct pathway SPNs express D1 dopamine receptors, substance P and dynorphin, whereas indirect pathway SPNs express D2 dopamine receptors and enkephalin (Gerfen et al., 1990). However, irrespective of the dopamine receptor type and neuropeptides being expressed, all SPNs demonstrate a very characteristic electrophysiological profile: SPNs have very hyperpolarized resting membrane potential, express inwardly and outwardly rectifying potassium channels that result in a highly non-linear membrane voltage-current relationship (Nisenbaum and Wilson, 1995). At rest, potassium conductance mediated by G-protein coupled inwardly rectifying K$^+$ channels ($G_{IRK}$) is the major influence keeping the SPNs at very negative membrane potentials. But even when SPNs are depolarized beyond the voltage activation range of $G_{IRK}$, depolarization-activated K$^+$ conductances emerge which further clamp the SPNs below the action potential threshold. These characteristics of the SPN electrophysiology, as a result, require a high degree of coherence among excitatory inputs onto SPNs for them to reach action potential threshold (Wilson and Kawaguchi, 1996; Gruber and O'Donnell, 2009). Thus, the interaction between
intrinsic membrane conductances and excitatory afferent innervation gives rise to two distinct membrane voltage states in SPNs: up-state and down-state (Wilson and Groves, 1981; Wilson, 2004). Bouts of up states interrupting synaptically silent down states are characterized by barrage of synchronous synaptic input and up-states last anywhere between couple of hundred milliseconds to a couple of seconds. This is the only time SPNs show phasic action potential firing (Murer et al., 2002; Tseng et al., 2001). Although up- and down-state transitions were first discovered in vivo, membrane voltage fluctuations reminiscent of up- and down- state transitions were also indentified in SPNs in organotypic cultures (Plenz and Aertsen, 1996), and such in vitro activity could be uncovered in acute striatal slices by NMDA application (Carrillo-Reid et al., 2008), which highlight the importance of excitatory drive for the initiation of membrane state transitions in SPNs.

This mechanism of bistable membrane voltage fluctuation of SPNs described above dictates that when the excitatory influences impinge in a coordinated fashion onto SPNs of the striatum, activation of these otherwise quiescent GABAergic projection neurons takes place (Kasanetz et al., 2008; Wilson and Kawaguchi, 1996). This in turn leads to phasic changes in tonically active basal ganglia output nuclei (Kita, 2001; Kita et al., 2006); the substantia nigra pars reticulata and internal segment of the globus pallidus. However, even when an SPN enters the up-state, the pattern of its firing activity shows considerable variability. This is because when integration of inputs in the striatum is considered, more than just cortical and thalamic afferents come into play. Two of the factors that will be discussed are local inhibitory microcircuitry and neuromodulatory influences arising predominantly from midbrain dopaminergic neurons.
1.3 Striatal interneurons and striatal information processing

Striatal interneurons make up a minority of the entire striatal mosaic accounting for less than 5% of the entire striatal neural population in rodents (Kawaguchi et al., 1995; Oorschot, 2010; Rymar et al., 2004). In mature striatum, the established interneuron types are (1) cholinergic, (2) parvalbumin (PV)-expressing, (3) calretinin (CR)-expressing, and (3) NADPH-somatostatin (SOM)-nitric oxide synthase (NOS) and neuropeptide-Y (NPY) co-expressing interneurons (Kawaguchi, 1993; Kawaguchi et al., 1995; Tepper, 2010; Wu and Parent, 2000). With the exception of cholinergic interneurons, all striatal interneurons are GABAergic (Kawaguchi, 1993; Kawaguchi et al., 1995; Koós and Tepper, 1999; Tepper, 2010).

Significant advances have been achieved in deciphering striatal interneuron diversity using other genetically engineered mice that allow reliable and immediate identification of these cells in slices based on reporter fluorescent protein expression (Gong et al., 2003, Valjent et al., 2009). Two more classes of striatal GABAergic interneurons were recently described using transgenic line of mice (Ibanez-Sandoval et al., 2010; 2011). A novel NPY-expressing interneuron has recently been described in striatum that lacks co-expression of SOM and NOS and displays a distinct morphology and connectivity patterns with SPNs. This has been termed as neurogliaform NPY-expressing (NPY-NGF) interneurons as it bears resemblance to NPY+ neurogliaform neurons of the cortex and hippocampus (Ibáñez-Sandoval et al., 2011). In addition, striatal TH+ neurons were recently established as a novel class of striatal interneurons on the basis of anatomical and electrophysiological characterization using BAC transgenic mice (Ibáñez-Sandoval et al., 2010). The distinction of striatal interneuron classes based on neurochemical identity was
originally defined by electrophysiological and morphological characterization in seminal line of studies by Yasuo Kawaguchi and colleagues (Kawaguchi et al., 1993; 1995; 1997; Kawaguchi et al., 1995; Kubota et al., 1993a; Kubota and Kawaguchi, 1994).

Developmentally, striatal interneurons arise from the medial ganglionic eminence (MGE) and express a common constellation of neurodevelopmental markers (Anderson et al., 1997; Olsson et al, 1998; Marin et al., 2000). The homeo-domain transcription factors NKX2.1, DLX1-2 and LHX6-7-8 and the basic helix-loop-helix protein, Mash1, are the critical for determining MGE precursors destined to be striatal interneurons (Marin et al., 2000).

These specifics of the developmental make-up of striatal interneurons have been recently exploited for identification of striatal interneurons using BAC transgenic strategies in mice (Gittis et al., 2010; Gittis et al., 2011). Using LHX6-eGFP mice, fast-spiking and persistent low threshold-spiking interneurons could be identified in acute striatal slices, but not other types of interneurons, which indicate that there is a combinatorial code of developmental markers determining striatal interneuron identity. However, one must bear in mind that the MGE is the embryonic proliferative zone for both cortical and striatal interneurons. The difference in migratory path has been linked to semaphorin 3A and 3F signaling, which is chemorepulsive for neurophilin-expressing cortical progenitors (Marin et al., 2001). Despite the common origin and developmental factors, the final make-up and localization of striatal interneurons after they complete a tangential migratory journey is far from being uniform. First, they form distinct anatomical and neurochemical clusters and second,
the striatal distribution varies drastically among interneuron classes (Luk and Sadikot, 2001; Rymar et al., 2004; Gerfen and Bolam, 2010).

Our stereological estimates indicate that there are ~15,000 cholinergic neurons, ~12,000 PV-expressing neurons, ~17,000 NPY-expressing neurons (~14,000 NPY-PLTS type and ~3,000 NPY-NGF type), ~10,000 CR-expressing neurons and ~3,000 TH-expressing neurons residing in the caudate-putamen and nucleus accumbens combined in mice (Ibanez-Sandoval et al., 2010; 2011; Unal et al., 2011 and Unal et al., this thesis). These data show that striatal interneurons make up around 3% of the entire striatal population when our stereological estimates of striatal interneurons are measured against the stereological estimates of total striatal counts in mice (Peterson et al., 1999; Diaz-Hernández et al., 2005).

Cholinergic neurons were the earliest identified interneuron subtype in striatum due to their very distinct morphological appearance. They are sometimes referred to as giant neurons of Kölliker, named after the German anatomist who first identified these neurons in the corpus striatum (as reviewed in Tepper et al., 2010). The elongated soma of striatal cholinergic cells can be 3 times larger than a SPN and give rise to 2-4 primary dendrites that moderately branch out as they extend farther away from the soma. The axonal arborization of the cholinergic neurons is very dense; they represent the sole source of acetylcholine in the striatum and for this reason acetylcholinesterase, the degrading enzyme for acetylcholine, is an extremely efficient biomarker for delineating the borders of the striatum. Electrophysiologically, most of the cholinergic neurons are identified by the intrinsically generated tonic firing they exhibit both in vivo and vitro, prominent voltage sag when hyperpolarized
and long duration action potentials that are followed by a long lasting after hyperpolarization (Kawaguchi, 1992; 1993; 1997; Kawaguchi et al., 1995; Wilson et al.; 1990). Most intriguingly, from the behavioral point of view, motivationally salient stimuli, such as learned contingencies between unconditioned stimuli and reward come to elicit pause response in cholinergic neuron tonic firing (Aosaki et al., 1994; 1995; Kimura et al., 1984)

A second well-characterized striatal interneuron is the medium-sized aspiny PV-expressing GABAergic interneurons. Striatal PV-expressing interneurons are electrophysiologically identified by their low input resistance, hyperpolarized resting membrane potential and their ability to sustain repetitive firing that maximally reaches to ~ 300Hz while showing minimal spike frequency accommodation. These neurons are referred to as fast-spiking interneurons (FSIs), like their counterparts in cortex (Markram et al., 2004) and hippocampus (Klausberger and Somogyi, 2008). In addition, FSIs are characterized morphologically by an ovoid soma from which 5 to 8 primary dendrites emanate and branch up to 3rd or 4th order filling up the 200-300 µm radius around the soma more or less homogeneously (Koós and Tepper, 1999). Their extremely dense axon collaterals exceed the field of dendritic arborization.

PV-expressing FSIs are a powerful source of feedforward inhibition onto SPNs (Koós and Tepper, 1999) owing to their heightened cortical responsiveness (Mallet et al., 2005) and special geometry of their axonal contacts with the SPNs (Koós and Tepper, 1999; Koós et al., 2004; Kubota and Kawaguchi, 2000; Taverna et al., 2007; Tepper et al., 2008). In addition to their local synaptic actions, the presence of gap-junction mediated electrical coupling among fast-spiking neurons also contributes to
their network wide effect (Koós and Tepper, 1999; VandeCastelee et al. 2002; Fukuda, 2009).

Vincent and Johansson, (1983) and Vincent et al. (1983) found that somatostatin (SOM) expressing interneurons in the striatum also express NADPH-diaphorase immunoreactivity, which was later identified as the identical enzyme to nitric oxide synthase (NOS) (Dawson et al., 1991). Co-expression of avian pancreatic polypeptide, the precursor for neuropeptide Y (NPY) was also identified in this population (Vincent and Johansson, 1983; Vincent et al., 1993). Thus, co-existence of these three markers were thought to reflect one uniform population of striatal interneurons until Figueredo-Cardenas et al. (1996) showed a separate subpopulation that only expresses NPY without SOM or NOS.

Using BAC transgenic mice, definitive anatomical and electrophysiological evidence was put forth showing that NPY-expressing interneurons are indeed a dichotomous population. NPY-SOM-NOS co-expressing cells correspond to persistent and low threshold spiking (PLTS) neurons described by Kawaguchi (1992; 1993; 1997) with their high input resistance and typical spontaneous activity in the slice (Ibáñez-Sandoval et al., 2011; Partridge et al., 2009). These neurons have an elongated bipolar soma, and modestly branching dendritic trees that show only marginal overlap with their sparse axonal arborizations (Ibáñez-Sandoval et al., 2011; Partridge et al., 2009; Tepper et al., 2008; Tepper et al., 2010). The other subtype of NPY-expressing neurons displays completely distinct electrophysiological and morphological characteristics that entail very low input resistance, lack of spontaneous activity, and somatic current injection elicited non-adapting train of
spikes with prominent spike after-hyperpolarizations (Ibáñez-Sandoval et al., 2011). Neurochemically, this second type of NPY-expressing interneuron lacks the co-expression of NOS, and SOM (Ibáñez-Sandoval et al., 2011), unlike the NPY-PLTS interneurons. Morphologically, these NPY-only neurons bear a striking resemblance to NPY+ neurogliaform cells of the cortex and hippocampus (Capogna, 2011; Karagiannis et al., 2009); they emit seven primary dendrites on the average, which ramify heavily within 200 µm of the soma. Moreover, the axonal plexus of this second type of NPY-expressing interneuron is very dense and extends beyond the dendritic field of the neuron (Ibáñez-Sandoval et al., 2011). Activation of NPY-NGF interneurons was found to lead to IPSCs with slow kinetics in more than 85% of the neighboring SPNs (Ibáñez-Sandoval et al., 2011).

Of all the striatal interneuron subtypes, CR neurons are the class that we still do not know much about. Up to now, the identification of CR-expressing neurons has been limited to immunocytochemical methods (Bennett and Bolam, 1993). Based on somatic immunoreactivity with CR antibody, there is indication that there are multiple morphologically distinct subtypes with differing gradients with the striatum (Bernacer et al., 2012; Mura et al., 2000a; Tepper et al., 2010). Previous studies and a part of the experiments in this thesis show that a small proportion of CR-immunoreactive cells (~5%) co-express TH (Mazloom and Smith, 2006; Darmopil et al., 2008, Huot et al., 2007).

Although the discovery of TH-expressing cells in the striatum dates back to Dubach et al. (1987), the definitive experimental evidence revealing their GABAergic interneuronal identity was presented recently. Using transgenic mice that express
eGFP under the control of TH promoter, Ibáñez-Sandoval et al. (2010) characterized striatal TH+ neurons electrophysiologically and anatomically. It was shown in Ibáñez-Sandoval et al. (2010) that not only did striatal TH-eGFP neurons display distinct electrophysiological characteristics that set them apart from SPNs and other striatal interneurons, but also show within-group electrophysiological diversity that allowed us to categorize them into four electrophysiologically distinct subtypes, denoted as Type I, II, III and IV.

The electrophysiological criteria used to group TH-eGFP neurons into distinct subtypes are discussed in detail in Ibáñez-Sandoval et al. (2010). Briefly, Type I TH-eGFP interneurons were the most common subtype encountered in the intact striatum. They were characterized by their very high input resistance, and moderate firing in response to low intensities of depolarizing somatic current injections. However, as they are depolarized further, they emit a brief burst of action potentials followed by a complete cessation of firing (Fig. 1.1A). Type II TH-eGFP neurons had lower input resistances and were able to sustain firing with minimal spike frequency adaptation in response supra-threshold somatic current injections (Fig. 1.1B). Type III TH-eGFP neurons had the most hyperpolarized resting membrane potential, and low input resistance, and they readily showed a pronounced spike frequency adaptation in response to high levels of somatic depolarization (Fig. 1.1C). Type IV TH-eGFP neurons were identified by the low-threshold spiking (LTS) which entails a brief wave of depolarization on which a burst of action potentials rides in response to supra-threshold somatic current injections (Fig. 1.1.D). It was further shown that a subgroup of TH-eGFP neurons displayed spontaneous activity (Fig. 1.1E). Striatal TH-eGFP neurons were found to form sparse GABAergic synaptic connections with
neighboring SPNs and a subgroup of these connections were bidirectional, indicating that SPNs can also innervate local interneurons, a finding which was later corroborated by optogenetic methods (Chuhma et al., 2011). Interestingly, in TH-eGFP neurons another striking electrophysiological characteristic detected in more than 50% of the entire population was the L-type calcium sensitive depolarizing plateau potentials that can hold the membrane at a highly depolarized state for several hundreds of milliseconds (Ibáñez-Sandoval et al., 2010).

**Figure 1.1. Characterization of different subtypes of TH-eGFP interneurons in the intact mouse striatum.** A representative example of each electrophysiological subtype of striatal TH-eGFP neurons is shown. Insets show the recorded TH-eGFP somata under epifluorescent illumination and voltage (V)- current (I) relationship for the shown subtype. (A) Striatal Type I TH-eGFP interneuron. (B) Striatal Type II TH-eGFP interneuron. (C) Striatal Type III TH-eGFP interneuron. (D) Striatal Type IV interneuron. Asterisk shows the LTS in Type IV neuron (E) A subgroup of Type I and Type II TH-eGFP neurons were spontaneously active. Modified with permission from Ibáñez-Sandoval et al., 2010.

Morphologically, TH-eGFP neurons issue moderately branching 2-4 primary dendrites from a multipolar medium-sized soma, which are sparsely spine-laden.
Axonal arborizations of TH-eGFP neurons are largely restricted to the extent of the dendritic field (Ibáñez-Sandoval et al., 2010; Tepper et al., 2010).

Just as with SPNs, there is anatomical and physiological evidence that striatal GABAergic interneurons also are critical recipients of cortical innervation (Bennett and Bolam, 1993; 1994; Gruber et al., 2009; Ibáñez-Sandoval et al., 2010; 2011; Lapper et al., 1992). Cholinergic interneurons, on the other hand, receive a significant excitatory input from centromedian/parafascicular complex of the thalamus (Lapper and Bolam, 1992; Smith et al., 2004) but limited cortical innervation (Thomas et al., 2000). Therefore, when excitatory inputs phasically excite SPNs, they also initiate a feed-forward inhibitory drive through striatal GABAergic interneurons directly (Gruber et al., 2009; Plenz and Kitai, 1998; Tepper et al., 2004; 2008) or indirectly through cholinergic interneurons (English et al., 2011; Sullivan et al., 2008). Furthermore, striatal interneurons play a permissive role for certain forms of synaptic plasticity that occur at cortico-striatal synapses (Calabresi et al., 2007; Centonze et al., 1999; Gittis et al., 2010; Partridge et al., 2002; Sammut et al., 2010) owing to their neurochemical make-up, which additionally expands and ramifies their realm of action. Combined electrophysiological and behavioral approaches further demonstrated that the activation of certain groups of striatal interneurons occur at distinct phases of motivated behavior which suggest that the dynamic organization of the striatal microcircuitry relies on temporally differential recruitment of interneuron classes (Berke, 2009; Gage et al., 2010; Lansink et al., 2010).
1.4 Dopaminergic innervation of striatum

1.4.1 Sources and developmental origins

Dopamine was discovered as a novel neurotransmitter, depletion of which is responsible for akinesia in reserpine-treated mice (Carlsson et al, 1957; 1958). Later on, the striatum was found to receive the densest dopaminergic innervation among other brain regions and other basal ganglia nuclei (Bertler and Rosengren, 1959). Therefore dopamine is regarded as a major pillar of striatal information processing. The striatum receives input arising from the ventral tegmental area (VTA) (A10), substantia nigra pars compacta (A9) (SNc) and retrorubral field (RRF) (A8) (as reviewed in Ikemoto, 2007; Wise, 2004; Björklund and Dunnett, 2007; Smith and Kieval, 2000). In mice, the size of dopaminergic populations were estimated to be around 20,000 unilaterally where 8,000 to 10,000 TH+ cells reside in SNc and remaining dopaminergic cells distributed across VTA and RRF (Baquet et al., 2009; Prasad and Richfield, 2010; Zaborszky and Vadasz, 2001; Vadasz et al., 2007). Furthermore, dopamine is regarded as the main currency in the brain for reward and action (Schultz et al., 1997; Surmeier et al., 2009), therefore, any dysfunction in dopaminergic systems has major consequences for psychomotor, cognitive and emotional functioning as in the case of PD, schizophrenia, attention deficit and hyperactivity disorder and obsessive compulsive disorder among many others.

