ABSTRACT OF THE DISSERTATION

Basal forebrain topography as defined by afferents and efferents: Separable circuits to support distinct functions

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The basal forebrain (BF) is a region of heterogeneous neurons, some of which send axon terminals to the cerebral cortex. The main source of acetylcholine in the cortex arises from the cholinergic basal forebrain (BFc). Historically the BFc has been alternately described as diffuse and discrete, which has contributed to incompatible views of the system across and within scientific disciplines. The known anatomical details of the BF are not sufficient to explain the variety of functions it achieves in the cortex.

This thesis describes three experiments that further investigate the anatomical details of BF cell topography, outputs, and inputs in the rat. The first utilizes retrograde tracing to show that cells projecting to visual and motor cortices are mostly found in the anterior diagonal bands and posterior basal forebrain, respectively. The BF topography of these two projection populations partially overlaps. There is also a segregation and overlap based on neurotransmitter content. The second experiment queries the BF topography of local afferents to BFc cells via monosynaptic viral tracing in ChAT-cre transgenic rats. BFc cells do not receive afferents from fellow BF cells spread across the entire BF volume. Instead, presynaptic cells coinhabit smaller pockets in which
their postsynaptic cholinergic targets are found, suggesting the potential for modular control of portions of the BFc at the local level. The final study describes the inputs to BFc cells on the basis of their outputs to four different cortical targets utilizing the same viral tracer as above. It is possible to infect transgenic basalocortical cells via monosynaptic tracing vector injection in the cortex, at the site of axon terminals, thereby only labeling transynaptically those afferents contacting corticopetal BFc cells projecting to a particular cortical region. Subpopulations of basal forebrain cholinergic cells, that send their efferents to different cortical areas, did not receive homogeneous input, but rather received differing complements of synaptic inputs.

The connectivity described herein likely serves as the anatomical basis for the differential control that the BFc exerts over its outputs. Along with this novel architecture comes a number of testable hypotheses put forth in the general discussion.
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Abbreviations

3V: third ventricle
AA: anterior amygdaloid area
AAV: adeno-associated virus
ac: anterior commissure
Acb: nucleus accumbens
ACh: acetylcholine
AP: anterior-posterior stereotaxic domain
Aq: cerebral aqueduct
ASt: amygdalo-hippocampal transition area
BAOT: bed nucleus of the accessory olfactory tract
BF: basal forebrain
BFc: cholinergic basal forebrain
BLA: basolateral amygdaloid nucleus, anterior part
BLC: basolateral complex of the amygdala
BM: basomedial nucleus of the amygdala
BNST: bed nucleus of the stria terminalis
BNST-AL: bed nucleus of the stria terminalis, anterolateral part
Cc: corpus callosum
CeA: central nucleus of the amygdala
CeC: central nucleus of the amygdala, capsular part
CeL: central nucleus of the amygdala, lateral part
CeM: central nucleus of the amygdala, medial part
ChAT: choline acetyltransferase
Cl: claustrum
Cl: caudal interstitial nucleus of the medial longitudinal fasciculus
Co: cortical amygdalar nuclei
CPu: caudate putamen
cre: cre recombinase
Cy3: a cyanine dye with red emission
Dk: nucleus of Darkschewitsch
DG: deleted rabies glycoprotein gene
DLG: dorsal lateral geniculate
DMTg: dorsomedial tegmental area
DP: dorsal peduncular cortex
DTT: dorsal tenia tecta
DV: dorsal-ventral stereotaxic domain
EA: extended amygdala
ec: external capsule
eGFP: enhanced green fluorescent protein
EM: electron microscopy
EnvA: envelope glycoprotein for avian sarcoma leucosis, subgroup A
EnvA-ΔG-rabies-eGFP: G-protein-deleted rabies pseudotyped with EnvA
f: fornix  
FB: fast blue  
FG: FluoroGold  
fi: fimbria  
fm: forceps minor of the corpus callosum  
fr: fasciculus retroflexus  
Fr3: frontal cortex, area 3  
GABA: γ-aminobutyric acid  
GFP: green fluorescent protein  
GP: globus pallidus  
HDB: horizontal limb of the diagonal band  
Hypothal: hypothalamus  
l: intercalated nuclei of the amygdala  
ic: internal capsule  
ICMS: intracortical microstimulation  
IL: infralimbic cortex  
IP: intraperitoneal  
IPAC: interstitial nucleus of the posterior limb of the anterior commissure  
LC: locus coeruleus  
LH: lateral hypothalamus  
LHA: lateral hypothalamic area  
lo: lateral olfactory tract  
LOT: nucleus of the lateral olfactory tract  
LSD: lateral septal nucleus, dorsal part  
LSI: lateral septal nucleus, intermediate part  
LV: lateral ventricle  
M1: primary motor cortex  
M1FL: primary motor cortex, forelimb-responsive area  
M2: secondary motor cortex  
mCherry: a fluorophore (reporter protein) with red emission  
Me: medial amygdaloid nucleus  
MG: medial geniculate nucleus  
ML: medial-lateral stereotaxic domain  
MPA: medial preoptic area  
mPFC: medial prefrontal cortex  
MS: medial septum  
mt: mammillothalamic tract  
NBM: nucleus basalis  
nCh: noncholinergic  
NPY: neuropeptide Y  
och: optic chiasm  
OFC: orbitofrontal cortex  
opt: optic tract  
PAG: periaqueductal gray  
PB: parabrachial nucleus
PB: phosphate buffer
pc: posterior commissure
PF: parafascicular thalamic nucleus
PFC: prefrontal cortex
Pir: piriform cortex
Po: posterior thalamic nuclear group
PrL: prelimbic cortex
R: raphe nucleus
Rab: rabies
Ret: reticular formation
RG: rabies glycoprotein
RRF: retrorubral field
Rt: reticular nucleus of the thalamus
S1f: primary somatosensory cortex, forelimb region
Sap: 192-IgG-saporin or ME20.4-saporin
SI: substantia innominata
sm: stria medullaris
SN: substantia nigra
SO: supraoptic nucleus
STh: subthalamic nucleus
STN: subthalamic nucleus
SupColl: superior colliculus
Tu: olfactory tubercle
TVA: avian sarcoma leucosis receptor, subgroup A
TVAmCherry: fusion protein of TVA receptor and mCherry reporter
V1: primary visual cortex
V1B: primary visual cortex, binocular
V1M: primary visual cortex, monocular
V2: secondary visual cortex, medial areas
V2ML: secondary visual cortex, mediolateral area
V2MM: secondary visual cortex, mediomedial area
VA: ventral anterior thalamic nucleus
VDB: vertical limb of the diagonal band
Vis: visual cortex
VL: ventrolateral thalamic nucleus
VO: ventral orbital cortex
VP: ventral pallidum
VPL: ventral posterolateral thalamic nucleus
VTA: ventral tegmental area
WGA-HRP: wheat germ agglutinin-horseradish peroxidase
CHAPTER I: INTRODUCTION
1.1 Overview

The cholinergic basal forebrain (BFc) plays multitudinous roles in cortical functions, modulating different cortical areas at different timepoints and over different timescales. However, the known anatomy of the basal forebrain (BF) and its connections do not adequately explain the spatiotemporally heterogeneous functions this structure subtends. For instance, the literature tells of nearly no back-projection from sensory cortex to BFc, yet cells in the basal forebrain are physiologically responsive to sensory information. Lack of explanatory evidence is accompanied by the persistence of a major hypothesis from the 1980s claiming that the BFc acts as a diffuse activating system (Saper, 1987; Sarter and Bruno, 1997), even though this seems unlikely in the wake of data collected in the decades since (for review see Záborszky et al., 2015b).

The studies in this document addresses the gaps in anatomical explanation for the functions of the basal forebrain cholinergic system in three ways: by defining the topography of BF cell populations projecting to different cortical areas, by defining the architecture of the local inputs to the cholinergic cells from within the BFc, and by defining the differential inputs to those cholinergic neurons that target different cortical areas.