Developmentally, dopaminergic precursors arise from the isthmus -the midbrain-hindbrain border- around embryonic day 12 (E12); then differentiate, grow neurites and start innervating striatal targets between E12.5 and E20 (Gates et al., 2006; Smidt and Burbach, 2007; van den Heuvel and Pasterkamp, 2008). Several proteins are crucial for the successful formation of midbrain dopaminergic neurons. Nurr1 has
been identified as the crucial transcription factor for the expression of TH, vesicular monoamine transporter 2 (vMAT2) and the dopamine transporter (DAT). In addition, Engrailed1-2 and Pitx3 were identified as necessary proteins for proper differentiation and survival of dopaminergic neurons (Smidt and Burbach, 2007). Just as in interneuron migration, semaphorin 3A signaling was found to be critical for proper migration of dopaminergic fibers to the striatum, but with an intriguing twist: semaphorin 3A proteins are chemorepulsive throughout the journey of dopaminergic fibers in the medial forebrain bundle but act as chemoattractant for the fibers destined to innervate cortical targets (Kolk et al., 2009). Furthermore, SNc and VTA dopamine neurons also express ROBO receptors, which bind to chemorepulsive Slit proteins. ROBO-slit interaction not only prevent midline crossing of dopaminergic fibers, but also is thought to contribute to patchy early innervation of striatum forming dopamine islands as striatal cholinergic neurons express Slit-1 (Hu et al., 2004; Smidt and Burbach, 2007). In addition, the chemorepulsive interaction between EphrinB1 receptor expressed in SNc dopamine neurons and its ligand ephrinB2 concentrated in the ventral striatum has been implicated in differential dopaminergic patterning of the dorsal and ventral striatum by SNc and VTA dopamine neurons, respectively (Yue et al., 1999).

The dopaminergic innervation of the striatum is incredibly dense (Matsuda et al., 2009; Moss and Bolam, 2010) but the dopaminergic patterning of the striatum nevertheless displays an elaborate set of developmental and topographical organizational principles. The dorsal striatum receives preferentially its dopaminergic innervations arising from SNc, whereas the VTA sends projections to ventral striatum, cerebral cortex, hippocampus and amygdala. The RRF, on the other hand, is
thought to innervate dorsal and ventral aspects of the striatum equally (Joel and Weiner, 2000; Bjorklund and Dunnett, 2007). However, closer anatomical examinations reveal that SNc and VTA dopaminergic cells are organized into dorsal and ventral tiers among themselves, and each tier shows a distinct axonal arborization domain in the striatum (Gerfen et al., 1987a; Gerfen et al., 1987b). Interestingly, the dopaminergic tiers have different maturational timelines (Gerfen et al., 1987b; Smidt and Burbach, 2007). The dopaminergic precursors in the ventral tier differentiate, mature (Smidt and Burbach 2007) and innervate their targets earlier than the dorsal tier neurons (Gerfen et al., 1987a; Gerfen et al., 1987b). What is more interesting is that striatal regions innervated by different dopaminergic populations also correspond to different striatal compartments referred as the patch-matrix organization of the striatum (Gerfen et al., 1985; 1987a; 1987b; Joel and Weiner, 2000). The striatal patches receive input from ventral tier dopamine cells, whereas the later born dorsal tier dopamine neurons arborize in the matrix compartment. Furthermore, it has been documented that different dopaminergic populations show differential susceptibility to disease-related or toxin-induced degeneration which may further accentuate the fact that anatomical differences are only one aspect of the diversity of the functions subserved by distinct dopaminergic populations (Granado et al., 2010).

1.4.2 Effect of dopamine on SPNs

Striatal dopamine has a firmly established role in differential recruitment of direct and indirect pathway SPNs expressing G-protein coupled D_1- or D_2- group dopamine receptors (D1R and D2R, respectively) in these two populations (Albin et al., 1989; Gerfen et al., 1990; Gertler et al., 2008; Kebabian et al., 1972). It is mainly regarded that D1R-mediated intracellular cascades promote excitability and D2R activation on
the other hand activate inhibitory pathways. D1R activation leads to hydrolysis of the alpha subunit of olfactory G-protein, which potentiates adenylyl cyclase activity and leads to an increase cyclic AMP (cAMP) levels. Elevation in cAMP levels triggers protein kinase A (PKA) activation. On the other hand, D2R activation leads to overall inhibition by two parallel signalling cascades: (1) inhibitory alpha subunit, which dampens adenylyl cyclase and PKA (Stoof and Kebabian, 1984) and (2) beta-gamma subunits, which activate the phospholipase C that splice phosphatidylinositol phosphate 2 into diacylglycerol and IP3, then leads to protein kinase C activation and mobilization of endoplasmic calcium stores, respectively. Furthermore, Greengard and colleagues discovered that in SPNs these distinct pathways differentially recruited by D1R and D2R signaling alter the activity of one critical effector molecule, termed dopamine and cyclic AMP -regulated phosphoprotein 32 (DARPP-32). D1R signaling promotes the phosphorylation of DARPP-32, which in turn inhibits protein phosphatase 1 (PP1) and potentiates glutamate and calcium channel currents. On the flip side, D2R signaling dephosphorylates DARPP-32, thus increasing the PP1 activity and dampening the excitatory conductances and increasing GABA transmission onto SPNs (as reviewed in Greengard, 2001). These cellular cascades of dopamine-triggered actions support the initial simplification of dopamine's effect on D1- and D2-expressing striatal neurons as excitatory and inhibitory, respectively. However, it was elegantly demonstrated that intrinsic membrane properties of SPNs, in turn, shape and determine the post-synaptic actions of dopamine (Hernandez-Lopez et al., 1997). Hernandez-Lopez et al. (1997) showed that, when membrane voltage of SPNs was close to the potassium equilibrium (around -80 mV), D1R agonists reduced the number of action potentials evoked by somatic current injections by a GIRK-dependent mechanism. On the contrary, when SPN membrane voltage is held around -
60mV, D1R agonist application increases the number of action potentials induced by somatic current injection by potentiating an L-type Ca2+ conductance. This dichotomy of the effects of D1R activation indicates that dopamine can create thresholds for incoming inputs, and once that threshold is met, can increase signal to noise ratio by recruiting other conductances (Hernandez-Lopez et al., 1997; Murer et al., 2002). Thus, the effects of dopamine in striatum must be evaluated beyond the receptor type involved, and a thorough consideration within the context of electrophysiological make up of the neuron, afferent inputs and network states (i.e., up- and down- states) is necessary.

However, differential yet simultaneous recruitment of direct and indirect SPNs by dopamine is only one of the ways to balance inhibition and excitation in the striatum. Besides its effects on single SPNs, dopamine is also a critical modulator of synaptic plasticity in the striatum. Endogenous dopamine is thought to regulate bidirectional homeostatic plasticity by promoting calcium-dependent long-term potentiation (LTP) in D1R-expressing striatonigral SPNs. Conversely, Kreitzer and Malenka (2005, 2007) demonstrated that the expression of long-term depression (LTD) in D2R-expressing striatopallidal neurons relies critically on the production of endocannabinoids, which act retrogradely to dampen the glutamate release probability onto SPNs as D2R-triggered intracellular cascade produces two types of endocannabinoids, anandamide and 2-arachidonylglycerol through the diacylglycerol metabolism as mentioned above.

In addition, dopamine can potentiate or weaken the feedback inhibitory network among SPNs in a pathway- and connection strength-dependent manner (Tecuapetla et
Thus, dopamine exerts effects at multiple levels from single neurons, to synaptic pairs and to networks.

There is a tight relationship between SPNs and dopamine. It has already been mentioned that dopaminergic innervation of the striatum forms a dense network. Most of the dopaminergic neurons display spontaneous activity, thus providing the striatum a constant dopamine influx up to an ambient dopamine concentration around 100 nM (Cheer et al., 2007; Kuhr et al., 1987). During bouts of burst activity of dopaminergic neurons due to the arrival of unexpected reward or reward-predicting stimuli (Schultz et al., 1997), the striatum experiences brief waves of several fold increases in dopamine release (Cheer et al., 2007). In the rat striatum a single dopaminergic axon has total length of 46.7 cm with an average diameter of 0.8 µm (Matsuda et al., 2009). Considering these neurochemical and neuroanatomical features, we can begin to appreciate that dopamine is not only a major neuromodulator source but also an integral structural element for the striatum. Consequently, in Parkinson’s disease (PD) in which the major biological marker is the loss of striatal dopamine due to the death of mesencephalic dopamine neurons (Hornykiewicz and Kish, 1986), and in experimental models for striatal dopamine loss, the changes on striatum and basal ganglia are multifaceted.

A common observation in post-mortem tissue from PD patients and experimental models is that the striatal SPNs express a 20% to 50% decline in dendritic spine density (Arbuthnott et al., 2000; McNeill et al., 1988). It has been found that both direct and indirect pathway neurons are similarly impacted (Arbuthnott et al., 2000; Deutch et al., 2007; Smith et al., 2009) although there is also evidence that the
mechanism of spine loss in direct and indirect pathway SPNs is different (Day et al., 2006; Surmeier et al., 2010). The decline in spine density was associated with a significant decrease in number of asymmetric synapses onto SPNs (Arbuthnott et al., 2000), indicating that dopamine-depletion alters excitatory afferents originating from cortex and/or thalamus. The source of changes was further investigated by taking advantage of the restricted expression of vesicular glutamate transporter 1 (vGlut1) and 2 (vGlut2) in corticostriatal and thalamostriatal terminals, respectively (Smith et al., 2009). Although there is an overall decrease in the numbers of vGlut1+ and vGlut2+ terminals in striatum, further structural plasticity takes place particularly in remaining corticostriatal terminals such as increased spine volume, perforations of the postsynaptic density and increase in structural complexity of the spine apparatus (Smith et al., 2009). In addition, expansion of astroglial support around the remaining axo-spinous excitatory synapses in dopamine-depleted striatum has also been documented (Villalba and Smith, 2011). Curiously, dopamine-replacement treatments do not reverse the spine loss on striatal loss (Stephens et al., 2005; Zaja-Milatovic et al., 2005) indicating that the structural alterations in SPNs due to loss of dopamine in PD exceed beyond the lack of dopamine receptor signaling and involve other structural and signaling changes.

Taken together, these findings show that there is an overall decrease in spine density in SPNs and increased complexity and structural support for remaining excitatory synapses. Intriguingly, the dopamine depletion-induced decline in spine density is prevented when cortex is removed at the time of dopamine lesion in organotypic cultures (Neely et al., 2007). Thus, it has been argued that reduction in spine density in fact reflects an increase in excitatory drive onto SPNs (Deutch, 2006;
Neely et al., 2007). In line with this argument, in dopamine-denervated striatum, intracellular recordings showed that there is an increase in the frequency of glutamate-mediated excitatory post-synaptic potentials onto SPNs (Calabresi et al., 2000) indicative of hyperactivity of corticostriatal terminals. Furthermore, in dopamine-deprived striatum it has been demonstrated in vivo and in vitro that the overall activity of SPNs increase (Jáidar et al., 2010; Tseng et al., 2001). In the intact striatum, SPNs exhibit intermittent peaks of correlated activity and form sequential activity patterns as monitored by calcium imaging (Jaidar et al., 2010). After dopamine depletion, the correlated activity within the striatum dramatically increases, and striatal neurons get trapped in a limited set of dominant activity states (Jaidar et al., 2010). This network level alteration in SPNs is also reflected as overcoupling to cortical slow rhythm, where discharge rate of SPNs during up-states dramatically increase (Dejean et al., 2012; Tseng et al., 2001). These changes in SPNs in dopamine-depleted states also propagate to striatal targets, which also start to show overcoupling to slow cortical oscillations in the form of increased phasic burst discharges in globus pallidus and SNr in chronic dopamine-depleted state (Murer et al., 2002).

In summary, the striatum sustains major alterations anatomically and electrophysiologically when it is deprived of dopaminergic afferents. However, except for a few recent studies, dopamine-depletion induced changes in striatal interneuron functioning are largely overlooked. This is a critical missing piece in this puzzle, as interneurons powerfully shape striatal output. Furthermore, it would be naïve to assume they all give the same type of response to dopamine depletion as classes of striatal interneurons are functionally, neurochemically and morphologically very distinct from each other. However, the complexity in composition of striatal
interneurons may provide critical insights as to which combination of factors may confer resistance or susceptibility for dopamine-depletion induced changes in the striatum. Therefore, in the next section the effect of dopamine on interneurons is going to be discussed.

1.4.3 Effect of dopamine on striatal interneurons

Dopamine can also alter the responsiveness in striatal interneurons; including heightening the excitability in FSI (Bracci et al, 2002) and LTS (Centonze et al., 2002) interneurons while dampening the activity in cholinergic interneurons via a D2 mediated mechanism (Abercrombie and DeBoer, 1997; Guevera et al., 1996; Yang et al., 1997). Interestingly, using BAC transgenic mice that express eGFP under the control of the promoter for D1 or D2 dopamine receptors, somatic expression of D2 receptors was confirmed only for cholinergic interneurons but no colocalization has been detected for GABAergic interneurons (Valjent et al., 2009) which suggests that other subtypes of dopamine receptors mediate the reported effects of dopamine on striatal GABAergic interneurons (Centonze et al., 2003; Rivera et al., 2002a).

Dopamine-mediated effects on striatal interneurons were more strikingly uncovered when dopamine was omitted from the picture. For instance, Dehorter et al. (2009) has reported LTS interneuron-mediated generation of massive barrages of inhibitory inputs onto SPNs in the dopamine-denervated striatum. Dopamine also appears to be critical for the balanced recruitment of direct and indirect pathway SPNs via its actions on interneurons. It was shown by Mallet et al. (2006) in dopamine-depleted striatum that an imbalanced feedforward inhibition targeting direct pathway SPNs is likely to be one of the underlying causes of motor deficits. In addition it was
shown by Salin et al. (2009) that following 6-OHDA lesions, there is a reduction of cholinergic and PV+ interneuron synapses onto striatonigral SPNs, whereas connectivity between cholinergic and striatopallidal neurons increase (Salin et al., 2009). Recently, it was documented that chronic but not acute (i.e., via reserpine) loss of striatal dopamine increases fast-spiking interneuron connections onto indirect pathway SPNs which was accompanied by a robust increase in axonal arborization of FSI's from dopamine-depleted striatum (Gittis et al., 2011). Conceivably, the most interesting and puzzling finding comes from a recent neural graft approach. Martinez-Cerdeno et al. (2010) found that in unilaterally dopamine-lesioned rats, implantation of fetal MGE grafts that contain striatal interneuron precursors ameliorate the motor deficits. Whether the observed effect is due to functional integration of MGE precursors in host striatal microcircuitry to exert a balancing influence or rely on a non-specific neurotrophic action has yet to be determined. But, clearly these experiments underline the fact that dopamine depletion-induced aberrant striatal activity and experimental scenarios towards a potential rescue of this situation should involve striatal interneurons.

### 1.5 Striatal TH+ interneurons: Where the worlds of interneurons and dopamine collide

TH+ interneurons stand out as a key element in our understanding of the striatal microcircuitry and involvement of striatal dopamine in it owing to several characteristics of their make-up: (1) tyrosine hydroxylase, the molecule on which the identity of these neurons is based is the rate limiting enzyme in the synthesis of catecholamines; (2) they display anatomical and electrophysiological characteristics of striatal interneurons; (3) the most agreed upon observation regarding these neurons
is the rise they show in number in response to experimentally-induced or PD-related dopamine loss (Betarbet et al., 1997; Darmopil et al., 2008; Dubach et al., 1987; Jollivet et al., 2004; Mao et al., 2001; Meredith et al., 1999; Mazloom and Smith, 2006; Palfi et al., 2002; Tande et al., 2006; Tashiro et al., 1989a; 1989b).

The neurochemical characterization of striatal TH+ neurons suggests that at least a fraction of these cells possesses components of the machinery necessary to synthesize, store and release dopamine. There are multiple lines of studies indicating co-expression amino acid decarboxylase (AADC) (Lopez-Real et al., 2003; Mura et al., 1995; Mura et al., 2000b; Tashiro et al., 1989), dopamine transporter (DAT) (Betarbet et al., 1997; Cossette, 2005a) and dopamine (Meredith et al., 1999) in striatal TH+ neurons. Moreover, the lack of dopamine beta hydroxylase (Betarbet et al., 1997; Tande et al., 2006), the enzyme that is responsible for the conversion of dopamine to norepinephrine, further supports the idea that some of striatal TH+ interneurons might be dopaminergic. Single cell RT-PCR analysis also showed expression of vesicular monoamine transporter (Ibáñez-Sandoval et al., 2010). However, it must be noted that immunohistochemical studies also identified the expression of some of the abovementioned elements crucial for neurotransmitter action of dopamine in isolation (Weihe et al., 2006). Therefore, at this point TH expression in these neurons does not appear to be a sufficient factor to attribute a dopaminergic identity to the entire population. Indeed, recent data from our laboratory utilizing selective optogenetic activation of striatal TH-expressing interneurons in acute slices in conjunction with voltammetry did not detect dopamine-release from TH-expressing interneurons (Xenias et al., 2012). Furthermore, no immunoreactivity for dopamine, AADC and
DAT could be detected in striatal TH-eGFP neurons (Xenias et al., 2012). This data indicates that most or all of striatal TH-eGFP neurons are not dopaminergic.

Besides the ongoing debate on dopaminergic aspects of the make-up of striatal TH+ neurons as alluded to in the previous paragraph, ultrastructural, immunocytochemical and electrophysiological characterization established a GABAergic interneuronal identity in these neurons. At the ultrastructural level, they display an invaginated nuclear envelope and terminal expression of GABA (Mazloom and Smith, 2006). Immunohistochemically, they express an interneuron specific isoform of glutamic acid decarboxylase (Cossette et al., 2005b; Dubach et al., 1987; Huot and Parent, 2007; Huot et al., 2007). Electrophysiologically, they give rise to bicuculline-sensitive GABAergic inhibitory post-synaptic potentials and currents in post-synaptic SPNs (Ibáñez-Sandoval et al., 2010). Striatal TH+ interneurons possess a medium soma size and sparsely spiny neurites. In addition they do not project to GPe and SNr. Thus overall, these anatomical and electrophysiological characteristics of striatal TH+ neurons set them apart clearly from SPNs and establish their identity as a novel and distinct class of striatal GABAergic interneuron (Betarbet et al., 1997; Ibáñez-Sandoval et al., 2010; Mazloom and Smith, 2006; Meredith et al., 1999; Tande et al., 2006).

Across species investigations indicate that there is an increased number of striatal TH+ neurons following striatal dopamine depletion (Huot and Parent, 2007; Huot et al., 2007; Lopez-Real et al., 2003; Meredith et al., 1999; Tande et al., 2006). Moreover, the dopaminergic control of TH+ neuron number seems to be bidirectional as L-DOPA-treated PD patients and Huntington Disease patients display a reduction
in the number of these neurons (Huot and Parent, 2007; Huot et al., 2007; Huot et al., 2008). Although striatal dopamine appears to affect synaptic connectivity and firing properties of other striatal interneurons (Dehorter et al., 2009; Ding et al., 2011; Gittis et al., 2011; Mallet et al., 2006; Salin et al., 2009), striatal TH+ interneurons appear to be the only group of striatal interneuron showing a discernible increase in number after dopamine-depletion (Muramatsu et al., 2003; Tashiro et al., 1989).

The stereological estimates obtained in our laboratory using the TH-eGFP mice consistently indicate a population of ~3000 per striatum per hemisphere (Ibáñez-Sandoval et al., 2010). With respect to stereological estimates of other striatal interneuron types in the rat (Oorschot, 2010; Rymar et al., 2004; Sadikot and Sasseville; 1997), TH+ interneurons appear to be the smallest class of striatal interneurons. On the other hand, midbrain dopaminergic counts are around 8000-9000 in the mouse brain unilaterally (Baquet et al., 2009; Prasad and Richfield, 2010). On the bases of this anatomical perspective and aforementioned studies indicating a dual GABA-DA phenotype (Mazloom and Smith, 2006; Meredith et al., 1999; Tashiro et al., 1989a; Tashiro et al., 1989b), it can be argued that there is ~1/3 of a substantia nigra pars compacta potential embedded in the striatal microcircuitry which can be uncovered when midbrain dopaminergic flow is disrupted. However, there are several limitations in this assumption that necessitate alternative routes of understanding to be developed. The lack of perfect co-localization among different elements of dopamine synthesis, store and release machinery in the striatal TH+ neurons is one of the counter-arguments towards such a stance. In addition, anatomical reconstructions of TH+ interneurons show only a modest axonal arborization pattern compared to a
midbrain dopaminergic neuron (Betarbet et al., 1997; Ibáñez-Sandoval et al., 2010; Matsuda et al., 2009).

Regardless of the debate whether they release dopamine in addition to GABA, what is established by now about TH+ interneurons is that they are well integrated in the striatal circuitry as GABAergic interneurons and display a striking within-group electrophysiological diversity (Ibáñez-Sandoval et al., 2010). Therefore, understanding the precise role they play in striatal processing in normal and Parkinsonian brain calls for a systematic assessment of their striatal distribution with respect to their electrophysiological diversity.

1.6 Why is putting TH+ interneurons into an anatomical perspective in the striatum important?

It has already been mentioned previously that the striatum is anatomically divided into several components. Anatomical partitioning of the striatum is relevant to the functional roles ascribed to the structure. Behavioral investigations point out that striatum is a functionally heterogeneous structure that can be broken down into four domains as dorso-lateral striatum, dorso-medial striatum, nucleus accumbens-core and nucleus accumbens-shell (Yin et al., 2008; Yin and Knowlton, 2006). Despite the anatomical and functional heterogeneity, what is interesting is that cytoarchitectural components in separate domains are the same: SPNs make up the majority, and all interneuron types are present in each functionally distinct division (Hussain et al., 1996; Jongen-Relo et al., 1993; Kubota and Kawaguchi, 1993; Kubota et al., 1994b; Smith and Bolam, 1990; Voorn et al., 2004).
In addition to these functional divisions of the striatum, patch/striosome-matrix compartmentalization adds further complexity to neurochemical organization and afferent and efferent patterning of the striatum (Gerfen, 1984; Gerfen et al., 1985; Gerfen et al., 1987a; 1987b; Graybiel et al., 1981; Graybiel et al., 1987; Holt et al., 1997, Langer and Graybiel., 1989). Although the function of this mosaic organization remains elusive, it has a critical organizing effect in striatal cytoarchitecture. The orientation and trajectory of SPN dendritic and axonal arborization obey compartmental boundaries (Kawaguchi et al., 1990), whereas processes of cholinergic and GABAergic interneurons do not (Chesselet and Graybiel, 1986; Cowan et al., 1990; Kawaguchi, 1992). Furthermore, the somata of cholinergic and NPY interneurons are preferentially situated at the compartmental boundaries (Bernácer et al., 2005; 2007; Kubota and Kawaguchi, 1993; Martone et al., 1994; Saka et al., 2002). These data collectively suggest the existence of important differences in signal processing in patch/striosome and matrix domains in which certain classes of striatal interneurons may play a crucial role (Saka et al., 2002).

Thus, diverging cortical and dopaminergic innervation patterning is one way that separate striatal domains can achieve different computations using the same cytoarchitectural components. However, an additional critical factor that must be taken into account is the distribution of interneurons. Anatomical mapping studies indicate that different classes of striatal interneurons are non-homogeneously distributed in the striatum (Bennett and Bolam, 1993; 1994; Gerfen and Bolam, 2010; Kubota and Kawaguchi, 1993; Kubota et al., 1993b; Rymar et al., 2004; Saka et al., 2002). For instance, in the rat striatum, PV-expressing interneurons show pronounced dorsolateral to ventromedial gradient, while NPY-SOM-nNOS neurons oppose the
distribution of fast-spiking neurons by displaying a higher density in the ventromedial aspect of the striatum (Gerfen and Bolam, 2010). In addition, CR-expressing interneurons were found to show a strong rostro-caudal gradient with the highest density of interneurons located in the dorsomedial pre-commissural striatum (Rymar et al., 2004). Additionally, although cholinergic interneurons appear homogeneously distributed throughout the striatum, when compartmental organization of the striatum is taken into account, cholinergic somata were found to be situated at patch-matrix border (Kubota and Kawaguchi, 1993; Martone et al., 1994; van Vulpen and van der Kooy, 1998). The positioning of striatal interneurons at the compartmental boundaries leads to attribution of relay function to cholinergic interneurons across the striatal compartments (Jabourian et al., 2007). A similar preferential localization at patch/striosome border was also the case for NPY-expressing interneurons (Kubota and Kawaguchi, 1993; Saka et al., 2002). Although the dendritic arborizations of SPNs are confined to the compartment where the somata is located, dendrites of cholinergic and NPY-expressing interneurons invariably extend into the neighboring compartment indicating that they may play an integral role in inter-compartmental communication (Kubota and Kawaguchi, 1993; Saka et al., 2002). In fact, when cholinergic and NPY-expressing interneurons were ablated by a specific saporin-toxin conjugate targeting tachykinin 1 receptor, patterned early gene activation in response to dopaminergic stimulation was disrupted and behavioral deficits arose. Taken together, these findings lead to the thinking that there might be local variability in interneuron-mediated effects, which may be a major contributing factor in the functional specialization within the striatum.
Therefore, uncovering how TH+ interneurons fit in this complex anatomical organization of the striatum will clearly deepen our understanding of the role of TH+ neurons in regulation of the striatal microcircuitry.

1.7 What really happens to TH interneurons following striatal dopamine loss?

Since their discovery in the primate striatum (Dubach et al., 1987), striatal TH+ neurons have sparked a great deal of interest, as the identifying marker, TH, is the rate-limiting enzyme in dopamine synthesis. Consequently, a number of studies have investigated the developmental and molecular origin of striatal TH+ neurons (Busceti et al., 2008) and their fate in experimentally induced and/or Parkinson’s disease-related loss of striatal dopamine (Huot and Parent, 2007; Huot et al., 2007; Mazloom and Smith, 2006; Meredith et al., 1999; Tande et al., 2006; Tashiro, 1989). A common observation in these studies is that there is a marked increase in number of these neurons following striatal dopamine loss. The most widely accepted explanation for the increase in the number of striatal TH+ interneurons is phenotypic conversion of a population of pre-existing striatal neurons, since the possible role of neurogenesis has been refuted (Darmopil et al., 2008; Tande et al., 2006). Nevertheless, these studies rely on immunocytochemical detection of the somatic TH protein, which was shown to be below the detection limits in most of the striatal TH-eGFP neurons by immunocytochemistry that necessitated experimental augmentation of somatic protein levels by colchicine injection (Ibáñez-Sandoval et al., 2010). Therefore, since we cannot be certain of the numbers of the actual resident population of striatal TH+ interneurons by immunocytochemistry, it appears vague that how much of the observed increases after dopamine denervation indeed reflects a striatal neural population appearing de novo. In this vein, it is plausible to argue that several folds of
increase in number of TH+ neurons in dopamine depleted striatum (Darmopil et al., 2008; Tande et al., 2005; Tashiro et al., 1989b) may in fact reflect an increase in somatic TH protein levels allowing detection by immunostaining of neurons that were already there. Indeed, ambient striatal dopamine is a well-known transcriptional inhibitor of TH protein (as reviewed in Daubner et al., 2011; Kaushik et al., 2007). Therefore, re-evaluation of the magnitude of dopamine-loss induced changes on the number of striatal TH+ interneurons is imperative using transgenic mice endowed with sensitive reporter gene activity for establishing a reliable baseline to which effect size of striatal dopamine denervation can be compared.

In addition, there has been no previous electrophysiological characterization of TH+ neurons in the dopamine-depleted striatum. Furthermore, there is controversy regarding the anatomical identity of these neurons with respect to what degree striatal TH+ neurons resemble spiny projections neurons or striatal interneurons following dopamine loss (Darmopil et al., 2008; Ibáñez-Sandoval et al., 2010; Masuda et al., 2011).

Therefore, characterizing electrophysiological and anatomical changes in TH-eGFP neurons would not only lend further insight into role of TH+ interneurons in the striatum, but also help us understand the adaptations taking place in dopamine-depleted striatum.

1.8 Goals of the Thesis
Understanding the anatomical organization and electrophysiological properties of the components of striatal microcircuitry is imperative for interpreting disease-induced changes in basal ganglia and probing therapeutic approaches. There is ongoing controversy about striatal TH+ neurons regarding their neurochemical identity; to what degree they resemble SPNs and how their number changes as a result of nigrostriatal dopamine loss. Significant advances have been made in these respects with the advent of transgenic mouse lines ascertaining that in the intact striatum most, if not all, striatal TH+ neurons are a distinct class of GABAergic interneurons (Ibáñez-Sandoval et al., 2010; Tepper et al., 2010). However, there is still a dearth of information regarding striatal distribution of TH+ interneurons with respect to anatomical organization of the striatum. This goal will be pursued in the first experiment of this thesis. The anatomical distribution of TH+ interneurons will be investigated with respect to their divergent electrophysiology, different layers of striatal anatomical organization (Chapter 3). In order to find out whether TH-eGFP interneurons are differentially distributed across striatal territories, first, mapping of electrophysiologically-characterized TH-eGFP neurons will be carried out. Second, TH-eGFP neurons will be mapped with respect to the patch/striosome-matrix mosaic following μ-opioid receptor (MOR) immunofluorescent labeling of the striatum to distinguish different striatal compartments. Third, compartmental distribution of different subtypes of TH-eGFP will be investigated in young mice, where striatal TH-eGFP cell bodies will be targeted for electrophysiological characterization and patches/striosomes will be simultaneously identified in the form of TH-eGFP fiber rich dopamine islands in young TH-eGFP mice as described earlier in Miura et al. (2007) and Miura et al. (2008).
In the second experiment, anatomical and electrophysiological changes in TH-eGFP neurons after nigrostriatal dopamine loss will be investigated (Chapter 4). In unilaterally 6-OHDA treated mice, striatal TH-eGFP neuron number, electrophysiology, and morphology will be compared in dopamine-depleted and vehicle-treated hemispheres. The findings gathered while investigating TH-eGFP neurons in dopamine-depleted striatum will also lend valuable insights for understanding as to how re-organization of the dopamine-deprived striatal network takes place and how GABAergic interneuronal circuitry might be involved.
Chapter 2

General Methods
2.1 EXPERIMENT 1

2.1.1 Subjects

The progeny of hemizygous Tg(Th-EGFP)DJ76Gsat/Mmnc (GENSAT, http://www.mmrrc.org/strains/292/0292.html) mice obtained from the Mutant Mouse Regional Resource Center at University of North Carolina, Chapel Hill crossed to FVB mice were used in the experiments. Mice of both sexes aged between 2 to 10 months were used in all experiments. The animals were kept in a temperature and humidity controlled AAALAC accredited animal facility and maintained on a 12/12 dark light cycle with light onset at 7 a.m. Prior to experiments, post-weaning mice were genotyped from tail snips to confirm that they expressed eGFP. All experimental protocols were in accordance with Rutgers University Institutional Animal Care and Use Committee and the NIH Guidelines to the Care and Use of Laboratory Animals. Utmost effort was shown to minimize the number of animals that were used and any pain and discomfort the animals underwent.

2.1.2 In vitro electrophysiology

Following intraperitoneal (i.p.) injection of ketamine (100 mg/kg) (Ketajaect, Henry Schein, Melville, NY), TH-eGFP mice were transcardially perfused with ice-cold modified Ringer’s solution that contained (in mM) 124 Choline Cl, 2.5 KCl, 26 NaHCO$_3$, 3.3 MgCl$_2$, 1.2 NaH$_2$PO$_4$, 10 glucose or 248 sucrose, 2.5 KCl, 7 MgCl$_2$, 23 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 7 glucose, 1 ascorbate, and 3 pyruvate. Subsequently, 250 to 350 µm coronal or 10° parahorizontal striatal sections were obtained using a vibrating microtome (Vibratome™ 3000, St. Louis, MO). Slices were transferred initially into a
slice chamber that contained Ringer’s Solution (in mM: 124 NaCl, 2.5 KCl, 26 NaHCO₃, 1.3 MgCl₂, 1.2 NaH₂PO₄, 10 glucose, 2 CaCl₂, 1 ascorbic acid, 3 pyruvate, 0.4 myoinositol) maintained at 33°C and later kept at room temperature until the time of recording. During the recordings slices were continuously perfused with normal Ringer’s solution at a flow rate of 2ml/min, which was maintained at 33°C via TC-324B inline heater system (Warner Instruments, Hamden, CT). TH-eGFP neurons were identified by infrared DIC and epifluorescence visualization with a 40X objective using a BX50-W1 Olympus microscope. Whole-cell patch clamp recordings in current clamp mode were obtained using glass pipettes (3-7 MΩ) filled with (in mM): 130 KMeSO₃, 10 NaCl, 10 HEPES, 1 EGTA, 0.1 CaCl₂, 2 MgCl₂, 3 ATP, 0.3 GTP, pH adjusted to 7.3. Biocytin (0.2%) was added to the intracellular solution in order to label the recorded neuron for later identification and anatomical investigation of recorded striatal TH-eGFP neurons.

Recordings were acquired with a Neurodata IR-283 current clamp amplifier and digitized at 10-40 kHz via a Micro 1401 Mk II data acquisition unit and transferred to a PC using Signal™ v.4 software (CED, Cambridge, UK) for offline analysis. To examine the basic membrane properties of the TH-eGFP neurons, a sequence of 500 msec hyperpolarizing and depolarizing current steps from -200 pA to +200 pA were injected. Current-voltage (I-V) curves were constructed by averaging 10 msec epochs within the last 100 msec of the 500 msec current injection that was devoid of action potentials or spontaneous synaptic potentials. Membrane input resistance was estimated from calculating the slope of the I-V relation at 0 pA current injection (resting membrane potential). Action potential measurements were taken from the first action potential evoked by injection of threshold depolarizing current. Action
potential half-width was determined by measuring the time between points of half-maximal action potential amplitude. The spike afterhyperpolarization amplitude was measured by subtracting minimum membrane potential following the AP from the AP threshold value. Finally, the membrane time constant (τ) was defined as the time it takes the membrane to fall to 63% of the steady state value by fitting an exponential to the initial phase of the membrane response to a -20 pA current injection.

2.1.3 Biocytin cytochemistry

Following electrophysiological characterization, sections containing biocytin filled neurons were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) overnight at 4° C and transferred next day into 0.1 M PB. Sections were then washed for 3 X 10 minutes, then treated with 0.1% Sodium borohydride in 0.1 M PB followed by 10% methanol and 3% H₂O₂ in 0.1 M PB for 15 minutes. Next, sections were incubated with avidin-biotin-peroxidase complex (Vector Laboratories; 1:200) and 0.1% Triton X-100 initially at room temperature for 2 hours followed by overnight at 4° C. After washing 6 X 10 minutes in 0.1 M PB the sections were reacted with 3,3’ diaminobenzidine (0.025%) and H₂O₂ (0.0008%) in PB with nickel intensification (2.5 mM nickel ammonium sulfate and 7 mM ammonium chloride) to visualize the biocytin-stained neuron for later reconstruction using Neurolucida™ (MBF Bioscience, VT). Slices were later postfixed in osmium tetroxide (0.1% in PB) for 30 minutes, dehydrated through a graded series of ethanol, followed by wash with xylene. Air-dried sections were then mounted in Depex (Electron Microscopy Sciences, PA) and coverslipped.
In a subset of sections (n=10), instead of DAB, biocytin was visualized by a Texas Red streptavidin conjugate (1:200, overnight at 4°C). This allowed visualization of the intrinsic fluorescent TH-eGFP signal (from somata and proximal neurites) along with detection of the biocytin fill by Texas Red.