Before reviewing the anatomy of the BFc, I will provide a brief definition of the structure, historical perspective, and summary of relevant functions involving the mammalian BFc, with particular focus on the rat, being the experimental model for all aims of this thesis.
1.2 Defining the basal forebrain

The basal forebrain (BF) is a subcortical region providing cholinergic and noncholinergic innervation of the cerebral cortex and subcortical areas (for review, see Semba, 2000). In this document, the BF is defined as the space containing cholinergic corticopetal projection neurons, which includes the classical cytoarchitectonic subdivisions of the medial septum and vertical diagonal band (MSVDB), horizontal diagonal band (HDB) which here includes the magnocellular preoptic area, ventral pallidum (VP), substantia innominata/extended amygdala (SI/EA), globus pallidus (GP), and internal capsule (ic). The MS and VDB are combined due to the reluctance to draw an artificial line between the two structures that form a continuum as observed under Nissl stain: such a line would vary between subjects. The term basal forebrain (BF) will refer to the full volume (containing cell bodies of cholinergic projection neurons, noncholinergic projection neurons, and interneurons), while the term cholinergic basal forebrain (BFc) will refer to only the cholinergic projection component.

1.3 Historical context and general basal forebrain functions

Interest in the BF increased following the finding of BF cholinergic cell loss as a hallmark of Alzheimer's disease (Whitehouse et al., 1981). The idea became popular that the loss of cholinergic cells in the BF was precipitating disease progression in a potentially causal manner, known as the 'cholinergic hypothesis' (Bartus et al., 1982). On the pharmacological side of things, a number of studies investigated the effects of cholinergic agonists and antagonists administered
systemically and locally, while behaviorists lesioned the BF electrolytically, chemically, or via aspiration to observe subsequent changes in function (for review see Robbins et al., 1997; Détári, 2000; Hasselmo and Sarter, 2011; Baxter and Bucci, 2013). Unfortunately, each of these techniques came with a major drawback. Pharmacological manipulations on the cholinergic system could not differentiate between effects on terminals of BFc cells and effects on the cholinergic projections arising from the brainstem. Nonselective lesion studies could not differentiate between effects on cholinergic versus noncholinergic cells in the BF, although they advanced the understanding of the area at the time, for instance indicating BF lesions could affect attention while sparing some abilities of learning and memory.

The tide for understanding functions of the BFc changed with the introduction of immunolesion techniques (Wiley et al., 1991), namely the targeted immunotoxins 192-IgG-saporin (Sap) and ME20.4-saporin (Sap). With administration of the correct concentration of one of these compounds, one could selectively remove cholinergic cell bodies from the BF while leaving other cell types in the same region intact, depleting most of the cholinergic innervation of cortex and creating deficits of learning memory and attention that were less severe than deficits resulting from nonselective BF lesions (Chiba et al., 1995; Turchi and Sarter, 1997; McGaughy et al., 2002; Risbrough et al., 2002). In some studies, the compound injected into the ventricles, again confounding results by lesioning other cells (e.g. Purkinje cells as in Wrenn et al., 1999). Others injected the compound into the BF, yielding effects more subtle than the nonspecific BF
lesions (e.g. electrolytic lesions). Even so, care needed to be used to infuse the correct concentration of Sap, and to verify the health of noncholinergic cells following lesion, since higher doses of this compound have nonselective lesioning properties. The most useful application of this compound eventually proved to be cortical injection at cholinergic axon terminals, retrogradely transporting saporin to the cell body, thereby allowing for a lesion selectively depleting a particular cortical area of its cholinergic inputs, while leaving the rest of cortex with its cholinergic innervation intact. This particular technique allowed investigators to answer pointed questions regarding the functions of acetylcholine in cortex (for review see Baxter and Bucci, 2013).

1.4 Cortical functions of acetylcholine

A number of studies have investigated the effects of cholinergic deafferentation of a single cortical area, sparing cortical cell bodies and sparing cholinergic fibers innervating the remainder of cortex. Specific deficits observed are dependent on which cortical area is denervated. For instance, Sap injections in motor forelimb cortex disrupted forelimb map reorganization in response to a skilled motor learning task, and disrupted the learning as well, while Sap directly in the BF created a larger deficit in learning (Conner et al., 2010). No such effects were observed following medial prefrontal (mPFC) Sap injections. Prefrontal Sap injections were shown to disrupt processes of attention and spatial working memory (Turchi and Sarter, 1997; Gill et al., 2000; Dalley et al., 2004; Newman and McGaughy, 2008; Croxson et al., 2011). In orbitofrontal cortex, Sap injections impair social transmission of food preference (Ross et al., 2005),
replicating the effect of Sap injections in the BF (Berger-Sweeney et al., 2000; Vale-Martínez et al., 2002). Visual recognition memory is impaired following Sap injection in rhinal cortex (Turchi et al., 2005) while visual cortical Sap injection impaired acquisition of a visual rate discrimination task, but did not impair performance of the same task learned preoperatively (Minces et al., 2013). Similar effects were found in another sensory modality, as auditory cortex Sap injections impair sound sequence learning (Kudoh et al., 2004). In barrel cortex, Sap injections blocked the plasticity normally resulting from whisker pairing (Sachdev et al., 1998) and in posterior parietal cortex, Sap injections disrupt attentional increases produced by stimulus-outcome uncertainty (Bucci et al., 1998).

The fact that acetylcholine is responsible for such a variety of functions in these different cortical areas is made more interesting yet when one considers the dynamics of this innervation: cholinergic projections to different cortical areas are differentially controlled (for review see Muñoz and Rudy, 2014). Sensory stimulation in the auditory, visual (vis), or somatosensory domain can evoke selective ACh efflux in the corresponding cortical regions respectively (Rasmusson, 1993). Neither visual nor somatosensory stimuli change ACh efflux in medial prefrontal cortex (Fournier et al., 2004; Laplante et al., 2005). This effect was highly robust, yet there remains no explanation of how the BF is differentially modulating its outputs. A review of anatomical descriptions of BF efferents is one starting place in this search.
1.5 Anatomy of basal forebrain efferents

The diverse functions of ACh in cortex can be viewed as dependent on the diversity of BFc efferents to various cortical areas. The whole cortical mantle and amygdala receive cholinergic projections, arising almost entirely from the BF. The BF also sends projections to other places (e.g. hippocampus, thalamus, hypothalamus, and brainstem) although this will not be highlighted here (for comprehensive review see Semba, 2000). Among the cell bodies innervating cortex, reports of proportions with a given transmitter content varies, but generally about half of projection cells are cholinergic, half GABAergic, and a small amount (~3%) are glutamatergic. These cell bodies can be divided into groups of further specificity, and display a banded topography regarding the location of their cell bodies in the BF (for review see Záborszky et al., 1999, 2005), although the functional meaning of this topography (analyzed irrespective of projection target) remains a mystery.

The distribution of axons can be visualized via BF injections of anterograde tracers. When hundreds of cells are labeled in this manner, the projections from HDB, SI/EA, and 'nucleus basalis' (NBM) appear diffuse: from each of these areas arise fibers terminating in nearly all cortical areas (Lamour et al., 1984; Luiten et al., 1985). If one were to look at this data alone, and assume that cells in each BF subregion are homogenous in their projection, then one could guess the BF projection system to be diffusely innervating cortex. However, this assumption would be incorrect, since BF projection neurons with cell bodies next to one another can innervate wildly different cortical targets. A
number of experiments have shown that when two different retrograde tracers are applied to two different cortical areas in the same subject, labeled cell bodies in the BF can be in close proximity, yet the proportion of double-labeled cells in the BF for these cases is often on the order of 5% or less (Bigl et al., 1982; Price and Stern, 1983; Saper, 1984; Okoyama et al., 1987; Baskerville et al., 1993). Furthermore, analysis of the axonal fields of single cholinergic cells confirms that individual projection neurons innervate one or two discrete cortical areas to the exclusion of the rest of the cortex (Price and Stern, 1983).

There is an additional level of complexity to consider, as the same neurons projecting to discrete cortical areas are collateralizing extensively within the BF itself (Záborszky and Duque, 2000; Duque et al., 2007). This collateralization, along with the various types of interneurons forming a network with ChAT cells in the BF, is understudied and will be partially addressed experimentally in this document.

The cell bodies that send efferents from the BF to cortical areas can be viewed as mostly separate subpopulations, as grouped by cortical injections of retrograde tracers. Injection of different retrograde tracers in two cortical areas in the same animal commonly results in few double-labeled cells projecting to both areas. However, depending on which cortices are injected, these mostly-separate subpopulations may have cell bodies that inhabit the same space in the BF, as is the case when comparing projections to motor and somatosensory cortices (Baskerville et al., 1993), or may lie in different compartments of the BF entirely, as is the case when comparing projections to retrosplenial and temporal
cortices (Price and Stern, 1983). This is potentially an important difference, as cholinergic cell bodies found in the same BF subregion tend to have similar dendritic orientations, and thus may share many inputs (Záborszky et al., 2002, 2015b). If one considers the specific results of retrograde tracers placed in medial prefrontal or visual cortex, it can be seen that retrogradely labeled cell bodies in the BF lie in the same specific subregions, namely the HDB and anterior SI/NBM (Rieck and Carey, 1984; Saper, 1984).

However, as previously mentioned, visual stimuli elicited a 75% increase in efflux of ACh in visual cortex (vs baseline) while eliciting no change in ACh efflux in mPFC over the same interval (Fournier et al., 2004). Can it be assumed that these two projection populations, comingling their cell bodies in the same BF subregions, are receiving the same inputs if their outputs can be controlled in this differential manner? This constitutes an apparent contradiction between the physiology and anatomy, and begs a more detailed look at afferents coming in to the BF.

1.6 Anatomy of basal forebrain afferents

While BF outputs are well documented in terms of the topography of projecting cell bodies, BF inputs are less well studied (for review see Záborszky et al., 1991). A number of studies used anterograde tracers and BF immunostaining followed by light microscopy to identify putative contact sites. However, this is not sufficient for describing a network, as many putative contact sites, once further investigated at the level of the electron microscope (EM), turned out not to form a synapse at all. Still, anterograde and retrograde tracing
studies have identified many putative inputs to the BF, including brainstem (Woolf and Butcher, 1986), BNST, fundus striati (Arluison et al., 1990), amygdalostriatal transition area (Jolkkonen et al., 2001), thalamus, VP, substantia nigra, hippocampus, subthalamic nucleus, lateral septum, and insular cortex (Carnes et al., 1990). Additionally, it has been confirmed that the HDB receives tyrosine hydroxylase positive synapses, likely arising from brainstem areas (Semba et al., 1988; Losier and Semba, 1993).

Arguably the most well-known input is the PFC. Its various regions innervate different portions of the BF, as analyzed via anterograde and retrograde tracers at the light microscopic level (Grove, 1988; Sesack et al., 1989; Carnes et al., 1990; Gaykema et al., 1991; Gabbott et al., 2005). This projection was verified at the electron microscopic level, although no contacts onto cholinergic cells were confirmed, suggesting that PFC preferentially synapses onto noncholinergic BF cells (Záborszky et al., 1997).

Additional afferents, confirmed at the EM level, arise from the hypothalamus (Cullinan and Záborszky, 1991), nucleus accumbens (Záborszky and Cullinan, 1992), intercalated cell mass of the amygdala (Paré and Smith, 1994), striatum (Henderson, 1997), and the remainder of the amygdala, with heavy inputs from its central nucleus (Záborszky et al., 1984; Jolkkonen et al., 2002). It should be noted that the hypothalamus, amygdala, and striatum all exhibit some topography in their innervation of various BF compartments.

Making a full picture of BF afferents from these disparate data points validated through time-consuming EM work is difficult. In some of the EM cases,
the identity of the BF target neuron being innervated remained unknown. In other cases, such as prefrontal projections to the BF, a number of putative contacts onto cholinergic cells were seen but none verified to be a synapse. An anterograde survey of every brain region, followed up with immunohistochemistry and EM confirmation of contact sites in the BF would take so much time as to be infeasible. Studying BF afferents with retrograde tracers on the other hand is troublesome, as every retrograde tracer appears to exhibit at least some nonselective uptake by fibers of passage, and the BF is rich in fiber bundles, most notably the internal capsule and medial forebrain bundle. Furthermore, in every study of BF afferents thus far, the cortical targets of BF cholinergic cells has been unknown due to the nature of classical tracing methodologies. This document details the novel application of recently developed viral tracing methods in order to address this very problem and define the input-output relationship for BF cholinergic cells.

1.7 Thesis statements

The various functions of the basal forebrain cannot be fully explained by the existing anatomical literature. More specifically, three related problems are addressed in this document, outlined here and recanted in more detail in the introductory sections of corresponding chapters III, IV, and V:

1) Discrete basalocortical projections influence sensory processing and a variety of learning and memory functions subtended by their cortical targets, and there is evidence to suggest that these discrete projections can be differentially modulated (Fournier et al., 2004). If one assumes that
neighboring BF cells another receive similar afferents, the anatomical
distribution of cells projecting to different areas becomes critical for
understanding how the BF modulates multiple cortical areas differentially.
Although the gross topography of the BF has been described, it would be
useful to detail the anatomical background by which the BF may
accomplish its proposed cortically-integrative functions (Záborszky et al.,
2015a).

2) Critical to the function of ascending cholinergic projections are the inputs
to these cholinergic cells. Of these inputs, the least well studied are local
afferents arising from within the BF. While the synaptic inputs to
cholinergic BF cells include terminals positive for somatostatin and
neuropeptide Y (NPY) (Záborszky, 1989, 1992), and while cholinergic
cells have been shown to contact one another (Ünal, 2012), the intrinsic
BF connectivity is almost totally unknown. Given that cholinergic dendrites
are often long (reconstructed up to 800uM in Duque et al., 2007) and
parallel in one given BF subregion (Záborszky et al., 2002), local synaptic
inputs to cholinergic BF cells may play a role in connecting distant portions
of the BF. To test this possibility, it would be important to study the
topography of cell bodies supplying local synaptic afferents to cholinergic
cells.

3) The sources of synaptic inputs to cholinergic cells are mostly unknown. A
rough topography of BF innervation was previously reported, yet the
relationship of these inputs to the system's outputs has never been
studied. Prior studies have shown that the BF sends cholinergic projections to a variety of cortical areas and modulates these projections independently of one another. Yet some cholinergic cells are interconnected, and many of these cells may receive diffuse inputs. Current knowledge about afferents to basolocortical cholinergic cells cannot explain the various functions these discrete projections subserve. These three sets of problems are addressed in chapters III, IV, and V, respectively.

Chapter III characterizes the distribution of basolocortical projection neurons to two physiologically-identified cortical areas via retrograde tracer injections in each: visual cortex (V1/V2) and the forelimb representation of motor cortex (M1FL). I use both a within-subject design to characterize the spatial relationship between BF neurons projecting to these two cortical areas, additionally identifying these cells as immuno-positive or -negative for ChAT. This experiment is designed to create a map showing the absolute position of these distributions of target-identified cell bodies (via same-section nissl staining), useful as a descriptive study and for the planning of future experiments targeting the cell bodies of this co-projection.

Chapter IV characterizes those cell bodies in the BF synaptically connected to cholinergic cells residing in a small BF subregion, utilizing monosynaptic viral tracing in ChAT-cre transgenic animals (Wickersham et al., 2007; Wall et al., 2010; specific methods appear in chapter IV). Several
subregions of local cells afferent to cholinergic cells are identified and compared. This experiment is designed to investigate the distribution of cells sending inputs to small groups of cholinergic cells, and show the size and variability of this distribution across different BF subregions. To my knowledge, this is the first study randomly sampling the topography of local cells afferent to BF cholinergic neurons.

Chapter V characterizes the input-output relationship of cholinergic BF cells projecting to a specific cortical area. To this end I will infuse a cre-dependent helper viruses in the BF and a helper-dependent monosynaptic tracing virus in the cortex of ChAT-cre transgenic animals (specific methods appear in chapter V). This experiment is designed to probe the similarities and differences among cell body populations afferent to BF cholinergic cells that specifically target motor cortex, medial prefrontal cortex, ventral orbital cortex, or the amygdala.
CHAPTER II: GENERAL MATERIALS AND METHODS
2.1 Ethical treatment of experimental animals

Animals involved in these studies were treated in accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals." Experiments were performed with the approval of the Institutional Animal Care and Use Committee of Rutgers University.

2.2 Animal subjects

Male and female adult (> 3 months of age) rats of a Long-Evans background were utilized, and were either transgenic - expressing cre recombinase in choline acetyltransferase cells (ChAT-cre, courtesy of Dr. Karl Deisseroth, Stanford University), or wild-type (Harlan). All subjects underwent either one or two surgeries to intracranially deposit either conventional fluorescent or viral neural tracers. One week after the final survival surgery, animals were anesthetized deeply and transcardially perfused. Table 2.1 summarizes experimental subjects and manipulations. Experimental details specific to each aim appear in each specific methods section.