2.1.4 Mu-opioid receptor immunocytochemistry

Adult TH-eGFP mice were sacrificed following deep anesthesia with i.p. Ketamine (200 mg/kg). Mice were then transcardially perfused with chilled Ringer’s solution followed by fixation with freshly prepared 4% paraformaldehyde added to 15% saturated picric acid in 0.1M PB. After decapitation, brains were stored in the same fixative for overnight fixation. On the next day, following several washes with PBS, 60 µm free-floating sections were obtained using a VibratomeTM 1200. Sections were pretreated with 1% sodium borohydride followed by 10% methanol and 3% H₂O₂ in PBS prior to incubation in 10% normal donkey serum, 2% bovine serum albumin, and 0.5% Triton X-100 for one hour. Next, sections were incubated in a solution containing 1:1000 rabbit anti-MOR monoclonal antibody (Immunostar Inc., Hudson, WI, #24216) along with 10% normal donkey serum, 2% bovine serum albumin, and 0.5% Triton X-100 (Sigma-Aldrich Co., St. Louis, MO) in PBS for 24 hours at room temperature. After washing 3 times for 10 minutes each in PBS, sections were transferred to a solution containing 1:500 Texas Red donkey anti-rabbit secondary (Molecular Probes, Inc., Eugene, OR, USA), 10% normal donkey serum, and 2% bovine serum albumin in PBS at 4°C overnight. After three 10 minute washes in PBS, sections were wet mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA), coverslipped and sealed with nail polish.
2.1.5 Stereology and anatomical mapping

For sections containing biocytin filled neurons, the slices were outlined using Neurolucida™. Briefly, section outlines, borders of the corpus callosum, anterior commissure and striatum were traced at 10X and a marker was placed at the location of recorded TH-eGFP somata. In order to account for variability in section condition (i.e. differential shrinkage), two sets of reference sections were cut at 100 µm in the coronal or 10° parahorizontal plane and Nissl-stained. Sections containing the biocytin-filled TH-eGFP somata were matched to the Nissl-stained reference sections at the closest z-depth and any deviations in dorso-ventral or medio-lateral aspects of the tissue were compensated for using the built-in shrinkage correction of the software. Registration to Nissl-stained reference sections thus enabled assigning individual z-depth values to recorded sections. A 45° line spanning from the most ventral end of the lateral ventricle to the most ventral end of the external capsule was drawn to parse the striatum into dorsal and ventral divisions.

In MOR-immunolabeled sections from TH-eGFP mice, every fifth section containing the striatum was traced at 4X following random determination of the initial section by the default workflow of the Optical Fractionator probe in StereoInvestigator™ v. 9 (MBF Biosciences, Williston, VT). The outlines of the entire section, the corpus callosum, the anterior commissure and the striatum were traced and visualized by different color contours. Additionally, the striatum was parsed into dorsal and ventral divisions based on the cell packing density and direction of fiber fascicles piercing the striatum, and with reference to the mouse brain atlas of Franklin and Paxinos (2001). The border between dorsal and ventral striatum
was further adjusted on the basis of strong MOR staining around the nucleus accumbens core segment.

Under epifluorescence illumination at 530 nm, patch/striosome and matrix compartments were outlined based on differential MOR immunoreactivity as it has been established that striosomes express stronger MOR-enkephalin immunoreactivity than the surrounding matrix in the dorsal striatum (Gerfen, 1989; Herkenham and Pert, 1981; Voorn et al., 1989). However, in the ventral striatum the afferent organization of MOR-rich domains resembles the dorsal striatal matrix (Jongen-Reilo et al., 1993; 1994).

The methodology for stereological cell counting procedure under epifluorescent illumination was adapted from previous reports using a similar approach (Prasad and Richfield, 2010; Henny and Jones, 2008). After virtual overlay of a 544 µm X 380 µm grid on the contoured serial sections, cell counting was performed at 40X under epifluorescence illumination at 380 nm to detect striatal TH-eGFP expressing somata. At each counting site, section thickness was measured, which yielded an average tissue thickness of 50.7 ± 1.14 µm indicating that there was ~16% shrinkage from the original cut thickness. Optical disector frame dimensions were set to 180 µm X 180 µm with 30 µm z-depth and 5% guard zones on top and at the bottom. Only those TH-eGFP somata falling entirely within the borders, i.e. falling in between and coming in contact with inclusion borders but not touching the exclusion borders were counted. The loci of the counted TH-eGFP somata with respect to MOR intensely immunoreactive regions were also noted during counting to determine the compartmental distribution of the cells. Since section thickness was measured at every
site, the numbers reported here are the stereological estimates weighed by section thickness. Raw data from biocytin filled cells and stereologically sampled TH-eGFP neurons were aligned and overlaid using Neurolucida™ Explorer and the Solid Modeling extension module (MBF Biosciences, Williston, VT).

2.1.6 Statistics

Data were analyzed using CED Signal™, Origin 7.0, Microsoft Excel™ and PAWS v. 18 software. Subtypes of TH-eGFP neurons were determined based on the electrophysiological criteria described in Ibáñez-Sandoval et al., (2010). For statistical comparisons, multivariate ANOVA and Bonferroni post-hoc tests performed in electrophysiological recordings. Independent samples t-test, Pearson’s chi-square test and Pearson’s correlation were performed on anatomical data sets. P values < 0.05 were accepted as statistically significant.

2.2 EXPERIMENT 2

2.2.1 Subjects

The progeny of hemizygous Tg(Th-EGFP)DJ76Gsat/Mmnc mouse were used as described for Experiment 1 in 2.1.1. A total of 86 mice were used in the current set of experiments.
2.2.2 6-OHDA lesion

Under isoflurane anesthesia, mice were fixed in a stereotaxic apparatus (Kopf Instruments). After a subcutaneous injection of Bupivacaine (0.5%, Henry Schein, Melville, NY), an incision was made on the scalp. Following anterior-posterior and medio-lateral flat skull adjustments, small burr holes were drilled on the skull overlying substantia nigra (3.4 mm posterior and 1.0 mm lateral with respect to bregma). The animals received desipramine (Sigma-Aldrich, St. Louis, MO) (25 mg/kg) i.p. 30 min prior to 6-OHDA infusions to prevent the uptake of toxin by noradrenergic terminals (Breese and Traylor, 1971). A glass micropipette (50 µm o.d. tip) was filled with freshly prepared 6-OHDA (Sigma-Aldrich, St. Louis, MO), 3.6 µg/µl 6-OHDA-HCl in 0.9% sterile saline containing 0.2% ascorbate) or vehicle. 6-OHDA or vehicle (0.5 µl) was pressure injected over 15 min into either SNc (4.3 mm ventral from the dura). Upon completion of injections, the glass capillaries were left in place for 3 additional minutes to allow diffusion before being slowly retracted. At the end of injections, the incision on the scalp was stitched using 4-0 surgical silk and the wound was infiltrated with Bupivacaine and bacitracin ointment. Animals were administered 1 ml of lactated Ringer’s solution and Buprenorphine (0.05mg/kg) s.c. and allowed to recover under a heat lamp, returned to their home cages following recovery from anesthesia, and kept there until use. The weight of the mice, and their food and water intake was monitored post-surgically and where needed, mice were provided with 10% sucrose solution and non-sweetened cereal pieces during recovery.

To evaluate the success of the unilateral 6-OHDA lesion, all TH-eGFP cells in SNc and VTA were counted in 6-OHDA and vehicle-treated hemispheres in 60 µm coronal midbrain sections taken from 3.3 to 3.5 mm posterior from bregma. The loss
of TH-eGFP cells in SNc and VTA was determined by dividing the numbers from 6-OHDA treated side by the numbers from the vehicle-treated side for SNc and VTA. In addition, forelimb use asymmetry was assessed using a cylinder test as described in Cenci and Lundblad (2007) prior to euthanasia. Mice were initially habituated to the experimental room for 5 min prior to behavioral phenotyping. Mice were placed inside a glass cylinder of 10 cm in diameter and 12 cm in height in a dimly lit room. Behaviors were recorded for 3 min with a digital camera. Behavioral sessions were analyzed offline frame-by-frame. For behavioral scoring, forelimb use asymmetry was calculated by dividing the full contacts of contralateral front paw placements on the cylinder wall by the sum of ipsi- and contra-lateral paw placements. 40% was regarded as a cutoff value indicating the success of the lesion (Lundblad et al., 2004).

2.2.3 Retrograde labeling

In a subgroup of unilaterally 6-OHDA lesioned mice (n=5) 0.1 µl of rhodamine microspheres (Lumafluor, Durham, NC) was pressure injected (Picospritzer, Intracel Ltd., UK) over 15 min bilaterally into the globus pallidus (0.4 mm posterior and 1.7 mm lateral with respect to bregma and 3.5 mm ventral to the dura) and substantia nigra pars reticulata (3.4 mm posterior and 1.4 mm lateral with respect to bregma and 4.5 mm ventral to the dura) in order to determine whether any of the TH-eGFP cells were projection neurons. Mice were sacrificed at 3, 7, 14 or 28 days post-lesion following deep anesthesia with i.p. ketamine (100 mg/ml) and transcardially perfused with chilled Ringer’s solution followed by 4% paraformaldehyde in 0.1M PB. Brains were kept overnight in 4% paraformaldehyde at 4°C and were subsequently transferred into 30% sucrose in 0.1M PB. Sixty µm coronal sections containing striatum and the injection sites were cut on a freezing microtome (Microm,
Heidelberg, Germany) and mounted on glass slides in Vectashield HardSet mounting medium (Vector Laboratories, Burlingame, CA) and coverslipped. Sections were analyzed for co-localization of TH-eGFP and rhodamine beads with an Olympus BX51 epifluorescent microscope.

2.2.4 Stereology

A group of TH-eGFP mice (n=16) underwent the same surgical procedures and were sacrificed at 3, 7, 14 or 28 days post-lesion, and coronal striatal sections were prepared as described above. Unbiased cell counting was done using the optical fractionator probe of StereoInvestigator (Microbrightfield v.10, Burlingame, VT). Briefly, after randomly determining the start level for the serial sections, every fifth section was contoured at 4X. Then a 544 µm X 380 µm grid was overlaid on the contoured serial sections and cell counting was performed at 40X under epifluorescent illumination at 380nm to detect TH-eGFP somata residing in the striatum. Disector frame dimensions were set to 180 µm X 180 µm with 30 µm z-depth and 5% guard zones on top and at the bottom. Only whole TH-eGFP somata falling within the borders, coming in contact with inclusion borders but not touching the exclusion borders were counted.

In mice which also had bilateral rhodamine infusion into GPe and SNr, striatal TH-eGFP somata were further investigated for colocalization of retrogradely transported rhodamine microspheres from GPe and SNr under epifluorescent illumination at 530 nm. These data were used to estimate the total number of TH-eGFP-only cells and double-labeled TH-eGFP/rhodamine+ cells in striatum.
2.2.5 Immunocytochemistry

Briefly, 60 µm coronal sections from mice 3 and 7 days post-6-OHDA-lesioning were pretreated with 1% sodium borohydride followed by 10% methanol and 3% H$_2$O$_2$ in PBS. Sections were then incubated in 10% normal goat serum, 2% bovine serum albumin and 0.5% Triton X-100 for 1h. Alternating serial sections were incubated with one of the following primary antibodies for 48h at 4°C: rabbit anti-tyrosine hydroxylase (1:1500, Millipore Cat. #: AB9983), rabbit anti-parvalbumin (1:1000, Immunostar Cat. #:24428), rabbit anti-NPY (1:3000, Immunostar Cat. #: 22940), rabbit anti-calretinin (1:1500; Millipore Cat. #: AB5054), goat anti-choline acetyl transferase (1:400, Millipore Cat. #: AB144) After rinsing 3 times for 10 minutes in PBS, sections were transferred into the solution containing 1:200 donkey anti-rabbit Alexa 594 or 1:200 donkey anti-goat Alexa 594 (Molecular Probes, Inc., Eugene, OR, USA, Cat. #s: A21207 and A11058) in PBS at 4°C overnight.

2.2.6 In vitro electrophysiology

A group of animals that underwent unilateral dopamine denervation were sacrificed at 3, 7, 14 or 28 days post-lesion. 250 µm thick coronal sections containing striatum were prepared for whole cell in vitro recording experiments as described in Experiment 1 in section 2.1.2.

Data were acquired with a Neurodata IR-283 for current clamp and with a Multiclamp 700B (Molecular Devices) for current clamp and voltage clamp recordings. Data were digitized at 10-40 kHz via a Micro 1401 Mk II data acquisition
Electrophysiological data were analyzed using CED Signal and spontaneous synaptic events were analyzed using Clampfit (v.10; Axon Instruments) using template based event detection. To obtain the basic electrophysiological parameters of the TH-eGFP neurons, a sequence of hyperpolarizing and depolarizing 500 msec current steps ranging from -200 pA to +200 pA with 20 pA increments were injected at 0.2 Hz. Current (I)-voltage (V) curves were constructed by averaging 10 msec epochs of the 500 msec current injection step that were devoid of action potentials or spontaneous synaptic potentials. Membrane input resistance was estimated from calculating the slope of the I-V relation at 0 pA current injection (resting membrane potential). Action potential (AP) measurements were taken from the first AP evoked by injection of threshold depolarizing current. AP threshold was defined as the point at which the slope of the voltage rise reached 10 mV/msec, AP amplitude was defined as the voltage difference between peak and the AP threshold. AP duration was determined by measuring the time between points of half-maximal action potential amplitude. AHP amplitude was measured by subtracting the minimum voltage following the first AP from the AP threshold value. The membrane time constant (τ) was defined as the time it takes membrane to reach 63% of the asymptote level and was measured by fitting a single exponential to the initial phase of the membrane response to -20 pA current injection. As reported previously (Ibáñez-Sandoval et al., 2010) some TH-eGFP cells exhibited a membrane sag in response to hyperpolarizing current injections. We quantified the sag ratio by dividing the minimum membrane potential at the beginning of a 500 msec current injection by the steady state potential.
at the end of the pulse, when the cells were hyperpolarized to -100 mV. Maximal firing rate was measured by 1/first interspike interval (ISI) during +200 pA current pulse. We also measured spike frequency adaptation in the first trace in which 3 or more spikes were elicited by current injection by dividing the last ISI by the first ISI. In addition, we quantified the amplitude and duration of the plateau potentials emerging at the offset of 0.5 sec of +100 pA depolarizing current by fitting a Boltzmann sigmoid \( y = (A_1 - A_2) / (1 + e^{(x-x_{50})/dx}) + A_2 \) where the difference between \( A_1 \) and \( A_2 \) was used to measure plateau potential amplitude and \( X_{50} \) was used to determine the time when plateau potential decays to half maximal amplitude.

For recording spontaneous EPSCs and IPSCs in voltage clamp, a Cesium-based internal solution was used that contained (in mM) (Mathur et al., 2011): 120 CsMeSO\(_4\), 5 NaCl, 10 TAE-Cl, 10 HEPES, 5 QX-314, 1.1. EGTA, 0.3 Na-GTP and 4 Mg-ATP which was pH adjusted to 7.3 with Cesium OH. Spontaneous EPSCs were isolated by holding the cell at -70 mV in the presence of bicuculline methiodide (10 \( \mu \)M, Sigma). Spontaneous IPSCs were isolated by holding the cell at 0 mV in the presence of AP-5 (25 \( \mu \)M, Tocris) and CNQX (10 \( \mu \)M, Tocris). We compared amplitude, rise \( \tau \), decay \( \tau \), inter-event intervals (IEI), coefficient of variation (CV) of IEIs and the frequency of spontaneous IPSCs and EPSCs isolated pharmacologically by sequential bath application of synaptic blockers (see above). The parameters for sIPSCs and sEPSCs were extracted from 1 min epochs of recordings from neurons held at 0 mV and -70mV holding potentials, respectively.
2.2.7 Biocytin cytochemistry and cell reconstruction

Biocytin-filled electrophysiologically identified TH-eGFP cells were reacted with Ni-DAB as described in Experiment 1. Sections were air dried on gelatin coated slides and coverslipped in Depex (EMS, Harfield, PA). A subset of these sections (3 from 6-OHDA- and 3 from vehicle treated striata) were later postfixfixed in osmium tetroxide (0.1% in PB) for 30 minutes, dehydrated through a graded series of ethanol and three additional 10 min washes with propylene oxide and then cured with Fluka Durcupan resin (EMS, Hatfield PA) 1 day at room temperature then at 60° C for 3 days.

For three-dimensional reconstructions of biocytin-injected neurons, stacks of images were taken with a 60X or 100X oil immersion lens with Olympus BX51 microscope using Neurolucida (v.10, MBF Biosciences, VT). Neurons were manually traced from virtual image stacks with Neurolucida and morphological quantification and Sholl analyses (Sholl, 1953) were later done with Neurolucida Explorer. Shrinkage corrections were done in z-axis as described in Marx et al., (2012).

2.2.8 Statistics

Statistical tests were done by IBM SPSS Statistics package (v20). Student’s two-tailed t-test, multivariate ANOVA, Chi-Square test were used for appropriate data sets. In cases where homogeneity of variance could not be assumed, Mann-Whitney and Kruskal-Wallis tests were used. Cell count data comparisons were done between 6-OHDA and vehicle-treated striata, therefore paired t-test was used. In addition, for morphological comparisons of TH-eGFP interneurons, data points on Sholl plots were statistically tested by repeated measure ANOVA. Data are expressed as Mean±SEM. Where possible, box plots were used to display the data, where boxes indicate the
interquartile range of the data and whiskers span the maximum and minimum value range of the data. Significance level was set at 0.05.
Chapter 3

Distribution of Striatal TH-eGFP interneurons with respect to different levels of anatomical organization of the striatum
3.1 Localization of electrophysiologically identified TH-eGFP cell types

Sixty-three electrophysiologically identified and biocytin-stained TH-eGFP neurons (n=31 in coronal and n=32 in parahorizontal orientations) were mapped as shown in Figure 1. As each of the recorded slices were assigned a unique z-depth value on the basis of the reference sections, the data from coronal and parahorizontal slices could be merged with each other by Neurolucida Explorer. In order to obtain a combined image and data set, parahorizontally mapped TH-eGFP cells were virtually rotated 90° along the anterior-posterior and 10° along the dorsal-ventral axes and merged with the cluster of TH-eGFP cells obtained in the coronal orientation (Fig. 3.1C).

Quantitative assessment of the distribution of electrophysiologically identified TH-eGFP neuron subtypes was analyzed in three dimensions (Fig. 3.1C). In the cumulative distribution for the coronal orientation, a 45° horizontal line was drawn dorsal to the anterior commissure extending from the ventral most point of the lateral ventricle to the ventral-most point of the external capsule (Voorn et al., 2004) in order to mark the division between dorsal and ventral striatum. On the basis of this division, it was estimated that 81% (51/63) of the recorded and filled neurons were in the dorsal striatum with the remaining 19% (12/63) within the territory of the ventral striatum. Pearson’s Chi-square analysis was performed in order to see whether any of the electrophysiologically distinct subtypes were disproportionately present in dorsal or ventral striatum. The results indicated that the distribution of TH-eGFP interneuron subtypes did not differ in the dorsal and ventral striatum (Pearson’s $\chi^2$ (3) = 6.088, p>0.05). In both dorsal and ventral striatum, Type I TH-eGFP neurons constituted the
majority of recorded neurons (48/63) (Fig. 3.1C), consistent with our previous observations (Ibañez-Sandoval et al., 2010). Sixty percent of the Type IV neurons in our sample were located in the ventral striatum, but this observation did not reach statistical significance, most likely because only a few of the mapped neurons belonged to this category (n=5).

3.2 Stereological cell counting and mapping with relation to MOR staining density

Striatal TH interneurons in a different set of sections were identified on the basis of their eGFP fluorescence irrespective of their electrophysiological profile, sampled using optical fractionator methodology and localized with regard to striatal MOR.
patches (Fig. 3.2A, 3.2B, 3.2C, 3.2D and 3.2E). In the dorsal striatum, dark areas of weaker TH-eGFP fiber fluorescence were observed to show a strong overlap with intensely MOR-labeled domains (Fig. 3.2A, 3.2B and 3.2C). The overall stereological estimates yielded an average of 2756 ± 192.4 (mean Gundersen’s Coefficient of Error=0.11) TH-eGFP neurons per striatum per hemisphere (n=10 hemispheres), in good agreement with values previously reported for striatal TH-eGFP interneurons (Ibáñez-Sandoval et al., 2010). Of the TH-eGFP neurons, 723.2 ± 44.9 were located in MOR-enriched domains corresponding to different compartments of striatal striosome-matrix organization in dorsal and ventral striatum combined. In addition, the proximity to the nearest striosome-matrix boundary was measured among 332 matrix-bound TH-eGFP somata. Slightly more than half of the entire population resided within 100 µm or less from the perimeter of the MOR-enriched regions in both dorsal and ventral striatum (Fig. 3.2F).

The numbers obtained from the stereological mapping were used in regression analyses in order to understand the relationship between the TH-eGFP neurons in MOR-enriched domains and the fraction of MOR-enriched domains with respect to the entire striatum. The regression analysis indicated that the fraction of TH-eGFP neurons falling within MOR-enriched islands was dramatically different in dorsal versus ventral striatum (Fig. 3.3A and 3.3B). Although the TH-eGFP neurons were present in MOR-enriched striosomes of the dorsal striatum at a chance level (\(r^2=0.069\)) (Fig. 3.3C), in the ventral striatum significantly more TH-eGFP neurons were located in MOR-enriched domains than what would be expected by chance (\(r^2=0.662, p=0.004\)) (Fig. 3.4D).
Figure 3.2. Identification of striosome-matrix compartmentalization with MOR immunohistochemistry in TH-eGFP mice. (A-C) Typical example of a Stereoinvestigator-generated montage of a single section from anterior striatum in TH-eGFP mice showing eGFP fluorescence (A), MOR immunofluorescence (B) and the merged overlay (C). Note that the dark areas (lower TH-eGFP fluorescence) in the dorsal striatum, show a strong overlap with the MOR-enriched domains (A, B and C). D. Higher magnification image of a different striatal section from another animal where TH-eGFP somata were marked on the basis of their compartmental distribution. Red arrowheads point to the TH-eGFP somata in matrix, and white arrowheads point to the TH-eGFP somata in MOR-enriched striosomes. E. Representative series of 60 µm sections at 300 µm intervals from one hemisphere showing MOR patches. F. The distance to the nearest MOR patch was measured among 332 TH-eGFP neurons in the matrix. The histogram shows that half of the matrix TH-eGFP neurons are located within 100 µm of the striosome-matrix borders. Scale bars = 250 µm. AC, anterior commissure, P, MOR patch/striosome.
Indeed, the regression between these parameters was found to be statistically significant, as the coefficients predicting the relationship in dorsal and ventral striatum (B=-0.332 on dorsal striatum vs. B=0.914 in ventral striatum) were found to differ significantly by t-test (t (1) = 3.935, p=0.001) (Figure 3.3C, 3.3D).

Figure 3.3 Distribution of TH-eGFP neurons with respect to MOR-enriched areas of the striatum. The raw data (n= 4 hemispheres) from stereological cell-counting procedures were aligned and overlaid for 3D visualization after two hemispheres from the same brain were collapsed on each other (A, B). Markers show the distribution of systematically random sampled TH-eGFP neurons in MOR-enriched and MOR-impoverished compartments of the striatum. The plots depict the percent distribution of TH-eGFP neurons in MOR enriched domains of dorsal and ventral striatum (C, D). The relationship between striatal MOR-enriched domains and distribution of TH-eGFP neurons with respect to these domains is significantly different in dorsal (C) and ventral (D) divisions of the striatum (p=0.001). Note the differences in the scale of y-axes in C and D.
The proportion of striatal area comprised of MOR-enriched domains did not differ between dorsal and ventral striatum (t (18) = -0.066, p=0.948) but it is interesting to note that in female mice (n=4 hemispheres), the striatal proportion of MOR-enriched domains in ventral striatum was significantly larger than in male mice (t (18)= -2.230, p=0.039). The stereological data pertaining to MOR-enriched regions are presented in Tables 3.1 and 3.2.

<table>
<thead>
<tr>
<th>Stereological estimates of striatal TH-eGFP neurons / hemisphere (Mean ± SEM)</th>
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<tr>
<td>Total number of striatal TH-eGFP neurons outside MOR-enriched domains</td>
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<tr>
<td>Total number of striatal TH-eGFP neurons inside MOR-enriched domains</td>
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<td>TOTAL striatal TH-eGFP neurons</td>
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<tr>
<td>Total number of dorsal striatal TH-eGFP neurons outside MOR-enriched domains</td>
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<td>Total number of dorsal striatal TH-eGFP neurons inside MOR-enriched domains</td>
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<tr>
<td>Total number of ventral striatal TH-eGFP neurons outside MOR-enriched domains</td>
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<tr>
<td>Total number of ventral striatal TH-eGFP neurons inside MOR-enriched domains</td>
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Table 3.1. The stereological estimates of the number of TH-eGFP neurons across striatal compartments in dorsal and ventral striatum. The stereological estimates of the number of TH-eGFP neurons from 60 µm serial sections at 300 µm intervals that were used for stereological counting and mapping of TH-eGFP neurons with respect to MOR-labeling.
3.3 TH-eGFP location with respect to TH rich patches/striosomes in young mice

Ten TH-eGFP interneurons were recorded from slices taken from young (postnatal day 30-33) TH-eGFP mice where striosome and matrix compartments could be identified under epifluorescent illumination on the basis of a differential TH-fiber density. In young animals, TH rich “islands” correspond to what will become the dopamine- and TH-poor striosome compartment in adults (Graybiel et al., 1987). Two of these neurons were located on the border between TH-rich dopamine islands and TH-poorer matrix, and both displayed the electrophysiological phenotype of Type IV TH-eGFP interneurons (Fig. 3.4A). Interestingly, the remaining 8 cells that were recorded within dopamine islands were all found to be Type I TH-eGFP interneurons (Fig. 3.4B). The maximum projection images of the neurons in Fig. 3.4A and 3.4B taken at 5 µm intervals indicate that in both cases, there are neurites that cross over

<table>
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<tr>
<th>REGION</th>
<th>Surface Area (µm²)/hemisphere</th>
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<tr>
<td></td>
<td>Males (n=6 hemispheres)</td>
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<tr>
<td>Total striatal area (A)</td>
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<tr>
<td>Total area of MOR enriched domains (B)</td>
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<td>(B)/(A)</td>
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<td>Total dorsal striatal area (C)</td>
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<td>Total area of dorsal MOR enriched domains (D)</td>
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<td>(D)/(C)</td>
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<td>Total ventral striatal area (E)</td>
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<tr>
<td>Total area of ventral MOR enriched domains (F)</td>
<td>1,716,929 **</td>
</tr>
<tr>
<td>(F)/(E)</td>
<td>16.7% **</td>
</tr>
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</table>

Table 3.2. The Cavalieri estimations of surface area measurements of striatal compartments across striatal divisions and sexes. The Cavalieri estimations of surface area measurements obtained from 60 µm serial sections at 300 µm intervals that were used for stereological counting and mapping of TH-eGFP neurons with respect to MOR-labeling. The independent t-test comparisons only indicated a significant difference for MOR-enriched domain estimations in the ventral striatum between the sexes. ** indicates, p<0.01.
into the neighboring compartments (Fig. 3.4C and 3.4D). Due to the very limited size of the sample, no statistics were performed on this subset of the data.

**Figure 3.4.** Distribution of electrophysiologically identified TH-eGFP neurons with respect to dopamine islands in juvenile TH-eGFP mice. A. Biocytin-filled Type I TH-eGFP interneuron in a dopamine island. Inset shows the I-V plot that identified the neuron as a Type I. B. Biocytin-filled Type IV TH-eGFP interneuron with the inset showing the I-V plot that identified the subtype. C. The maximal projection of an image stack obtained at 5 µm intervals of the Type I TH-eGFP neuron in (A). The boxed region corresponds to the contrast-enhanced inset showing the proximal neurite crossing a compartmental boundary. D. The maximal projection of the image stack obtained at 5 µm intervals of the Type IV TH-eGFP neuron in (B). The boxed region corresponds to the contrast-enhanced inset showing the distal neurites crossing a compartmental boundary. Scale bars, 50 µm. Note different scale markers in A and C, B and D.
Chapter 4

Anatomical and electrophysiological changes in striatal TH-interneurons after nigrostriatal dopamine loss.
4.1 6-OHDA lesion: Immunocytochemical and behavioral characterization of lesion extent

Unilateral intra-nigral infusion of 6-OHDA resulted in a profound loss of TH-eGFP expressing somata and neurites in the central and lateral segments of substantia nigra at all time points sampled, whereas the most medial aspects of SN and the VTA were spared (Fig. 4.1A, B, C). Vehicle infusion into the contralateral SN did not lead to any loss of TH-eGFP or TH immunofluorescence (Fig. 4.1A, B, C). In the SNC, the average loss of dopaminergic cells was 85%±3.3% whereas in VTA it was 54 %±7.8%. There was no significant effect of time point (3, 7, 14 or 28 days) on the average loss of TH-eGFP cells in SNC and VTA ($X^2 (8,3)$, $p=.884$ and $p=.212$, respectively).

Loss of inherent TH-eGFP signal and TH immunofluorescence originating from nigrostriatal axons was evident in the dorsal striatum ipsilateral to the intranigral 6-OHDA infusion (Fig. 4.1D, E, F). TH expressing fibers were evident in the ventral striatum (Fig. 4.1D, E, F). On the other hand, the contralateral striatum demonstrated strong TH-eGFP expression and TH-immunolabeling of axons in all fields (Fig. 4.1D, E, F). Previously, in striatum of intact adult TH-eGFP mice, we failed to find striatal TH-eGFP neurons immunoreactive for TH (Ibáñez-Sandoval et al., 2010). However, after intrastriatal colchicine injections, a subset of striatal TH-eGFP interneurons could be labeled with TH antibody indicating that somatic levels of TH protein in TH-eGFP interneurons are below detection limits by immunocytochemistry at baseline (Ibáñez-Sandoval et al., 2010). In the lesioned striatum, particularly in the
dorsolateral segment, several TH-eGFP somata were found to co-localize TH immunofluorescence (Fig. 4.1G, H, I) (17 out of 97 stereologically sampled striatal TH-eGFP cells). Colocalization of TH-eGFP and TH-immunoreactivity shows that after lesion, there is an increase in TH-eGFP production since in intact mice TH-eGFP cells do not show TH-immunoreactivity unless somatic TH protein levels are augmented by intrastriatal colchicine injection (Ibáñez-Sandoval et al., 2010).

In a subgroup of unilaterally 6-OHDA lesioned mice (n=11), offline analysis of behavior in the cylinder test showed a significantly higher number of ipsilateral forepaw contacts with the walls of the cylinder (t (1,10)=−4.66; p=0.001, data not shown). The mean front limb asymmetry score and total number of front limb contacts did not differ across different time points post lesion ((X^2(12,3), p=.748 and p=.350 respectively)
4.2 Effect of 6-OHDA lesion on the number and distribution of striatal TH-eGFP neurons

The stereological estimates of striatal TH-eGFP cells showed inter-subject variability. In the lesioned hemisphere the cell counts ranged from ~2100 to 4500 whereas in the vehicle-treated hemisphere the cell counts ranged from ~1800 to 3500 (Fig. 4.2A). Regardless, the increase in the total number of striatal (dorsal plus ventral striatum) TH-eGFP neurons ipsilateral to the nigrostriatal lesion was statistically significant (Vehicle: 2988±153 vs. 6-OHDA: 3428±253; paired t-test (13) = 2.94, p=0.012).

For a more reliable statistical measure that takes inter-individual variability into account, stereological estimates of TH-eGFP cell counts were standardized across subjects by expressing cell numbers as the % difference in the cell counts in the hemisphere ipsilateral to the nigrostriatal lesion with respect to the counts obtained from the vehicle-treated hemisphere (Fig. 4.2B). At the 3 day time point, the striatum ipsilateral to intranigral 6-OHDA treatment contained 30% more TH-eGFP cells than the vehicle-treated hemisphere (Fig. 4.2B), a difference that decreased with time after lesion. The counts in the ipsilateral striatum leveled off around control counts at 14 days post-lesion time point. However, when the changes in striatal TH-eGFP cell counts were investigated after parsing striatum into dorsal striatal (dSTR) and ventral striatal (vSTR) divisions with a 4 x 3 x 2 (days post lesion x striatal division x lesion/control) multivariate ANOVA (MANOVA), a different picture emerged. MANOVA revealed a significant main effect of lesioning (F (1, 30)=6.2, p=0.019). The three-way interaction between striatal division, days post-lesion and lesioning
was also statistically significant ($F(6, 30) = 2.529, p=0.042$). Post-hoc tests using LSD indicated that normalized TH-eGFP counts on post-lesion day 3 were significantly higher than at days 14 and 28 ($LSD_{3-14} = 28.1, p=0.037$; $LSD_{3-28} = 30.91, p=0.036$).

Cell counts of TH-eGFP neurons in dorsal striatum did not differ significantly as a function of time post-lesion ($F(3, 3) = 1.322, p = 0.412$). However in vSTR, cell counts at different post-lesion time points were significantly different ($F(3, 3) = 51.760, p=0.004$). At 3 days post-lesion in the ipsilateral vSTR, TH-eGFP neuron cell

**Figure 4.2. Changes in striatal TH-eGFP cell counts following unilateral 6-OHDA lesion.** (A) There was a significant increase in stereological estimated of striatal TH-eGFP neuron numbers after 6-OHDA lesion. (B) Increase in striatal TH-eGFP numbers normalized to vehicle treated side indicate that overall there was a 30 % increase in the earliest time points (3 and 7 days) but counts decline back to control levels within 2 weeks. (C) Change in dorsal striatal TH-eGFP numbers normalized to vehicle-treated side. (D) Change in ventral striatal TH-eGFP numbers normalized to vehicle-treated side.
counts were 2.1 times greater than counts from the contralateral side, a difference that decreased at subsequent time points (Fig. 4.2).

4.3 Retrograde labeling of striatal projection neurons by rhodamine bead injections into GPe and SNr in unilaterally dopamine denervated TH-eGFP mice

In a previous study (Ibáñez-Sandoval et al., 2010) we showed that none of the striatal TH-eGFP neurons in normal BAC transgenic control mice were retrogradely labeled following large injections of fluorescent beads into substantia nigra and globus pallidus (Ibáñez-Sandoval et al., 2010), consistent with electrophysiological
and morphological properties that were completely distinct from those of spiny projection neurons. However, the possibility remained that some of the TH-eGFP neurons that accounted for the increased number of striatal TH-eGFP neurons that we observed following ipsilateral destruction of the nigrostriatal dopamine input resulted from a phenotypic shift in which spiny projection neurons might start expressing TH (e.g., Darmopil et al., 2008). Thus we repeated the retrograde tracing experiments, making large (0.2 µl) injections of fluorescent beads into the substantia nigra and globus pallidus ipsilateral to a nigral 6-OHDA lesion in 5 mice. Just as in control mice, none of the striatal TH-eGFP cells was found to be double labeled (Fig. 4.3A, B, C, D) in dSTR (Fig. 4.3C) or vSTR (Fig. 4.3D), consistent with our previous findings that all of the striatal TH-eGFP neurons in both lesioned and control striata are in fact, interneurons.

4.4 Effect of 6-OHDA lesion on striatal TH-eGFP interneuron neurochemical markers

In a previous study of the neurochemical expression profile of TH-eGFP interneurons, we tested for co-localization of eGFP with PV, NOS and CR in control TH-eGFP mice. None of these substances was observed in TH-eGFP interneurons (Ibáñez-Sandoval et al., 2010). However, by the same logic discussed above, the possibility remained that following dopaminergic denervation, a phenotypic shift could occur that would lead to the expression of TH in other striatal GABAergic interneurons that do not normally express this enzyme.
Therefore, colocalization of TH-eGFP with PV, ChAT, NPY and CR was investigated by immunofluorescence in four unilaterally 6-OHDA lesioned TH-eGFP mice (3 days to 1 week post-lesion), by random sampling of TH-eGFP neurons using optical fractionator workflow in StereoInvestigator (v.10). Out of 159 TH-eGFP neurons none was immunopositive for NPY (Fig. 4.4A, B, C). In addition, none of the 312 TH-eGFP cells sampled in lesioned and vehicle treated striatum showed colocalization with ChAT (Fig 4.4D, E, F). None of 234 TH-eGFP cells sampled in lesioned or vehicle treated striatum co-localized PV immunoreactivity (Fig. 4.4G, H, I).