<table>
<thead>
<tr>
<th>AIM</th>
<th>Sex</th>
<th>Long-Evans Transgenic Strain</th>
<th>Method for Identification of Injection Sites</th>
<th>Retrograde Tracer Injections</th>
<th>AAV Helper Viruses Injections</th>
<th>EnvA-ΔG-Rabies-eGFP Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIM I</td>
<td>M</td>
<td>Wild-type</td>
<td>Anesthetized Physiology &amp; Microscopy</td>
<td>M1 forelimb and V1/V2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>AIM II</td>
<td>M + F</td>
<td>ChAT-cre</td>
<td>Microscopy</td>
<td>N/A</td>
<td>Local BF</td>
<td>Local BF</td>
</tr>
<tr>
<td>AIM III</td>
<td>M + F</td>
<td>ChAT-cre</td>
<td>Microscopy</td>
<td>N/A</td>
<td>Extensive BF</td>
<td>Cortex or Amygdala</td>
</tr>
</tbody>
</table>

Table 2.1 Summary of experimental subjects and manipulations. Details are available in specific methods sections appearing in subsequent chapters.
2.3 Survival surgeries

Surgical procedures were performed under deep general anesthesia, using 1-4% isoflurane inhalation in oxygen, or an intraperitoneal (IP) ketamine/xylazine mixture specifically during cortical physiological identification (100mg/mL ketamine: 20mg/mL xylazine). Once anesthetized, animals were placed in a stereotaxic apparatus and maintained at body temperature via a circulating water heating pad. Hair covering the scalp was removed partially by initially using a small electric beard trimmer, then completely by subsequently applying Nair hair removal. After 2 minutes, Nair was removed and the skin was washed with 0.9% saline. Betadine was then applied to the skin and allowed to dry. Eyes were protected by application of white petroleum ophthalmic ointment. A single incision was made along the sagittal midline, and fascia was gently prised back to expose the skull during liberal application of 0.9% saline.

In the case of virus injection surgeries, burr holes (<1mm in diameter) were made to access the dura in 1-4 locations, depending on the experiment. In the case of retrograde tracer injection surgeries, a craniotomy was hand-drilled only large enough to expose the right visual cortical regions (V1 and V2M), exposing approximately 3 x 3 mm of dura, and subsequent to visual recordings, a second craniotomy was made ipsilaterally over M1 forelimb, exposing approximately 3.5 x 4 mm of dura. Durotomy was performed and subsequent to this the cortex was kept wet with 0.9% saline.
Physiological recording and stimulation procedures, as well as stereotaxic coordinates and amounts of tracers and viruses injected appear in specific methods sections in the subsequent chapters.

Following successful injections, the incision was rinsed again with 0.9% saline and closed with sutures. Rats were observed until recovery from anesthesia in their home cages, and then received the non-steroidal anti-inflammatory Meloxicam (0.2 mg/kg) and the antibiotic cephalexin (50 mg/kg), both applied to Honey Nut Cheerios (General Mills) for oral administration. Animals were returned to the colony and administered cephalexin daily for four additional days.

2.4 Perfusion, fixation and tissue collection

Seven days following the final survival surgery, animals were anesthetized deeply via IP administration of Euthasol (pentobarbital) or ketamine. Rats were transcardially perfused with 300-500 mL of 0.1M phosphate buffer (PB) at 4°C, and subsequently with 300-500 mL of 4% paraformaldehyde in 0.1M PB. Brains were removed and post-fixed in 4% paraformaldehyde at 4°C between 4 and 12 hours, depending on initial firmness of the brain following perfusion. Brains were then transferred to 30% sucrose in 0.1M PB and left at 4°C until brains sunk. Brains were cut in the coronal plane at a thickness of 50 µM with a freezing microtome. Four series of sections were collected, such that further tissue processing occurred at a sampling rate of one section for every 200 µM of anterior-posterior (AP) distance unless otherwise noted. Following three 5-minute washes in 0.1M PB, sections were either mounted on gelatin-coated slides, dried
and subsequently coverslipped for microscopic analysis of fluorescence, or stored in cryoprotectant at -20°C for later immunohistochemistry.

2.5 Immunohistochemistry

In chapter III, sections were processed for ChAT immunohistochemistry. Following washing in 0.1M PB, sections were incubated for 24 hours at room temperature in monoclonal goat-anti-ChAT (Millipore, 1:500), in 0.1M PB with 1% blocking serum and 0.5% triton. Following three 5-min washes in 0.1M PB, the sections were further incubated in secondary antibodies for 3 hours at room temperature with donkey-anti-goat CY3 (Jackson Immuno, 1:200) in 0.1M PB with 1% blocking serum and 0.5% triton. Sections were then washed in 0.1M PB, mounted on gelatin-coated slides, dried, and coverslipped with DePeX.

2.6 Fluorescent microscopy

Cell bodies exhibiting fluorescent labeling (Fluoro-Gold, Fast Blue, eGFP, or CY3) were imaged on a Zeiss AxioSkop microscope retrofitted with a robotic stage controller and camera, and at the same time mapped at 20x magnification using Neurolucida software (MBF Bioscience). Maps were created in a 200µM series (sampling every fourth section, in Aims I and III) or in a gapless series (mapping every section, in Aim II). After mapping cell bodies and creating contours for some key markers (e.g. the section outline, white matter tracts, and ventricles), a few representative areas were imaged in a montage at 10x or 20x magnification for later use. Additionally, injection sites were mapped under fluorescence for later cytoarchitectonic identification under Nissl stain.
2.7 Generation of warped volumes

In cases where multiple animals are shown warped into a single volume, custom scripts were created to extract data from multiple subjects' Neurolucida files and convolve them into a single volume using MATLAB (MathWorks). Each subject was then linearly warped to the same "master brain," a gapless series containing an entire hemisphere of ChAT labeling in the BF, using a linear transformation in Neurolucida. After warping, all subjects were then taken together with another custom MATLAB script into the same volume, warped to the coordinate space of the master brain, for purposes of analysis and 3D video generation.

2.8 Cytoarchitectonic identification of structures

Following fluorescent mapping and imaging of sections, coverslips were removed in xylenes and sections remained on slides to be stained for Nissl substance with thionin. Following Nissl staining, slides were again coverslipped with DePeX medium and set back on the microscope. Under 2.5x and 10x magnification, the Nissl-stained sections were aligned with their virtual fluorescent map in Neurolucida, and contours were placed around cytoarchitectonic areas. With the exception of BF subregions, areas were traced and named according to The Rat Brain atlas, 6th edition (Paxinos and Watson, 2007), and when necessary cross-referenced with the Swanson atlas (Swanson, 1992). Using NeurolucidaR and Adobe Illustrator, maps of injection sites and fluorescent cell body locations were overlaid onto images of Nissl-stained sections taken at 10x magnification in order to compare subjects at various
anterior-posterior levels. Finally, maps of cell bodies belonging to identified cytoarchitectonic zones were converted into Microsoft Excel tables using Neurolucida Explorer (MBF Bioscience), yielding data that was analyzed in Microsoft Excel.
CHAPTER III: TOPOGRAPHY OF BF CHOLINERGIC CELLS PROJECTING TO MOTOR AND VISUAL CORTEX
3.1 Introduction

Individual BF cholinergic neurons innervate discrete slabs of cortex (Price and Stern, 1983; Duque et al., 2007). Each portion of cortex receives a cholinergic and noncholinergic projection from the BF, arising from a reproducible topographic distribution of BF cell bodies. Investigation of the common topography between two populations of basolocortical cell bodies is possible with application of retrograde tracers in cortex via iontophoretic injection (Schmued and Heimer, 1990), and the combination of retrograde tracers with immunohistochemical markers for identification of BF projection cell type has been available for some time (Wahle et al., 1984). When comparing two cortical areas each injected with their own retrograde tracer, two distributions of cell bodies in the BF appear, with differing spatial overlap depending on the cortices injected (e.g. Záborszky et al., 1999, page 347). If the area of BF overlap between these different basolocortical projections represents cells co-modulated by afferents to this common area, and this co-projection achieves some sort of integrative function between cortices innervated, as was hypothesized recently (Záborszky et al., 2015a), the area of BF overlap becomes important in further querying the nature of such integration in future studies. With this in mind, I set about studying BF projections to two areas of cortex of interest, namely motor and visual cortex.