Figure 4.4. Comparison of TH-eGFP expression with other striatal interneuronal markers. TH-eGFP cells did not show any overlap with NPY immunoreactivity (A, B, C), ChAT immunoreactivity (D, E, F) or with PV immunoreactivity (G, H, I). Scale bars, 50 µm.
However, six TH-eGFP neurons out of 138 sampled cells (4/75 in 6-OHDA-lesioned striatum (Fig. 4.5A, B and C), 2/63 in vehicle treated striatum (Fig. 4.5D, E and F) showed co-localization with CR indicating that ~5% of TH-eGFP cells are

Figure 4.5. Colocalization of CR immunoreactivity with a subset of TH-eGFP neurons. Colocalization of CR immunoreactivity with a subset of TH-eGFP neurons in striatum ipsilateral to intranigral 6-OHDA (A, B, C) or vehicle (D, E, F) treatment. Widefield image from 6-OHDA treated ventral striatum, where arrows point to TH_eGFP (A,C, D, F) cells that show CR immnoreactivity (B, C, E, F). CR+ TH-eGFP cells were also encountered in vehicle-treated striatum (D, E, F). Scale bars, 50 µm.
CR+ and that the proportion of this population does not appear to change after dopamine lesion. Overall, these experiments show that striatal TH-eGFP interneurons do not change their neurochemical expression profile after unilateral dopaminergic denervation.

4.5 Effect of 6-OHDA lesion on the passive and active electrophysiological properties of striatal TH-eGFP interneurons

We identified 4 electrophysiologically distinct TH-eGFP interneuronal cell types from dopamine-denervated (n=72) and vehicle-treated hemispheres (n=44) in whole-cell current clamp recordings. The most frequently encountered cell type in both lesioned and vehicle-treated hemispheres (81% and 72%, respectively), denoted as Type I (Fig. 4.6A) (Table 4.1), demonstrated a resting membrane potential around -70 mV, which is relatively more depolarized than SPNs and fast-spiking neurons (Ibáñez-Sandoval et al., 2010; Koós and Tepper, 1999; Wilson and Kawaguchi; 1996) (Fig. 4.6A). Typical Type I TH-eGFP neuron manifests a complete cessation of firing following an initial burst of action potentials at about 45 Hz induced by depolarizing current injection.

In a subpopulation of Type I neurons, plateau potentials were observed during membrane repolarization at the offset of supra-threshold depolarizing current injections (Fig. 4.6A). Furthermore, some of the Type I neurons showed I_h-like sag responses in response to hyperpolarizing current injections. Some of the Type I interneurons were found to be spontaneously active (11/72 ipsilateral to the 6-OHDA vs 12/44 ipsilateral to vehicle-treatment).
Table 4.1: Summary of the electrophysiological parameters of TH-eGFP neurons recorded from striatum ipsilateral to intranigral 6-OHDA and vehicle infusions. Values are expressed as Mean ± SEM.

<table>
<thead>
<tr>
<th>Type</th>
<th>(Vehicle)</th>
<th>N=28</th>
<th>Vrest</th>
<th>Rinj</th>
<th>AP threshold</th>
<th>AP amplitude</th>
<th>1st AP delay</th>
<th>AP amplitude</th>
<th>Membrane time constant</th>
<th>Membrane potential</th>
<th>Action potential half-width</th>
<th>Action potential accommodation</th>
<th>Spike frequency</th>
<th>Maximum firing frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Vehicle</td>
<td>N=58</td>
<td>-66.22 ± 1.41 mV</td>
<td>718 ± 50.73 MΩ</td>
<td>-35.97 ± 2.9 mV</td>
<td>45.7 ± 1.7 mV</td>
<td>173.14 ± 28.6 msec</td>
<td>16.32 ± 0.71 mV</td>
<td>41.17 ± 4.4 msec</td>
<td>1.03 ± 0.01</td>
<td>0.63 ± 0.04 msec</td>
<td>1.30 ± 0.18</td>
<td>81.51 ± 15.24 Hz</td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>6-OHDA</td>
<td>N=6</td>
<td>-68.76 ± 1 mV</td>
<td>641 ± 44.6 MΩ</td>
<td>-36.11 ± 2.3 mV</td>
<td>52.1 ± 1.56 mV</td>
<td>177.14 ± 36 msec</td>
<td>15.64 ± 0.66 mV</td>
<td>33.25 ± 2.5 msec</td>
<td>1.05 ± 0.06</td>
<td>0.56 ± 0.03 msec</td>
<td>1.28 ± 0.93</td>
<td>96.23 ± 26.34 Hz</td>
<td></td>
</tr>
<tr>
<td>Type II</td>
<td>Vehicle</td>
<td>N=6</td>
<td>-62.36 ± 2.7 mV</td>
<td>566.5 ± 63.5 MΩ</td>
<td>-39.82 ± 0.84 mV</td>
<td>56.37 ± 4.30 mV</td>
<td>109.75 ± 60 msec</td>
<td>14.93 ± 1.36 mV</td>
<td>35.02 ± 3.94 msec</td>
<td>1.03 ± 0.01</td>
<td>0.48 ± 0.03 msec</td>
<td>1.44 ± 0.23</td>
<td>158.6 ± 37.5 Hz</td>
<td></td>
</tr>
<tr>
<td>Type II</td>
<td>6-OHDA</td>
<td>N=4</td>
<td>-66.47 ± 4.2 mV</td>
<td>391.4 ± 61.1 MΩ</td>
<td>-40.1 ± 0.44 mV</td>
<td>57.53 ± 6.22 mV</td>
<td>77 ± 34 msec</td>
<td>15.77 ± 1.20 mV</td>
<td>25.47 ± 11.47 msec</td>
<td>1.03 ± 0.01</td>
<td>0.43 ± 0.03 msec</td>
<td>1.87 ± 0.42</td>
<td>108.8 ± 21.3 Hz</td>
<td></td>
</tr>
<tr>
<td>Type III</td>
<td>Vehicle</td>
<td>N=3</td>
<td>-73.74 ± 2.19 mV</td>
<td>313.60 ± 98.01 MΩ</td>
<td>-37.34 ± 2.35 mV</td>
<td>51.67 ± 25.14 mV</td>
<td>51.67 ± 25.14 msec</td>
<td>16.88 ± 2.65 msec</td>
<td>18.36 ± 1.02 msec</td>
<td>1.02 ± 0.01</td>
<td>0.45 ± 0.07 msec</td>
<td>1.14 ± 0.1</td>
<td>131.83 ± 31.97 Hz</td>
<td></td>
</tr>
<tr>
<td>Type III</td>
<td>6-OHDA</td>
<td>N=10</td>
<td>-69.53 ± 3.95 mV</td>
<td>219.77 ± 36.89 MΩ</td>
<td>-35.01 ± 5.35 mV</td>
<td>41.25 ± 18.92 mV</td>
<td>41.25 ± 18.92 msec</td>
<td>15.39 ± 0.93 msec</td>
<td>13.65 ± 1.03 msec</td>
<td>1.03 ± 0.01</td>
<td>0.75 ± 0.14 msec</td>
<td>1.80 ± 0.4</td>
<td>96 ± 10.61 Hz</td>
<td></td>
</tr>
<tr>
<td>Type IV</td>
<td>Vehicle</td>
<td>N=7</td>
<td>-69.23 ± 2.92 mV</td>
<td>983.24 ± 100.36 MΩ</td>
<td>-31.31 ± 11.03 mV</td>
<td>47.80 ± 4.18 mV</td>
<td>144.67 ± 33.11 msec</td>
<td>11.27 ± 2.61 mV</td>
<td>11.27 ± 2.61 msec</td>
<td>1.03 ± 0.01</td>
<td>0.92 ± 0.15 msec</td>
<td>0.72 ± 0.1</td>
<td>136.50 ± 6.50 Hz</td>
<td></td>
</tr>
<tr>
<td>Type IV</td>
<td>6-OHDA</td>
<td>N=0</td>
<td>---</td>
<td>---</td>
<td>---</td>
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Type II TH-eGFP neurons were comprised 6% and 10% of TH-eGFP interneurons in 6-OHDA- and vehicle-treated striata, respectively. In stark contrast with Type I TH-eGFP neurons, Type II neurons displayed repetitive firing in response to depolarizing current injection reminiscent of fast-spiking interneurons albeit the maximal firing rate was around 150 Hz. This is much lower than striatal PV+ FSIs (Koós and Tepper, 1999). Unlike Type I neurons, Type II TH-eGFP neurons did not manifest cessation of firing even at highest amplitudes of depolarizing pulses. Moreover, Type II neurons had a considerably lower input resistance and shorter action potential duration (Fig. 4.6B) (Table 1).
Type III TH-eGFP neurons were the second most prevalent cell type (14%) in 6-OHDA-treated striatum whereas in vehicle-treated hemisphere Type III neurons comprised 6% of TH-eGFP cells. Type III TH-eGFP interneurons were characterized by significantly more hyperpolarized resting membrane potential, and lower input resistance. Moreover, Type III neurons have the highest action potential threshold among all recorded TH-eGFP neuron types. Nevertheless, this type of neurons also manifested a cessation of spiking at higher amplitude depolarizing current injections similar to the Type I neurons (Fig. 4.6C) (Table 1).

Interestingly, Type IV neurons were only encountered in the control hemisphere, and they were characterized by low-threshold spiking bursts riding on a depolarization wave appearing both at the offset of hyperpolarizing pulses and suprathreshold current injections (Fig. 4.6D, please refer to the blue trace for LTS component) (Table 1). All these descriptions of the intrinsic electrophysiological properties of the TH-eGFP neurons are in good agreement with those originally reported by Ibáñez-Sandoval et al., (2010) and did not differ between control or dopamine-denervated striatum, except plateau potentials and spontaneous synaptic events that will be discussed in

A MANOVA indicated that the identified cell types differed from each other in terms of resting membrane potential ($V_{rest}$ (F (3, 75) = 3.479, p = 0.02), and input resistance ($R_{input}$ (F (2, 43) = 6.234, p= 0.001). Specifically, Type III TH-eGFP neurons had significantly more negative resting membrane potentials than Type I and IV neurons (LSD (Type I-Type III)=3.92, p=0.003; LSD (Type III-Type IV)=−5.98, p=0.011). Type III neurons also had significantly lower $R_{input}$, Type I neurons
(LSD_{input} (Type I-Type III) =379.11, p<0.0001) (Table 4.1) The main effect of lesion did not reach statistical significance for any passive and active membrane properties except plateau potential and spontaneous synaptic events (Table 1 and see below).

6-OHDA lesions of the substantia nigra altered the proportional distribution of the 4 TH-eGFP interneuron types ($\chi^2 (3) = 16.97, p=0.001$). A significantly higher proportion of Type III TH-eGFP neurons were found in dopamine-depleted striatum and type IV neurons were found only in vehicle treated striatum (Fig. 4.7). These results imply that TH-eGFP interneurons respond to loss of striatal dopamine by increases in number that appear to be mostly of Type I TH-eGFP interneurons.

![Image](image.png)

Figure 4.7. The proportions of different electrophysiological subtypes of striatal TH-eGFP interneurons ipsilateral to intranigral vehicle or 6-OHDA infusion. The proportions of different subtypes of TH-eGFP interneurons vary significantly between the groups (p<0.01)

4.6 Effect of 6-OHDA lesion on the plateau potentials in striatal TH-eGFP interneurons
There was a significant decrease in the percentage of TH-eGFP neurons that expressed plateau potentials in the dopamine-depleted striatum (70% in vehicle-treated STR vs 44% in lesioned STR, $X^2=3.625, p<0.05$). Furthermore in those cells that expressed plateau potentials (Fig. 4.8A, 4.8B) the plateau decay time was significantly smaller in ipsilateral to the nigrostriatal lesion compared to vehicle-treated striatum ($6$-OHDA= 544 ±62.4 msec, Vehicle= 1244±291 msec; $t (37)= 2.392, p=0.025$) (Fig. 4.8D) although their amplitudes did not differ ($6$-OHDA=22.85±1.8 mV, Vehicle=23.92±2.4 mV); $t(33)=0.351, p=0.728$) (Fig. 4.8C). Furthermore, in TH-eGFP neurons ipsilateral to vehicle treatment, spontaneously occurring plateau potentials were observed interrupting spontaneous firing (Fig. 4.8E).

**Figure 4.8. Plateau potentials in striatal TH-eGFP interneurons** (A) Plateau potential in a striatal TH-eGFP neuron from vehicle-treated side in response to +100 pA somatic current injection for 0.5 sec. (B) Plateau potential in striatal TH-eGFP neuron from 6-OHDA-treated side in response to +100 pA somatic current injection for 0.5. Inset shows the same neuron in expanded time scale. (C) Plateau potentials in striatal TH-eGFP interneurons from 6-OHDA and vehicle-treated sides did not differ in terms of amplitude. (D) Plateau potentials in striatal TH-eGFP interneurons ipsilateral to intranigral 6-OHDA treatment side is significantly shorter in duration in comparison to plateau potentials in striatal TH-eGFP interneurons from vehicle-treated side, $p<0.05$. (E) In the vehicle-treated side, in a subset of TH-eGFP interneurons spontaneously generated plateau potentials were also observed interrupting spontaneous firing, as indicated with the arrow.
The same differences were obtained in recordings using a cesium-based intracellular solution containing the membrane impermeable voltage-dependent Na+ channel blocker QX-314 (Fig. 4.9): TH-eGFP neurons from the vehicle-treated striatum (Fig. 4.9A) exhibited plateau potentials significantly longer in duration than ipsilateral to the 6-OHDA treatment (Fig. 4.9B) (6-OHDA=920±185 msec, Vehicle=1765±374 msec; t=2.197, p=0.048) (Fig. 4.9D) although the plateau potential amplitudes did not differ (Fig. 4.9C).

Figure 4.9. Ionic conductances underlying plateau potentials in TH-eGFP interneurons in vehicle-treated and 6-OHDA-treated striatum. Plateau potentials in striatal TH-eGFP interneurons ipsilateral to vehicle- (A) and 6-OHDA- (B) treatment recorded with Cesium-based intracellular solution containing QX-314 in response to +100 pA somatic current injection (C) Amplitude of plateau potentials did not differ in TH-eGFP interneurons from vehicle-treated and 6-OHDA-treated hemispheres. (D). Duration of plateau potentials in TH-eGFP interneurons were shorter in striatum ipsilateral to 6-OHDA compared vehicle-treated side, p<0.05.
Plateau potentials in striatal TH-eGFP interneurons were highly sensitive to L-type calcium channel blockade by bath application of nimodipine (10 µM, Sigma) (n=3, paired t-test=5.44, p=0.0016) (Fig. 4.10A, B). Taken together, these findings show that the plateau potentials in TH-eGFP neurons from dopamine-lesioned striatum rely on same ionic conductances as in intact striatum (Ibáñez-Sandoval et al., 2010). L-type calcium channels activate a calcium activated non-specific cationic conductance (I_{CaN}) which uses Na+ from TTX/QX-314)-insensitive persistent sodium channels as the charge carrier (Lee and Tepper, 2007). The plateau potential is a key electrophysiological characteristic in striatal TH-eGFP neurons that is particularly sensitive to neuromodulatory regulation by endogenous dopamine.

Figure 4.10. L-type Ca2+ channel involvement in generation of plateau potentials in striatal TH-eGFP interneurons from dopamine-depleted striatum.
In striatal TH-eGFP interneurons ipsilateral to intranigral 6-OHDA treatment, (A) plateau potential generation was highly sensitive to L-type Ca2+ channel antagonism by Nimodipine (10 µM). (B) Duration of plateau potential duration was significantly attenuated after bath application of Nimodipine (10 µM), p<0.002.

4.7 Effect of 6-OHDA lesion on spontaneous synaptic inputs onto striatal TH-eGFP interneurons

Voltage clamp recordings of spontaneous excitatory and inhibitory post-synaptic currents (sEPSCs and sIPSCs, respectively) were obtained using a Cesium-based internal solution containing voltage-gates Na+ channel blocker QX-314 1 week after
unilateral 6-OHDA lesion (N_{6-OHDA}=14; N_{vehicle}=10), a time point where the most drastic changes in TH-eGFP cell counts were observed (see above). The use of a Cesium-based intracellular solution containing QX-314 masked many of the passive and active membrane properties used in identification of the electrophysiological subtype of the TH-eGFP interneuron, therefore for the voltage-clamp experiments the electrophysiological subtype of TH-eGFP interneurons were not ascertained.

We found a significant increase in the frequencies of both sEPSCs (Fig. 4.11A and B) and sIPSCs (Fig. 4.12A and B) in TH-eGFP neurons after 6-OHDA lesion (sEPSC: 6-OHDA= 2.93 ± 0.66 Hz, Vehicle= 1.20± 0.32 Hz; t=2.371, p=0.033 and sIPSC: 6-OHDA= 2.19±0.37 Hz, Vehicle=1.02±0.25 Hz; t=2.567, p=0.019, respectively). However, the amplitudes (Fig. 4.11C and Fig. 4.12C) did not change (sEPSC: 6-OHDA= 16.2±2.49 pA, Vehicle= 14.25±2.60 pA; t=0.448, p=0.664 and
sIPSC: 6-OHDA = 9.21±0.6 pA, Vehicle = 10.04±1.3 pA; t=0.392, p=0.702, respectively).

The rise time constant ($\tau$) of sEPSCs of TH-eGFP neurons ipsilateral to the 6-OHDA lesion was significantly shorter than in the vehicle-treated hemisphere (t-test ($\tau_{\text{sEPSC Rise}}$) = 6.523, p<0.0001) (Fig. 4.13A, 4.13B). There was no significant difference between decay time constants of sEPSCs (Fig. 4.13C) and rise and decay time constants of sIPSCs in TH-eGFP interneurons from dopamine lesions in the vehicle-treated striatum (t-test ($\tau_{\text{sIPSC Rise}}$) = 0.136, p=0.892; t-test ($\tau_{\text{sIPSC Decay}}$) = 0.53, p=0.958) (Fig. 4.14B and 4.14C). In addition, the coefficient of variation of inter-event intervals (CV$_{\text{IEI}}$) of sEPSCs (Fig. 4.13D and E) but not sIPSCs (Fig. 4.14D) in lesioned striatum TH-eGFPs was significantly smaller than vehicle-treated striatum (t-test (CV$_{\text{IEI sEPSC}}$) = 2.83, p=0.017). This finding shows that the intervals between sEPSCs recorded in TH-eGFP interneurons from dopamine

Figure 4.12. Spontaneous IPSCs in striatal TH-eGFP neurons. (A) Sample traces from 3 separate cases from 1 wk post-vehicle and 6-OHDA treated hemispheres. Scale bars 5 pA, 0.5 sec (B) Frequency of spontaneous EPSCs increase significantly in striatal TH-eGFP interneurons after 6-OHDA treatment, p<0.05 (C) Amplitude of spontaneous EPSCs in striatal TH-eGFP interneurons do not differ in 6-OHDA treated side.
depleted-striatum show less variability. Such a reduction in CV of IEIs may indicate that there is an increase in firing regularity of excitatory afferents synapsing onto TH-eGFP neurons.