The cholinergic projection to motor cortex is important in motor learning, and critical for the motor cortex expansion resulting from specialized motor learning. Indeed, such expansion does not take place following cholinergic
deafferentation of motor cortex or full 192-saporin immunolesion of the BF (Conner et al., 2003, 2010). The cholinergic projection to visual cortex is important for visual perception. Optogenetically-evoked ACh release improves performance on a visual perception task (Pinto et al., 2013). Clearly, BF cholinergic projections to motor and visual cortices are important for motor and visual functions. However, there exists no detailed map of BF cholinergic cells projecting to motor or visual cortex; only descriptions or retrograde-only maps are published (Rieck and Carey, 1984; Saper, 1984; Laplante et al., 2005), or for the motor projection, a single coronal section is meant to represent the thousands of micrometers in the anterior-posterior space where these projection cells exist (Baskerville et al., 1993).

Therefore, I performed the following experiment in order to create detailed maps of the cholinergic and noncholinergic BF projection to motor and visual cortices, as well as identify the precise cytoarchitectonic locations of overlap between the neurons at the origin of these projections.

3.2 Specific methods

Six adult male wild-type Long-Evans rats (415-460g) were anesthetized with ketamine/xylazine and, following craniotomy and durotomy, a micropipette filled with 3M NaCl (tip diameter approximately 20 µM) was inserted into the right cerebral cortex to detect visually evoked potentials in response to strobe light stimulation of the contralateral eye. Visually-responsive areas were noted. Then, a separate pipette (tip diameter approximately 35 µM) was inserted into the same cortical area at two or more such responsive locations, and the retrograde tracer
Fast Blue (2% in 0.1M NaCl) was deposited iontophoretically (via 5.7 µA current) at a depth of up to 1.3mm ventral to pia, for 7 minutes per site.

Following Fast Blue deposition in visual cortex, a micropipette filled with 3M NaCl (tip diameter approximately 20 µM) was inserted into right frontal cortex for applying intracortical microstimulation (ICMS) to determine the location of the primary motor forelimb area (M1FL). Stimulation was delivered in 20ms trains of 20µs pulses at 333Hz, with an inter-train-interval of 1.2 seconds, for a maximum of 20 trains in a row, whilst steadily increasing the current from 0 up to a maximum of 400uA until a contralateral response was detected. Areas eliciting no motor activity at 400µA were deemed unresponsive. Responsive areas were marked and a separate pipette (tip diameter approximately 35 µM) was inserted into the same cortical area at three or more such responsive locations, and the retrograde tracer Fluoro-Gold (2% in 0.1M NaCl) was deposited iontophoretically (via 5.7 µA current) at a depth of up to 1.5mm ventral to pia, for 7 minutes per site. Areas eliciting any motor activity other than the contralateral forelimb were not injected with tracer. Motor forelimb responses are summarized in figure 3.1.

Injection sites were traced using Neurolucida, and their cytoarchitectonic location verified following re-staining the same sections for Nissl. All other methods employed in this experiment are detailed in the General Methods chapter.
Successful motor forelimb stimulation was achieved reliably in this approximately 1mm² area of M1\textsubscript{FL}.

3.3 Results

3.3.1 Retrograde tracer placement and efficacy

Injection site imaging under fluorescent microscopy and subsequent imaging of the same sections following Nissl stain confirmed the placement of retrograde tracers. Fluoro-Gold was limited to M1, while Fast Blue was limited to visual cortex, mostly in V1M and V2ML, with some incursions in V1B and V2MM (fig 3.2). Cortical areas were confirmed via prominence or absence of cortical layers and granularity (Paxinos and Watson, 2007; Sefton et al., 2015).
Figure 3.2 Location of cortical injections sites in the six subjects of Aim I. Injection sites made by iontophoretic application of Fluoro-Gold in primary motor cortex and Fast Blue in visual cortex were traced under darkfield fluorescent microscopy, and the cytoarchitectonic locations of these sites confirmed following subsequent nissl staining of the same sections. Injections from subjects 36, 40, 45, 59, 60, and 61 are projected on representative figures of the rat atlas (Paxinos and Watson, 2007).
Efficacy of retrograde tracers in these cortical areas was corroborated by the presence of cell bodies labeled with Fluoro-Gold in the motor-projecting ventrolateral thalamic nucleus and those labeled with Fast Blue in the visual-projecting dorsal lateral geniculate thalamic nucleus.

3.3.2 Distribution of BF neurons projecting to cortex

The distribution of cell bodies projecting to motor and visual cortex across the BF is not uniform. Moreover, afferent BF neurons express different transmitters depending on their location. Figures 3.3 through 3.8 show a sampling of sections mapped at six representative anterior-posterior levels. The anterior portions of the BF have a larger presence of cholinergic and non-cholinergic projections to visual cortex and weaker, almost entirely non-cholinergic projections to motor cortex (figures 3.3 through 3.5). These anterior somata are found much more commonly in the VDB and HDB than in the MS and VP. In the middle-posterior portions of the BF, abundant projections to motor cortex display a gradient such that the cholinergic portion lays preferentially dorsomedial to its noncholinergic counterpart, while projections to visual cortex are less abundant and appear in clusters (figures 3.6 and 3.7). In the most posterior reaches of the BF, there are more cholinergic cells projecting to either cortical area in a mixed topography (figure 3.8).

Of particular note is the limited spatial overlap of cholinergic neurons projecting to either cortical area. Such overlap was only seen in the anterior internal capsule, as seen in figure 3.6. Additionally, there are very few double-labeled cells found in any subject.
Figure 3.3 BF maps of six subjects from Aim I. In the anterior BF, there are a fair amount of cell bodies projecting to visual cortex (Vis), both cholinergic (ChAT) and noncholinergic (nCh). In contrast, at this anterior level few noncholinergic and no cholinergic cells project to primary motor forelimb cortex (M1). MS, medial septum; VDB, vertical diagonal band of Broca; VP, ventral pallidum; Tu, olfactory tubercle; Acb, nucleus accumbens; ac, anterior commissure; CPu, caudate and putamen. Atlas insert from Paxinos and Watson, 2007.
Figure 3.4 BF maps of six subjects from Aim I. Immediately anterior to the HDB, there are cells in the VDB projecting to visual cortex (Vis), both cholinergic (ChAT) and noncholinergic (nCh). In contrast, at this level few noncholinergic and no cholinergic cells project to primary motor forelimb cortex (M1). MSVDB, medial septum and vertical diagonal band of Broca; VP, ventral pallidum; Acb, nucleus accumbens; ac, anterior commissure; CPu, caudate and putamen. Atlas insert from Paxinos and Watson, 2007.
Figure 3.5 BF maps of six subjects from Aim I. At the crossing of the anterior commissure, cholinergic (ChAT) and noncholinergic (nCh) cells projecting to visual cortex (Vis) or motor forelimb cortex (M1) inhabit a similar space. HDB, horizontal diagonal band of Broca; SI, substantia innominata; VP, ventral pallidum; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; ac, anterior commissure; CPu, caudate and putamen. Atlas insert from Paxinos and Watson, 2007.
Figure 3.6 BF maps of six subjects from Aim I. Posterior to anterior commissure, the cholinergic (ChAT) to noncholinergic (nCh) ratio increases, and the motor forelimb-projecting (M1) cells become more numerous than the visual-projecting (Vis) cells. HDB, horizontal diagonal band of Broca; SI/EA, substantia innominata and extended amygdala; GP, globus pallidus; ic, internal capsule; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; LHA, lateral hypothalamic area; sm, stria medullaris; f, fornix; Rt, reticular thalamic nucleus; CPu, caudate and putamen. Atlas insert from Paxinos and Watson, 2007.
Figure 3.7 BF maps of six subjects from Aim I. At the level of the anterior central amygdala (CE), the motor forelimb-projecting (M1) cells dwarf the visual-projecting (Vis) cells in number, the cholinergic containing cells of which (ChAT) prefer a dorsomedial position relative to their noncholinergic (nCh) counterparts. SI/EA, substantia innominata and extended amygdala; GP, globus pallidus; ic, internal capsule; LH, lateral hypothalamus; Rt, reticular thalamic nucleus; CPu, caudate and putamen. Atlas insert from Paxinos and Watson, 2007.
Figure 3.8 BF maps of six subjects from Aim I. At the posterior reaches of the BF, the cholinergic (ChAT) to noncholinergic (nCh) ratio is highest, with projections to visual cortex (Vis) and motor forelimb (M1) in similar proportion. SI/EA, substantia innominata and extended amygdala; GP, globus pallidus; ic, internal capsule; opt, optic tract; CeA, central amygdala; BLA, basolateral amygdala, anterior part; VPL, ventral posterior thalamic nucleus; Rt, reticular thalamic nucleus; CPu, caudate and putamen. Atlas insert from Paxinos and Watson, 2007.
These results describe a BF projection system that is uniform neither in its neurotransmitter content nor in the modality of cortical areas it innervates. The general pattern of results show that while visually-projecting BF cells are located predominately in the anterior regions of the BF, the motor forelimb projection is more spread out across the BF, and lacks a cholinergic component in the most rostral regions. Results based on the BF as subdivided into classical cytoarchitectonic areas tell of dramatic differences when comparing BF projection to these modalities, across transmitter type. Of particular note are the similarities or differences, depending on BF subregion, between the cholinergic-noncholinergic ratio of each basalocortical projection (figure 3.9). Differences were found in MSVDB, HDB, GP, and IC between labeled cell types, (Kruskal-Wallis, p<.01) and for each of these regions, individual pairwise Mann-Whitney U tests are shown in figure 3.9.