![Figure 4.1](image-url)

**Figure 4.13. Kinetics of spontaneous EPSCs in striatal TH-eGFP neurons.** (A) Single sEPSCs in TH-eGFP neurons isolated from 60sec of recordings from 1 wk post-vehicle vs 6-OHDA treated hemispheres. Gray traces show the individual events and averaged traces are shown in black (vehicle -treatment) and red (6-OHDA treatment) and in overlay (B) Rise time constant of sEPSCs in TH-eGFP neurons is significantly smaller in 6-OHDA treated side vs vehicle-treated side, p<0.0001 (C) There was no statistical difference in decay time constants. (D, E) Inter-event intervals and CV of IEIs were significantly smaller after 6-OHDA treatment in TH-eGFP neurons, p<0.05.

Taken together changes in spontaneous synaptic currents recorded in TH-eGFP interneurons show that there is an increase in frequency of both sEPSCs and sIPSCS after dopamine depletion. However, the kinetic properties of only sEPSCs, but not sIPSCs, change in TH-eGFP interneurons in dopamine depleted striatum indicating that sEPSCs in TH-eGFP interneurons are subject to both pre-synaptic and post-synaptic changes following striatal dopamine loss.
4.8 Morphological changes in TH-eGFP neurons after dopamine depletion

Biocytin filling of electrophysiologically identified subtypes of TH-eGFP neurons from striata ipsilateral to intranigral 6-OHDA and vehicle treatment showed that TH-eGFP interneurons had the same morphological characteristics as in intact mice (Ibáñez-Sandoval et al., 2010, Tepper et al., 2010) (Fig. 4.15A, B, C, D). All four types of TH-eGFP interneurons possessed medium-sized somata (range: 10.7 µm-14.1 µm) that gave rise to 2-4 sparsely spiny primary dendrites. The axon initial segment emerged in some cases directly from the soma and in remaining cases from one of the primary dendrites (Fig. 4.15A, B, C, D). As the number of cases for Type II, III and Type IV TH-eGFP interneuron subtype from dopamine-depleted versus vehicle-treated hemisphere were low, morphological comparisons were done on Type
I striatal TH-eGFP interneurons from dopamine-depleted (n=11) and vehicle-treated hemispheres (n=8) (see below).

Figure 4.15. Reconstructions of different types of TH-eGFP interneurons. Reconstructions of different types of TH-eGFP interneurons recorded from striatum ipsilateral to intranigral 6-OHDA (A, B, C) or vehicle infusion (D). (A) Type I TH-eGFP neuron (B) Type II TH-eGFP neuron, (C) Type III TH-eGFP neuron (D) Type IV TH-eGFP neuron. Insets show the epifluorescent image of the reconstructed neuron taken during recording. Soma and dendrites are indicated in blue, axonal arborization is shown in red. Scale bars 50 µm.

Neurolucida reconstructions of 11 Type I striatal TH-eGFP interneurons from 3 days to 1 week 6-OHDA ipsilateral to intranigral 6-OHDA infusion (Fig. 4.16B) were compared to 8 Type I TH-eGFP neurons ipsilateral to intranigral vehicle infusion (Fig. 4.16A).
**Figure 4.16. Morphological changes in TH-eGFP interneurons after striatal dopamine depletion.**

(A, B) Reconstructions of representative striatal Type I TH-eGFP neuron ipsilateral to vehicle- or 6-OHDA- treatment. (A1, B1 and B2) Inset shows photomicrograph taken at a single z-depth of the biocytin-NiDAB image of the reconstructed neuron at the indicated sites. Quantification of dendritic length (C), dendritic branching (D) and dendritic spine density (E) by Sholl analysis. (E) Dendritic spine density in TH-eGFP neurons from dopamine-depleted striatum showed a significant increase within 100 µm perimeter of the soma. Soma and dendrites are indicated in blue, axonal arborization is shown in red. Scale bars, 50 µm, * p<0.05.
Sholl analyses were performed for dendritic arborizations by superimposing concentric circles originating from soma at 10 µm intervals. In terms of dendritic arborization, MANOVA indicated the absence of significant effects of 6-OHDA lesion on dendritic length or branching (F=1.447, p=0.233 and F=0.068, p=0.795, respectively) (Fig. 4.16C and 4.16D) but there was a strong effect on the density of spine-like formations on dendritic trees (Fig. 4.16E)(F=12.038, p=0.001), particularly between 30 to 180 µm from the soma. Finally, in terms of the soma measurements of TH-eGFP neurons none of the variables tested (perimeter, area, aspect ratio and roundedness) show difference in lesioned vs. vehicle-treated striatum (p=0.957; p=0.446; p=0.498; p=0.638).
Chapter 5

Discussion
5.1 OVERVIEW

The experiments undertaken in this thesis were driven with the hypothesis that striatal interneurons are important sites of action for striatal dopamine and show discernible changes electrophysiologically and morphologically when the striatum is deprived of dopamine. I have focused on a particular class of striatal interneuron namely, TH+ interneurons that were identified in acute or fixed striatal slices for electrophysiological and anatomical experiments, respectively with the aid of eGFP expression under the control of TH promoter in transgenic mice.

In the first set of experiments, initially the locations of different electrophysiologically defined TH-eGFP subtypes within the striatal volume were determined. Second, striatal TH-eGFP neurons were mapped with respect to striosome and matrix compartments defined on the basis of MOR immunofluorescence. Finally, a small subset of TH-eGFP neurons was recorded in different compartments as identified by the intensity of TH-eGFP-TH fluorescence in young mice. Our results show that electrophysiologically identified subtypes are equally prevalent in all regions of the striatum. However, when the finer level anatomical organization of the striatum is taken into account, a different pattern emerged in terms of TH-eGFP neuron distribution in the dorsal-ventral axis of the striatum. In the dorsal striatum, TH-eGFP neurons are diffusely spread out with most of them residing in the matrix compartment as expected from proportion of patch/striosome over matrix domain whereas, in the ventral striatum, there is a disproportionately higher presence of TH-eGFP neurons in MOR-enriched regions.
In the second set of experiments, anatomical and electrophysiological changes in striatal TH-eGFP interneurons were investigated following nigrostriatal dopamine denervation by unilateral 6-OHDA lesion. Our results revealed the occurrence of significant electrophysiological and morphological changes as a result of dopamine denervation, and suggest that striatal TH-eGFP interneurons depend on endogenous dopamine derived from the nigrostriatal pathway for maintenance of their normal electrophysiological and morphological characteristics.

Our previous anatomical characterizations indicated that there is a resident population of around 3000 striatal TH-eGFP neurons per hemisphere and none were found to be projecting to substantia nigra pars reticulata, or to the globus pallidus external segment- the targets of the direct and indirect pathway SPNs, respectively (Ibáñez-Sandoval et al., 2010). Here, in intact and vehicle-treated striatum, we have obtained stereological estimates that were in good agreement with the numbers reported in Ibáñez-Sandoval et al., 2010. After nigrostriatal dopamine denervation, only a marginal but significant increase (~30%) in numbers of striatal TH-eGFP was detected. As in intact mice, in unilaterally dopamine-denervated mice none of the TH-eGFP neurons were projecting to GPe or SNr. We have observed the same electrophysiological heterogeneity in TH-eGFP neurons from dopamine-depleted striatum as formerly reported by Ibáñez-Sandoval et al. (2010) in intact striatum. Although most of the electrophysiological parameters did not show any changes following striatal dopamine loss, we have found a significant reduction in proportion of TH-eGFP cells showing plateau potentials and a significant reduction in plateau potential duration. Finally, a significant increase in number of dendritic spine-like appendages was observed in TH-eGFP neurons following striatal dopamine loss.
which was accompanied by a significant increase in frequency of spontaneous excitatory and inhibitory evens. Taken together, these findings bolster previous findings that TH-eGFP neurons are an electrophysiologically heterogeneous cluster of striatal interneurons and in our hands we found no morphological and electrophysiological resemblance of these interneurons to striatal SPNs or to other groups of striatal interneurons in dopamine-intact or in dopamine-depleted striatum. Striatal TH-eGFP interneurons show particular anatomical and electrophysiological changes after dopamine-depletion that further sets them apart from other striatal neurons. In conclusion, these results collectively suggest that TH-eGFP interneurons may play an important compensatory role not by virtue of dopamine synthesis as previously thought (Xenias et al., 2012) but by being differentially recruited as a source of GABAergic feedforward inhibition onto SPNs in the dopamine-depleted state.

5.2 Distribution of TH-eGFP neurons with respect to anatomical heterogeneity of the neostriatum

The first set of experiments was undertaken to investigate the anatomical distribution of striatal TH-eGFP interneurons. In the first part, anatomical mapping done with electrophysio logically identified and biocytin-filled TH-eGFP neurons indicated that none of the TH-eGFP subtypes showed a dorsal-ventral or anterior-posterior patterning. In the second set, electrophysio logically unidentified TH-eGFP neurons were stereologically sampled and mapped with respect to the density of MOR immunolabeling in the striatum. The results indicate that there is a selective distribution of TH-eGFP cells in the MOR-enriched domains in ventral striatum but not in dorsal striatum. TH-eGFP neurons were found in ventral striatal MOR-enriched
domains 1.6 times more frequently than what would have been expected by chance. The current results do not indicate a distribution gradient of the different subtypes of TH-eGFP neurons across the dorsal-ventral divisions of the striatum. However, cortical and local stimulation experiments and paired-recordings indicate that TH interneurons are well-integrated elements of striatal circuitry (Ibáñez-Sandoval et al., 2010). Therefore, the difference in anatomical localization with respect to MOR-enriched domains in the dorsal and ventral striatum suggests that striatal TH interneurons may differentially influence local network dynamics in ventral versus dorsal striatum.

5.3 Why is the anatomical distribution of Th-eGFP interneurons different in the dorsal and ventral striatum?

What is the significance of these differences in anatomical localization? The clues to the answers may lie within the rules that govern the developmental organization of the dorsal and ventral striatal divisions. It has been shown that striatal SPNs and PV-expressing interneurons follow different timelines for maximal embryonic birthrate and post-mitotic maturation in dorsal striatum versus nucleus accumbens (Sadikot and Sasseville, 1997). Furthermore, van Vulpen and van der Kooy (1998) found a curious relationship between birthdates and compartmental localization of cholinergic interneurons: the earliest born cholinergic neurons were more likely to be situated in the striosome compartment whereas later born cholinergic neurons had a higher likelihood to wind up in the matrix. It is possible that ventral striatal TH-eGFP neurons have earlier birthdates than dorsal striatal ones, which may account for different compartmental localization of these neurons across the dorsal-ventral axis of the striatum. Currently, the only available findings from a developmental stance come
from the early postnatal maturation of striatal TH+ neurons (Busceti et al., 2008), which indicates the preferential localization of striatal TH+ neurons in close vicinity of pioneering dopaminergic nigrostriatal fibers forming the dopamine islands. Further experiments are needed to examine the embryonic development and anatomical migratory patterns of TH-eGFP neurons.

Apart from the possibility that there are differences in birthdates, one also must take into account the fact that compartmental organization reflects a complex molecular patterning requiring the orchestrated action of multiple molecules including retinoic acid (Liao et al., 2008), notch signaling (Mason et al., 2005), certain transcription factors (Arlotta et al., 2008) cell adhesion (Redies et al., 2002) and guidance molecules (Hamasaki et al, 2001, Janis et al., 1999). Differential sensitivity to these factors in dorsal and ventral striatum may have given rise to the differences in compartmental localization of TH-eGFP interneurons. In particular, it has been shown that the repulsive action of netrins is integral for proper migration of matrix-bound neuronal precursors (Hamasaki et al., 2001). Subsequently, it was demonstrated that netrin-1 shows a pronounced ventral to dorsal gradient, and that cholinergic interneurons co-expressing netrin-1 largely fall within the ventral striatum (Schatzmiller et al., 2008). The same study also found that SOM-expressing GABAergic interneurons co-localized netrin-1 whereas PV and CR-expressing GABAergic interneurons did not. Since PV and CR interneurons do not show compartmental preferences within the striatum (Cowan et al., 1990; Rymar et al., 2004) and cholinergic and NPY-SOM neurons respect compartmental boundaries (Kubota and Kawaguchi, 1993; Martone et al., 1994; Saka et al., 2002), the possibility arises that expression of netrins and/or netrin receptors by TH-eGFP interneurons may
be a determining factor of the compartmental localization in the dorsal and ventral striatum.

Although the current results show that there is a disproportionately higher number of TH-eGFP interneurons in MOR enriched domains of the ventral striatum (~40% versus ~25% in the dorsal striatum), the majority of the TH interneurons reside in MOR-impoverished areas. A closer examination of matrix TH-eGFP neurons indicated that half of the population dwells within 100 µm or less of the striosome-matrix borders. This is consistent with results reported by Busceti and colleagues (Busceti et al., 2008) in which the majority of striatal TH neurons clustered near the pioneering nigral dopamine fibers commonly referred as “dopamine islands”, which are destined to form striatal striosomes in the adult (Graybiel et al., 1981; Miura et al., 2007; Miura et al., 2008).

These data suggest that even if they do not reside in striosomes, striatal TH neurons may still participate in intercompartmental communication in a manner similar to that of cholinergic and NPY interneurons (Kawaguchi, 1992; Kubota and Kawaguchi, 1993; Saka et al., 2002). In order to better understand such a functional role full reconstructions of axonal and dendritic arborization of biocytin-filled TH-eGFP neurons with respect to striosome and matrix compartments in mature mice are necessary.
5.4 Subtype-specific localization of TH interneurons to TH-eGFP patches / striosomes in juvenile striatum

In terms of the overall anatomical distribution of electrophysiological subtypes of TH-eGFP interneurons, no preferential regional localization was observed. However, one must bear in mind that there were only a few examples from some electrophysiological subtypes available for anatomical mapping. With an increased number of cases representing each TH-eGFP interneuronal subtype, a clearer picture of any preferential anatomical distribution might be revealed. Still, the preliminary results in this thesis in which electrophysiological characterization of TH-eGFP neurons was carried out along with simultaneous compartmental localization based on TH fiber intensity in young TH-eGFP mice (n=10 neurons) suggest that there is chance that subtypes are localized in different compartments. It is interesting that eight of 10 such labeled neurons in TH-rich islands displayed characteristics of Type I neurons, the most abundant electrophysiological subtype (>50% of entire recorded population, Ibáñez-Sandoval et al., 2010), whereas the remaining two somata that were located in the TH-poor zones displayed the phenotype of Type IV neurons, the second most prevalent subtype (~25%, Ibáñez-Sandoval et al., 2010). We would have expected to see just the opposite – the more abundant phenotype present in the more abundant striatal matrix compartment - if indeed TH-fiber intense zones correspond to striosomes as suggested (Miura et al., 2007; 2008). The mice from which these recordings and biocytin labeling obtained were aged between PN30-P33, and it is possible that this is a developmental range in which differences in TH fiber density could be detected but compartments had already started maturing. Thus, TH-fiber rich zones in PN30-33 mice might correspond to the matrix whereas TH-poor(er) zones might be the striosome precursors. It has been documented in adult cat, monkey and
human brains that striosomes show reduced TH immunoreactivity (Graybiel et al., 1987). Indeed, in the MOR-immunolabeled striatal sections from adult TH-eGFP mice (>3 months), we have noticed that the dorsal striatal zones showing a lower TH-eGFP fiber density consistently overlapped with the MOR-enriched domains (Fig. 3.2A).

Therefore, it can be argued that depending on the degree of post-natal maturation, TH fiber-enriched domains in TH-eGFP mice may correspond to patches/striosomes or matrix. However, in either case, juvenile or fully mature, the intensity of the TH-eGFP fiber background can be a distinguishing feature of striatal compartments in these mice. It is interesting to note that at least for Type I and Type IV neurons, striatal TH-eGFP interneurons have processes that cross the boundaries between the TH-enriched regions and the rest of the striatum. To the extent that the TH-enriched regions are in fact the precursors to the adult striosomes, this finding indicates that the axons and dendrites of striatal TH interneurons mediate intercompartment communication.

5.5 TH-eGFP interneurons, the striosome-matrix mosaic and striatal function

Although the exact significance of the mosaic organization of striatum is poorly understood, there is evidence that each compartment may be involved in different aspects of striatal functioning. For instance, the matrix was found to show higher metabolic responses as a result of unrestrained activity, somatosensory stimulation and brief restraint (Brown et al., 2002). In addition, concurrent dopamine receptor stimulation, cocaine/apomorphine induced stereotypy, and behavioral sensitization lead to differential activation of striatal compartments as measured in terms of early
immediate gene responses (Saka et al., 2002). Furthermore, it has been shown by targeted ablation of NPY-SOM\textsuperscript{+} and cholinergic populations using a saporin toxin conjugate against a tachykinin receptor subtype that these interneurons are essential for compartmentally distinct patterns of activation to emerge in the striatum (Saka et al., 2002). Therefore, striatal TH interneurons may be working in parallel with NPY-SOM and cholinergic interneurons in the establishment of regionally different striatal activity patterns. Further anatomical and electrophysiological studies are imperative to determine whether there is a special relationship among these interneurons across striatal compartments.

Finally, the current anatomical results will be essential to interpret commonly reported changes in striatal TH-expressing neuron number following experimentally-induced or disease-related loss of striatal dopamine (Betarbet et al., 1997; Dubach et al., 1987; Jollivet et al., 2004; Mao et al., 2001; Meredith et al., 1999; Mazloom and Smith, 2006; Palfi et al., 2002; Tande et al., 2006; Tashiro et al., 1989a; 1989b). Whether the appearance of "novel" TH neurons as a result of dopamine loss follows a distinct anatomical pattern would be very interesting to uncover, as striatal compartments were shown to display separable degrees of susceptibility to toxins and disease-related degeneration (Granado et al., 2010; Lawhorn et al., 2008; Sato et al., 2008). The present findings indicate that TH-eGFP neurons follow a non-random, distinct pattern of distribution across striosome-matrix compartments throughout the dorsal-ventral axis of the striatum. These results provide further impetus for future studies as to whether and how TH-eGFP neurons may affect striatal information processing based their anatomical location.
5.6 Anatomical and electrophysiological changes in striatal TH-eGFP interneurons following 6-OHDA lesion of the nigrostriatal dopaminergic pathway

The main finding of the present set of experiments is that the electrophysiological and anatomical characteristics of striatal TH-eGFP neurons depend on the presence of endogenous nigrostriatal dopamine. There was a 30% overall increase in the number of TH-eGFP neurons after unilateral nigrostriatal 6-OHDA lesion, due largely to an increase in the number of TH-eGFP neurons in the vSTR. The increase in TH-eGFP neuron numbers was transient, and progressively declined back to control levels within 2 weeks following the lesion.

The lack of any retrograde labeling from GPe and SNr in TH-eGFP neurons after dopamine depletion, confirms previous findings that TH-eGFP neurons comprise a group of striatal GABAergic interneurons in intact eGFP mice (Ibáñez-Sandoval et al., 2010). The present data also show that it is highly unlikely that the increased number of TH-eGFP neurons seen after dopamine depletion arise from a novel expression of TH by spiny projection neurons (but see Darmopil et al., 2008).

As in intact TH-eGFP animals (Ibáñez-Sandoval et al, 2010), we identified four distinct electrophysiological subtypes of striatal TH-eGFP interneurons. The relative distribution of these subtypes was altered after dopamine depletion. Passive and active membrane properties were largely unaffected by 6-OHDA lesion, although there were significant reductions in the proportion of TH-eGFP interneurons expressing plateau potentials and in the duration of plateau potentials. In addition after 6-OHDA lesion, the frequency of spontaneous EPSCs and IPSCs in TH-eGFP interneurons increased,
as did the density of spine-like appendages on TH-eGFP interneurons. The proposed mechanism of these changes are discussed in the following sections.

5.6 Changes in the number of striatal TH-eGFP interneurons after dopamine denervation

Following unilateral intranigral infusion of 6-OHDA, there was an overall increase of 30% in the number of TH-eGFP neurons, which was mostly due to an increase in TH-eGFP neurons in the vSTR. The increased number of TH-eGFP neurons from the dopamine-depleted striatum progressively declined back to control levels within 2 weeks following the lesion. This is a much smaller increase in the number of striatal TH neurons than that previously reported by others following 6-OHDA lesions, which are in the range of 2-4 fold (Darmopil et al., 2008; Huot and Parent, 2007; Huot et al., 2007; Tande et al., 2006). We hypothesize that this is due to the greatly increased sensitivity of the BAC transgenic TH-eGFP compared to TH immunocytochemistry (Ibáñez-Sandoval et al., 2010).

The numbers of TH-expressing interneurons in the intact striatum that we report here and in Ibáñez-Sandoval et al. (2010) are significantly higher than those reported from studies that relied on immunocytochemical detection of TH (Betarbet et al., 1997; Darmopil et al., 2008; Dubach et al., 1987; Jollivet et al., 2004; Mao et al., 2001; Meredith et al., 1999; Mazloom and Smith, 2006; Palfi et al., 2002; Tande et al., 2006; Tashiro et al., 1989a; 1989b). This is likely due to the much greater detection sensitivity of using BAC TH-eGFP as a marker for TH-expressing neurons than immunocytochemistry. Indeed, in control TH-eGFP mice, it was necessary to
treat mice with colchicine to get reliable TH immunofluorescence in most TH-eGFP fluorescent interneurons, indicating that under normal or control conditions, the levels of the protein in the soma were below the detection limits of immunocytochemistry (Ibáñez-Sandoval et al., 2010).

In the present case, 1 week following dopamine depletion, about 15% of the TH-eGFP interneurons showed immunofluorescence for TH, a much greater proportion than seen in controls (without colchicine). This indicates that the DA depletion increased the amount of TH protein in the TH-eGFP interneurons. The increase in the number of TH-eGFP interneurons indicates that this was due, at least in part, to increased transcriptional activity of the TH gene, since this is what eGFP expression monitors (Gong et al., 2003). This suggests that the reason for the apparently smaller increase in the number of TH interneurons following dopamine denervation in our study is due to the increased sensitivity of the eGFP reporter compared to TH immunocytochemistry. That is, we suggest that the vast majority of the “new” TH immunoreactive neurons reported by others were already being detected by the more sensitive eGFP reporter in our mice.

Most of the increase in the TH-eGFP population occurs in the ventral striatum during the first week of dopamine loss. This is somewhat puzzling since the ventral striatum receives dopamine input primarily from the VTA, which is only partially lesioned in our paradigm (Fig. 4.1). Striatal TH+ interneurons may have an intimate relationship with TH afferents from midbrain. Busceti et al. (2008) showed that striatal TH+ somata are abundant between post-natal days (PN) 1-8: when TH fibers are limited to dopamine islands, and undergo a reduction in numbers between PN 8-
16 when the second wave of dopaminergic afferents begin to innervate the matrix. In addition, Darmopil et al. (2008) reported the emergence of TH+ neurons in regions that showed partial TH+ fiber loss but not in areas undergoing total TH+ fiber loss following 6-OHDA. It was hypothesized earlier that surviving dopaminergic fibers may exert trophic effects in the striatum (Huot and Parent, 2007).

In striatal cultures, it has been shown that GFAP and BDNF promote TH expression (Du and Iacovitti, 1995; Du et al., 1995). Thus, it is important to determine the nature of changes within surviving population of midbrain dopaminergic neurons to evaluate the changes in the striatum. For instance, using Aphakia mice, which lack the homeobox transcription factor, Pitx3, (essential for the formation of SNc dopamine neurons), Smits et al. (2005) found a significant increase in the activity of remaining VTA dopamine neurons. This study indicates that compensatory changes take place within the midbrain dopaminergic population when there is perturbation to the developmental plan. Thus, determining whether surviving VTA dopamine neurons also show transient or permanent changes in neural activity and/or neurotrophin expression soon after lesioning would help us understand better the concomitant changes in striatum.

Our results support the hypothesis that the TH-eGFP interneurons that appear following dopamine depletion are already present in the striatum prior to dopamine denervation, but simply begin expressing TH or increased levels of TH after lesioning. Tande et al. (2006) and Darmopil et al. (2008) failed to find 5-bromo-2-deoxyuridine incorporation in TH immunoreactive neurons following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment in monkeys or 6-OHDA
injections in mice, despite finding an increase in TH+ cell number. Co-expression of GAD-67 and TH, and previous electrophysiological studies also suggest strongly that these neurons are part of an already existing inhibitory neural population within the striatum (Meredith et al., 1999; Cossette et al., 2005b; Mazloom and Smith, 2006; Ibáñez-Sandoval et al., 2010). However, since we did not find any colocalization of TH-eGFP with PV or NPY, and only a marginal overlap with CR, we maintain that most or all of the TH-eGFP or TH+ interneurons that “appear” after lesion represent striatal TH neurons that were always present but simply express greater levels of TH or eGFP due to increased transcriptional activity of the TH gene (see below).

5.7 Electrophysiological changes in striatal TH-eGFP interneurons after 6-OHDA lesion

The relative abundance of the different TH-eGFP subtypes differed between lesion and vehicle conditions. Type IV neurons which are characterized by low-threshold spiking and make up ~20-25% of TH-eGFP population in the intact striatum (Ibáñez-Sandoval et al., 2010) were absent following unilateral nigral 6-OHDA at all 4 time points investigated. Parallel to this, we found that Type I neurons made up ~80% of the electrophysiologically identified cells. These findings suggest that there is a phenotypic conversion within the TH-eGFP interneuronal population, possibly due to altered ionic conductances, as a result of sustained dopamine loss.

We found a significant reduction in the proportion of TH-eGFP interneurons from lesioned mice that expressed plateau potentials. In the TH-eGFP interneurons that did express plateau potentials, their duration was significantly shorter on the dopamine-depleted side although the amplitudes did not differ. In some cases from control
striatum, we encountered spontaneous activity interrupted by spontaneously generated plateau potentials, something that was never observed in the dopamine-depleted side. Similar activity was observed when cells were patched with a cesium-based internal solution containing the voltage gated Na+ channel blocker, QX-314, but this was completely abolished by L-type channel blockers. These findings demonstrate that the plateau potentials are triggered by Ca2+ influx through L-type channels (Ibáñez-Sandoval et al., 2010), which in turn activates $I_{\text{CAN}}$ that uses Na+ influx from TTX-resistant persistent Na+ channels as the charge carrier (Lee and Tepper, 2007). Plateau potentials in TH-eGFP interneurons are potentiated by amphetamine (Ibáñez-Sandoval et al., 2010) consistent with the findings reported here that ionic conductances underlying the plateau potential are strongly modulated by endogenous dopamine.

5.8 6-OHDA-induced alterations in spontaneous EPSCs and IPSCs in TH-eGFP interneurons

There was a significant increase in the frequency of spontaneous EPSCs and IPSCs in TH-eGFP interneurons after 6-OHDA lesion. Interestingly, we found an unexpected significant reduction in the CV of excitatory events, which suggests that presynaptic excitatory drive may undergo significant changes after dopamine depletion. Dejean et al., (2012) recently showed increased coherence between cortex and striatum emerging around 1 week following 6-OHDA lesions. In addition, reduction in neural activity in the centromedian/parafascicular complex of the thalamus (Yan et al., 2008) and an increase in the density of corticospinal synapses was noted in MPTP treated primates (Raju et al., 2008). Changes in sEPSCs and
sIPSCs in striatal TH-eGFP interneurons after dopamine-depletion are further discussed in the next section within the context of morphological alterations.

5.9 Morphological changes in striatal TH-eGFP interneurons after 6-OHDA lesion

The only previous report on the detailed morphology of TH-eGFP interneurons following biocytin labeling indicated that these neurons are small- to medium-sized and very sparsely spiny neurons (Ibáñez-Sandoval et al., 2010), in agreement with the majority of reports on striatal TH-immunoreactive neurons (Betarbet et al., 1997; Darmopil et al., 2008; Dubach et al., 1987; Jollivet et al., 2004; Mao et al., 2001; Meredith et al., 1999; Mazloom and Smith, 2006; Palfi et al., 2002; Tande et al., 2006; Tashiro et al., 1989a; 1989b). There was no resemblance of these neurons to SPNs in intact or dopamine-lesioned striatum electrophysiologically or morphologically. Furthermore, no retrograde labeling from GPe and SNr was evident in TH-eGFP cells in 6-OHDA-treated and vehicle-treated striata. However, there are two reports arguing that a proportion of striatal TH+ neurons also show immunoreactivity for enkephalin, dynorphin, calbindin (Darmopil, 2008) and DARRP-32 (Masuda et al., 2011) -markers for striatal projection neurons-. In Darmopil et al., (2008) colocalization of TH immunoreactivity with enkephalin, dynorphin and calbindin was detected after L-DOPA treatment in dopamine denervated striatum which may involve additional phenotypic conversion. Masuda et al., (2011), on the other hand, derived TH-eGFP mice differently than the GENSAT mice used here and by Ibáñez-Sandoval et al., (2010). Furthermore, they restricted the age of the mice they were using to <1 month post-natal whereas we only used mice >2 months old since we waited until mice reached 20 gr body weight for 6-OHDA
surgeries. Another reason we refrain from using mice younger than this age is because nigrostriatal dopaminergic patterning, functional maturation of cortical and thalamic afferents is not complete by the end of 1st month post-natally (Tepper et al., 1998).

Somatic size, dendritic length and branching remained unchanged in TH-eGFP interneurons after 6-OHDA lesion, However, there was a significant increase in the number of proximal dendritic spine-like appendages on TH-eGFP interneurons following 6-OHDA lesion. The maximal increase in density of dendritic spine-like appendages in TH-eGFP neurons (2-4 spines/10 µm on the average in TH-eGFP interneurons in vehicle-treated side versus 7-8 spines/10 µm on the average after striatal dopamine depletion (Fig. 4.16E)) occured within 50 µm radius around the soma. But even with this increase, the dendritic spine counts in striatal TH-eGFP interneurons after 6-OHDA never exceeded couple of hundreds which is considerably lower than SPNs. It is widely regarded that in the brain dendritic spines are the sites where excitatory synapses form (as reviewed in Yuste and Bonhoeffer, 2004). Parallel to the increase in spine-like formations on TH-eGFP interneurons after striatal dopamine loss, there was a significant increase in frequency of sEPSCs in striatal TH-eGFP interneurons ipsilateral to intranigral 6-OHDA treatment. The time constant of sEPSCs of TH-eGFPs were significantly shorter in the dopamine-depleted side. Taken together with the proximal appearance of spine-like formations, faster rise kinetics of sEPSCs in TH-eGFP interneurons after dopamine-depletion can be argued to be the result of reduced electrotonic attenuation for proximally arriving synaptic input in somatic recordings (e.g. Gustafson et al., 2006; Maccaferri et al., 2000). In addition to proximally appearing dendritic spines, change in post-synaptic glutamatergic receptor composition can also be responsible for faster rise kinetics of sEPSCs. In fact, in the
developing hippocampus it was found that NR2B-NR2D subunits of the NMDA receptor are associated with slower EPSC kinetics and NR2A subunit confer faster EPSC kinetics (Kirson and Yaari, 1996; Ye et al., 2005). Similar possible changes in NMDA receptor composition in TH-eGFP neurons after striatal dopamine-depletion can be an alternative source for the reduction in rise time constants of sEPSCs reported here.

In addition, the coefficient of variation of inter-event intervals of eEPSCs was significantly smaller after dopamine depletion. This suggests, that the regularity of activity of presynaptic glutamatergic drive onto TH-eGFP interneurons increases after dopamine depletion. It has recently been elegantly demonstrated by Kozorovitskiy et al., (2012) that developmental perturbation of the balance of activity between direct and indirect pathway SPNs gave rise to dramatic and persistent changes in corticostriatal innervation of the striatum. Specifically, when D1R-expressing SPNs were silenced, excitatory events diminished and spine density decreased in SPNs whereas when D2R-expressing SPNs were silenced corticostriatal activity onto SPNs increased accompanied by pronounced increase in dendritic spine density. Dopamine-depletion is known to perturb the balanced recruitment of direct and indirect projection pathways of the striatum (Kravitz et al., 2010) therefore our findings show that in addition to SPNs, dopamine depletion striatal also takes its toll in terms of electrophysiological and morphological changes in striatal interneurons in dopamine depleted basal ganglia.

But what about the increase in sIPSCs in striatal TH-eGFP interneurons after dopamine depletion? Striatal GABAergic interneurons also receive corticostriatal
innervation (Bennett and Bolam, 1993; 1994; Gruber et al., 2009; Ibáñez-Sandoval et al., 2010; 2011; Lapper et al., 1992). Therefore it is conceivable to assume the over-activity in corticostriatal innervation after dopamine-depletion also enhances GABAergic interneuron-mediated feedforward inhibition in the striatum. In line with this reasoning, Gittis et al., (2011) recently showed that striatal FSI connections onto striatonigral SPNs increase and axonal arborization of FSIs expand after striatal dopamine loss. Systematic investigations on interneuron-to-interneuron interactions are relatively few in striatum. But recent approaches using optogenetic control and along with BAC-transgenic identification of distinct striatal interneuronal elements suggest that there is a selective connectivity pattern among striatal interneurons (English et al., 2011). How dopamine depletion alters such connectivity patterns among striatal interneurons is unknown. Striatal TH+ interneurons also receive GABAergic input from SPNs (Ibáñez-Sandoval et al., 2010). Therefore further experiments are needed to determine the exact source of GABAergic inputs onto TH+ interneurons.

We suggest a common mechanism for the various changes in TH-eGFP interneurons following striatal dopamine loss. A significant decline in plateau potential expression, increased synaptic inputs onto TH-eGFP interneurons and a parallel increase in spine-like formations on TH-eGFP interneurons are reminiscent of the opposite scenario described in Day et al., (2006). In dopamine-depleted striatum, striatopallidal SPNs undergo a marked loss of dendritic spines, which is associated with loss of antagonizing effect of D2-receptor mediated inhibition on L-type calcium channel activity. Mechanistically it was suggested that there is an increase in influx of Ca2+ from L-type channels leading to activation of calcineurin, which in turn
dephosphorylates myocyte enhancer factor 2 increasing its transcriptional activity and increase expression of proteins related to synaptic remodelling, Nurr77 and Arc (Surmeier et al., 2010; Tian et al., 2010). In the case of TH-eGFP neurons, a reduction L-type channel mediated Ca2+ influx would not only reduce the plateau potential, but also calcineurin activity and reduce spine pruning drive similar to indirect pathway SPNs (Day et al., 2006), enabling the formation of new spines and presumably also synaptic inputs. Our results further signal that dopamine loss indeed wreaks havoc in striatal circuit organization, not only excitatory afferents change targets but members of the inhibitory network also form new connections while losing others (Gittis et al., 2011; Salin et al., 2009; Taverna et al., 2008).

5.10 CONCLUSION

During the last 20 years, it has been established that striatal interneurons are key players in shaping of the striatal microcircuitry and they display striking neurochemical, electrophysiological and morphological diversity although they only make up a minority of the striatal population. Although striatal projection neurons have been heavily investigated within the context of dopamine particularly what happens in the absence of dopamine in Parkinson's disease and experimental models, striatal interneurons have received limited attention so far.

There is a wealth of information regarding morphological changes in SPNs following striatal dopamine denervation. Most of them converge on the observation that there is a 20-50% decline in spine density following dopamine loss (Arbuthnott et al., 2000; McNeill et al., 1988; Day et al., 2006; Smith et al., 2009; Villalba and
Smith, 2011). Striatal interneurons have recently started to be evaluated in detail in terms of changes to synaptic connections and morphological features in hemiparkinsonian models. Following 6-OHDA lesions, synaptic connections from cholinergic and PV+ interneuron onto striatonigral SPNs decline whereas connectivity of cholinergic to striatopallidal neurons increase as measured by retrograde transport of Rabies virus by striatal interneurons from direct and indirect pathway SPNs, respectively (Salin et al., 2009). As mentioned previously, Gittis et al. (2011) also found that the probability of synaptic connections between striatopallidal SPNs and PV+ fast-spiking interneurons increased in dopamine denervated striatum which was accompanied by a dramatic sprouting of axon collateral arborization of PV+ interneurons in dopamine depleted striatum.

Our findings here overall show that striatal interneurons also undergo significant reorganization synaptically and morphologically that is different, in our case with TH-eGFP interneurons opposite, to changes taking place in SPNs. By carefully characterizing changes in components of the striatal microcircuitry we can begin to understand how balance of striatal activity is perturbed in dopamine-depleted striatum and devise ways to restore it.
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