Figure 3.9 BF topography by projection. Subregional proportions (vertical axis) of cholinergic (ChAT) and non-cholinergic (nCh) cell bodies projecting to various cortical areas, grouped based on the BF subregion (horizontal axis) in which the cell bodies are located. * denotes p<0.01; Mann-Whitney U-test.
This joint topography is reproducible, as evidenced by maps of the BF across 6 subjects (figures 3.3-3.8) and represents a dataset uncommon among such maps, as cortical injection sites are selected based on physiological identification, and cell bodies are identified as part of a particular cytoarchitectonic area.

3.3.3 Summary of the main findings

a) Visually-projecting BF cell bodies are located predominately in the anterior diagonal bands, while motor forelimb-projecting BF cell bodies are located predominately in the posterior BF.

b) Cell bodies in the BF projecting to M_{FL} are predominately non-cholinergic in the diagonal bands, SI/EA and GP, while overwhelmingly cholinergic in the internal capsule.

c) BF subpopulations projecting to motor and visual cortex are reproducibly found in both separate and overlapping domains.
CHAPTER IV: DISTRIBUTION OF LOCAL BF SOMATA AFFERENT TO CHOLINERGIC CELLS
4.1 Introduction

Information regarding the local network within the basal forebrain is almost nonexistent. While the synaptic inputs to cholinergic BF cells include terminals verified as positive for somatostatin and NPY at the EM level (Záborszky, 1989, 1992), and while cholinergic cells have been shown to contact one another (Ünal, 2012), little is known about the location of local cell bodies synaptically connected to cholinergic cells. Early horseradish peroxidase retrograde tracing in monkeys and cats indicated, without EM confirmation of a synapse, that the septum and HDB may serve as afferent inputs to the NBM/SI (Irle and Markowitsch, 1986), although this tracer can be taken up by fibers of passage, and is thus no guarantee of termination onto a cholinergic cell or even the presence of terminals in the region of injection. A similar study placed wheat germ agglutinin-horseradish peroxidase (WGA-HRP) in the diagonal bands, SI, or peripallidal regions in rats, finding labeling of cell bodies in the MS/VDB and VP retrograde to SI and HDB injections, in the VDB, SI, and peripallidal regions retrograde to the HDB, and in the VDB, HDB and peripallidal regions retrograde to SI (Carnes et al., 1990). However, this tracer is also suspect when injected into areas rich in fibers of passage, such as the BF. Additional studies described GABAergic cells in the pallidum and SI projecting to the magnocellular preoptic area and SI (Fort et al., 1998).

Local BF circuits may play an important role in BF cholinergic outputs. However, technical limitations have hindered progress. For instance, selective immunotoxic removal of GABAergic BF cells, including projection neurons and
local interneurons, decreases ACh efflux (Roland et al., 2014). However, it is unclear whether GABAergic projection cells or interneurons are responsible for this effect.

The recent introduction of viral tracing methods appears to be one way of defining local circuits impinging on BF cholinergic cells. One can control the entry and spread of a replication-defective rabies virus such that entry and spread may only be achieved in cells expressing cre recombinase which have previously been modified with an adeno-associated virus (AAV) to express a special receptor and rabies glycoprotein (Wickersham et al., 2007; Wall et al., 2010). With this method, utilized in a transgenic rat line expressing cre recombinase in ChAT cells and detailed below, I demonstrate the topographical extent of local afferent cell bodies synapsing onto BF cholinergic neurons in small subregions of the BF, showing these intrinsic connections to be far more spatially restricted than previously thought.

4.2 Specific methods

Four ChAT-cre transgenic Long-Evans rats (2 males, 450-480g; 2 females, 250-300g) underwent two surgeries each: the first for administration of helper viruses to the BF, and the second for administration of a replication-deficient rabies virus to the same point in the BF. Using these methods, we were able to map the location of BF neurons monosynaptically connected to cholinergic BF cells.
4.2.1 Experimental design

We explored the local circuit of cells afferent to cholinergic cells in 3 BF subregions across 4 rats via local viral injections, as shown in figure 4.1. The helper viruses AAV-CA-FLEX-RG and AAV-eF1a-FLEX-TVAmCherry (Dr. Naoshige Uchida; UNC Vector Core) were introduced to a small subregion of the BF, and in a subsequent surgery the pseudotyped replication-deficient monosynaptic tracing vector, EnvA G-deleted Rabies-eGFP (Salk Institute) was injected at the same coordinate. The helper viruses successfully incorporate their genome into that of cells only in the presence of cre recombinase (thus only in ChAT+ cells in these animals) thereby allowing production of the rabies glycoprotein coat (RG) and the avian sarcoma leukemia receptor (TVA) in a fusion product with a red fluorescent reporter (mCherry). TVA allows the subsequently injected pseudotyped rabies vector to enter cells where it is present (in helper-infected ChAT cells), as the protein coat EnvA has no method of entry for wild-type mammalian cells, and RG then allows the replicated rabies virions, otherwise deficient of their mammalian protein coat (ΔG) to bud off the infected cholinergic cell and spread to those neurons having terminals synaptically connected to it, successfully labeling them with eGFP. It should be noted that

![Figure 4.1 Experimental schema, Aim II. Rats receive injections of the helper viruses, then after 3 weeks, injections of the modified rabies virus are made in the same location.](image)
these replication-deficient rabies vectors do not cause injury to the rats, do not cause them to suffer in the least, and in no way cause any of the symptoms of wild-type freely-replicating rabies. Viral constructs are represented in figure 4.2 and a schematic of this transfection paradigm appears in figure 4.3.

![Viral constructs diagram](image)

Figure 4.2 Viral constructs used. Modified from Watabe-Uchida et al., 2012 and Wall et al., 2010.
Figure 4.3 Monosynaptic transfection schema. Helper viruses AAV-CA-FLEX-RG (AAV-RG) and AAV-eF1a-FLEX-TVAmCherry (AAV-TVA) infect all cells, but only make viable products in cells expressing cre recombinase. The products RG and TVA-mCherry (TVA) are expressed, allowing entry of EnvA G-deleted Rabies-eGFP and monosynaptic spread of the viral progeny G-deleted Rabies-eGFP. After spreading, there is no method for further viral spread without the presence of helper viral products in the afferent neurons. (Modified from Wall et al., 2010.)
4.2.2 Helper virus surgery

Rats were anesthetized under isoflurane and placed in a stereotaxic apparatus. A single burr hole was created in the skull over the coordinate in the BF to be injected. Two separate micropipettes (35-45 µM tip diameter) were backfilled with the separate helper viruses, and each used to pressure inject (Drummond Nanoject II) these vectors at the same BF coordinate via separate injections. Injection volumes and coordinates, from bregma, ventral to pial surface, appear in table 4.1. Post-surgically, helper viruses were allowed 3 weeks for adequate expression of RG and TVA-mCherry.

4.2.3 Monosynaptic virus surgery

Three weeks following helper virus surgeries, rats were again anesthetized and placed in a stereotaxic apparatus. Burr holes were reopened at

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Sex</th>
<th>Injection coordinates (mm) from bregma &amp; pia</th>
<th>Helper virus volumes (µL) per vector</th>
<th>EnvA-ΔG-Rab-eGFP volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14109</td>
<td>F</td>
<td>-0.87 AP 2.3 ML 5.2 &amp; 6.5 DV</td>
<td>0.66 (total across two sites)</td>
<td>0.33 (total across two sites)</td>
</tr>
<tr>
<td>14110</td>
<td>F</td>
<td>-1.7 AP 3.4 ML -5.6 DV</td>
<td>0.44</td>
<td>0.22</td>
</tr>
<tr>
<td>14111</td>
<td>M</td>
<td>0.0 AP 1.65 ML -7.1 DV</td>
<td>0.44</td>
<td>0.22</td>
</tr>
<tr>
<td>14112</td>
<td>M</td>
<td>-1.72 AP 3.6 ML -5.8 DV</td>
<td>0.44</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Table 4.1 Local BF viral surgical species, amounts, and coordinates. DV: dorsal-ventral; ML: medial-lateral; AP: anterior-posterior. Note negative AP values signify distances posterior from bregma.
the same coordinate drilled previously in each subject. The pseudotyped vector EnvA G-deleted Rabies-eGFP (Salk Institute) was injected at the same coordinate where helper viruses were previously introduced, via the same injection method. Injection volumes and coordinates, from bregma, ventral to pial surface, appear in table 4.1. Post-surgically, this vector was allowed one week prior to transcardial perfusion to express eGFP in cells monosynaptically connected to those ChAT-cre cells expressing all three viral genomes.

Using fluorescent microscopy, cells were identified as expressing eGFP, mCherry, or both (figure 4.4). All eGFP-labeled BF cells in all sections were mapped in Neurolucida and placed in a 3D warped volume to be compared with a map of the entire cholinergic BF. All other methods employed in this aim are detailed in the General Methods chapter.

![Figure 4.4 Identification of transgenic cell types. BF cells were identified as containing ChAT and helper virus (red mCherry cells), or containing ΔG-rabies-eGFP (green cells) or containing all of these (indicated by arrowhead).](image)
4.3 Results

4.3.1 Validation of viable injection sites

The four subjects in this aim had injections centered in and adjacent to the BF. Due to the presence of cholinergic interneurons immediately outside the BF (e.g. in the accumbens and striatum), validation of transfection within the BF (and not outside) could not be achieved by injection site location alone. Therefore, all sections in and around the entire BF were evaluated in order to ensure there were no cholinergic cells outside the BF spreading the modified rabies virus.

It was thereby determined that, although some injection sites were centered just outside the BF (figure 4.5), the population of cholinergic cells expressing helper viral products and rabies products (i.e. candidates for monosynaptic rabies shedding) was entirely contained in the BF.
Figure 4.5 Injection site centers, Aim II. Injection centers are in or adjacent to the cholinergic BF. ac: anterior commissure; BNST: bed nucleus of stria terminalis; CPu: caudate putamen; EA: extended amygdala; f: fornix; GP: globus pallidus; ic: internal capsule; LPO: lateral preoptic area; Rt: reticular thalamus; SI: substantia innominata; sm: stria medullaris; VP: ventral pallidum.
4.3.2 Topography of local cells afferent to BF cholinergic cells

Cholinergic BF cells were found to have local BF afferents whose cell bodies lie in the immediate vicinity of the cholinergic cells capable of spreading the virus (figure 4.6). No afferent cells were found in the contralateral BF. The 3D view of figure 4.6 is useful to see the proportion of the BF taken up by intrinsic populations of neurons synapsing onto cholinergic cells in each subject are color coded: yellow is afferent to orange (subject 14112), light blue is afferent to dark blue (14110), light green is afferent to dark green (14109), and pink is afferent to red (14111). The x axis (red arrow) points laterally, the y axis (green arrow) points dorsally, and the z axis (blue arrow) points caudally. ac: anterior commissure; EA: extended amygdala; GP: globus pallidus; HDB: horizontal diagonal band; ic: internal capsule; MS: medial septum; opt: optic tract; SI: substantia innominata; VDB: ventral diagonal band.

Figure 4.6 Basal forebrain cholinergic cells receive inputs from proximal but not distal regions of the BF itself. An oblique view shows the full septo-pallidal axis of one hemisphere of the BF. A reference volume mapping every BF ChAT(+) cell (19,904 white dots) serve as the background delineating the entire space of the cholinergic BF. Local populations of neurons synapsing onto cholinergic cells in each subject are color coded: yellow is afferent to orange (subject 14112), light blue is afferent to dark blue (14110), light green is afferent to dark green (14109), and pink is afferent to red (14111). The x axis (red arrow) points laterally, the y axis (green arrow) points dorsally, and the z axis (blue arrow) points caudally. ac: anterior commissure; EA: extended amygdala; GP: globus pallidus; HDB: horizontal diagonal band; ic: internal capsule; MS: medial septum; opt: optic tract; SI: substantia innominata; VDB: ventral diagonal band.
network architecture, as well as the distance between double-labeled ChAT cells and their afferent cells relative to the entire length of the BF. These local afferent cells occupy a small volume relative to that of the whole cholinergic BF (table 4.2).

When cells were categorized by their cytoarchitectonic domain on the other hand, a different aspect of the results can be seen (table 4.3). In each experimental case, any cytoarchitectonic area containing eGFP(+)/mCherry(+)

Table 4.2 Volume containing local cells afferent to cholinergic BF cells. The volume of local afferent networks is compared to the volume of all cholinergic cells of the BF.

<table>
<thead>
<tr>
<th>Subject:</th>
<th>14111</th>
<th>14109</th>
<th>14110</th>
<th>14112</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of local afferents to cholinergic BF (mm$^3$)</td>
<td>0.51</td>
<td>1.43</td>
<td>1.9</td>
<td>1.83</td>
</tr>
<tr>
<td>Volume of cholinergic BF (mm$^3$)</td>
<td>11.41</td>
<td>11.41</td>
<td>11.41</td>
<td>11.41</td>
</tr>
<tr>
<td>Percent of BF volume occupied by local network</td>
<td>4%</td>
<td>13%</td>
<td>17%</td>
<td>16%</td>
</tr>
</tbody>
</table>

Table 4.3 BF cholinergic cells of individual subregions, and their afferent cells, cytoarchitectonically organized. A single cell in SI/EA is receiving local BF afferents arising from cells in the SI/EA itself and the VP, along with a much smaller proportion of afferent cells in the HDB, while no afferent cells are seen in MSVDB, GP, or ic. In three other subjects, cholinergic cells in ic and GP are receiving their local BF afferents from cells in the GP, IC, and to a lesser extent the SI/EA, while no afferent cells are seen in MSVDB, HDB, or VP.
double-labeled cells also contains some, yet not all of the eGFP-labeled cells afferent to these cells. In each case, there is one cytoarchitectonic area sending afferents to cholinergic cells found in other cytoarchitectonic area(s). Additionally, in each case, there are three whole cytoarchitectonic areas of the BF which send no afferents to the eGFP(+) /mCherry(+) double-labeled cells. Finally, for all subjects injected in the internal capsule, there appear double-labeled cells in the GP and ic, and eGFP-labeled afferent cell bodies in GP, ic, and SI/EA yet not in MSVDB, HDB, or VP.

4.4 Summary of the main findings

a) Cholinergic cells in the BF are innervated neither by the whole of the BF, nor by the whole of their home cytoarchitectonic domain.

b) Cholinergic cells in the BF are innervated by local cells in a distribution previously undefined, with afferent cell bodies lying across local cytoarchitectonic borders within the cholinergic BF.

c) Local BF innervation of ChAT cells is not constrained strictly on the basis of classical cytoarchitectonic zones, although a majority of labeled afferents appear to occupy the same area as their cholinergic targets.
CHAPTER V: TOPOGRAPHY OF CELLS SYNAPTICALLY CONNECTED TO CHOLINERGIC BF PROJECTION NEURONS
5.1 Introduction

Contrary to previous hypotheses, it is currently known that the ascending cholinergic projection of the basal forebrain is not diffuse (Sarter et al., 2009). The outputs of the cholinergic basal forebrain are heterogeneous and project to discretely innervated cortical areas (Price and Stern, 1983; Duque et al., 2007; for review see Semba, 2000). These various projections are differentially modulated such that acetylcholine efflux is increased in some cortical areas while not in others (Fournier et al., 2004). However, the mechanism by which these outputs are differentially controlled is the subject of some controversy. For instance, BF afferents from the PFC (Grove, 1988; Sesack et al., 1989; Carnes et al., 1990; Gaykema et al., 1991; Gabbott et al., 2005) have variously been thought to control cortical ACh efflux via sensory information relayed to the BF through the PFC (Golmayo et al., 2003) or not (Nguyen et al., 2015). Although one can describe some properties of a system physiologically, the conclusions made based on this are bounded in part by the anatomy of the system. The case remains that the relationship between the inputs and the outputs of the cholinergic basal forebrain is for the most part unknown.

Some BF inputs are known based on light or electron microscopy, as discussed in section 1.6 above. The contribution of each input to the BF output is unknown, as is the principal source of BF inputs for each region of cortex the BF sends outputs to. Which inputs are controlling which outputs? With the advent of viral tracing techniques this question can now be addressed. The experiment put forth in this chapter tests whether different BF cholinergic outputs receive the
same synaptic inputs, or if they differ, how. What emerges is a series of input
topographies specific to each cholinergic output.

5.2 Specific methods

Seven ChAT-cre transgenic Long-Evans rats (3 males, 420-560g; 4
females, 280-310g) underwent two survival surgeries each: the first for
administration of helper viruses to the BF, and the second for administration of a
replication-deficient rabies virus to the cortex or amygdala. By allowing the
pseudotyped rabies vector access to BF ChAT cells only via a particular field of
cortical or amygdalar axon terminals (similar to the distal application of rabies
vector as shown by Miyamichi et al., 2011), we were able to map the cell body
locations of afferents monosynaptically connected to cholinergic BF cells, which
themselves project to a specific cortical or amygdalar area.

5.2.1 Experimental design

We explored the differences in locations of cell bodies afferent to various
cortical-target-identified BF ChAT cell populations. This was achieved by first
injecting all subjects with both helper viruses mentioned in the previous chapter,
this time transfecting a large portion of the BF via 5 injection sites, and
subsequently injecting the same pseudotyped rabies vector mentioned in the
previous chapter into one specific field where ChAT terminals exist: either medial
prefrontal cortex (PrL/IL), orbitofrontal cortex (OFC; VO), motor cortex (M1/M2)
or the amygdala (BLA/CeC). The experimental design is further explained by the
schema in figure 5.1, and the explanation of viral spread appears in figure 5.2.
Figure 5.1 Experimental design, Aim 3. All subjects receive 5 injections of each helper virus across the BF. Three weeks following this, each subject receives an injection of the pseudotyped rabies vector into a particular region of cortex or amygdala as shown.
Figure 5.2 Viral system, Aim 3. Helper viruses AAV-TVA and AAV-RG are introduced across the basal forebrain, expressing TVA and RG only in cells expressing cre recombinase (here, ChAT+ cells). Pseudotyped Rabies-ΔG-eGFP is injected in cortex, where it enters the cholinergic axon terminal expressing TVA, allowing cells with all three viral genomes to produce and monosynaptically spread the Rabies-ΔG-eGFP progeny virions. Cells monosynaptically receiving this progeny express eGFP but can not spread the virus further without helper virus expression. Modified from Wall et al., 2010.
5.2.2 Helper virus surgery

Rats were anesthetized under isoflurane and placed in a stereotaxic apparatus. Four burr holes were created in the skull over the coordinates in the BF to be injected. Two separate micropipettes (35-45 µM tip diameter) were backfilled with the separate helper viruses, and each used to pressure inject (Drummond Nanoject II) these vectors at the same five BF coordinates via separate injections, aimed at all BF regions with the exception of the medial septum. Injection volumes totaled 2.66µL per helper virus per animal, and coordinates, from bregma, ventral to pial surface, appear in table 5.1. Post-surgically, helper viruses were allowed 3 weeks for adequate expression of RG and TVAmCherry.

5.2.3 Monosynaptic virus surgery

Three weeks following helper virus administration, subjects undergo a second survival surgery for cortical injection of the pseudotyped rabies vector. A single burr hole is created in the skull over the cortical or amygdalar coordinates to be injected. EnvA-Rabies-ΔG-eGFP is injected via micropipette at coordinates detailed in table 5.2. Post-surgically, this vector was allowed one week prior to transcardial perfusion to express eGFP in cells monosynaptically connected to those ChAT-cre cells expressing all three viral genomes.

All other methods employed in this aim are detailed in the General Methods chapter.
Table 5.1 Aim III helper virus injections. All stereotaxic coordinates (in millimeters) are taken anterior-posterior (AP) and medio-lateral (ML) from bregma, with injections made in the dorsal-ventral (DV) direction from pia.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sex</th>
<th>Helper injection coordinates</th>
<th>Each helper virus, volume per injection site</th>
</tr>
</thead>
<tbody>
<tr>
<td>14069, 14068, &amp; 13134</td>
<td>M</td>
<td>+0.5 AP, 1.05 ML, 6.7 DV; +0.0 AP, 1.65 ML, 7.1 DV; -0.87 AP, 2.3 ML, 5.3 &amp; 7.1 DV; -1.72 AP, 3.6 ML, 5.8 DV</td>
<td>0.44 µL, 0.66 µL, 0.44 µL &amp; 0.44 µL, 0.66 µL</td>
</tr>
<tr>
<td>14097, 14096, 14098, &amp; 14099</td>
<td>F</td>
<td>+0.5 AP, 1.05 ML, 6.0 DV; +0.0 AP, 1.65 ML, 6.5 DV; -0.87 AP, 2.3 ML, 5.2 &amp; 6.5 DV; -1.7 AP, 3.4 ML, 5.6 DV</td>
<td>0.44 µL, 0.66 µL, 0.44 µL &amp; 0.44 µL, 0.66 µL</td>
</tr>
</tbody>
</table>

---

Table 5.2 Aim III monosynaptic virus injections. All stereotaxic coordinates (in millimeters) are taken anterior-posterior (AP) and medio-lateral (ML) from bregma.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>EnvA-ΔG-Rab-eGFP injection site (as identified on slide)</th>
<th>EnvA-ΔG-Rab-eGFP coordinate: DV level taken from skull surface, with the exception of 13134, 14098 &amp; 14099, taken from pia</th>
<th>EnvA-ΔG-Rab-eGFP total volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>14069</td>
<td>M</td>
<td>PrL/IL</td>
<td>+3.15 AP, 0.5 ML, 3.9 to 4.7 DV</td>
<td>0.22 µL</td>
</tr>
<tr>
<td>14097</td>
<td>F</td>
<td>PrL/IL/DP/DTT</td>
<td>+3.15 AP, 0.5 ML, 3.9 to 4.7 DV</td>
<td>0.22 µL</td>
</tr>
<tr>
<td>14068</td>
<td>M</td>
<td>VO/Cl</td>
<td>+3.6 AP, 2.23 ML, 4.7 DV</td>
<td>0.11 µL</td>
</tr>
<tr>
<td>14096</td>
<td>F</td>
<td>VO</td>
<td>+3.6 AP, 2.23 ML, 4.7 DV</td>
<td>0.11 µL</td>
</tr>
<tr>
<td>14098</td>
<td>F</td>
<td>M1</td>
<td>+1.1 AP, 3.2 ML, 1.0 to 1.3 DV</td>
<td>0.11 µL</td>
</tr>
<tr>
<td>14099</td>
<td>F</td>
<td>M1/M2</td>
<td>+1.1 AP, 3.2 ML, 1.0 to 1.3 DV</td>
<td>0.11 µL</td>
</tr>
<tr>
<td>13134</td>
<td>M</td>
<td>BLA/CeC</td>
<td>- 2.16 AP, 4.756 ML, 7.55 DV</td>
<td>0.22 µL</td>
</tr>
</tbody>
</table>
5.3 Results

5.3.1 Injection site locations

Large injections of helper viruses resulted in satisfactory mCherry labeling throughout the BF in all subjects, with the exception of the MS, which was not injected in this study. In a subject with typical expression, 786 mCherry labeled cells were found, counting every fourth section. Extrapolated to the full hemisphere, it is estimated that 3144 cells (or roughly 16%) of cholinergic neurons are transfected with helper virus (786 * 4 / 19,904 ChAT cells per hemisphere). Care was taken that each subject has mCherry in VDB, HDB, SI/EA, GP and ic. Thus, in each subject, a sampling of the cholinergic volume becomes available for rabies transfection.

Subsequent cortical and amygdala injections of EnvA-ΔG-rabies-eGFP resulted in comparable pairs of subjects for injections to motor, prefrontal, and ventral orbitofrontal cortices, as well as a single subject with a good localization of virus between central and basal nuclei of the amygdala (figure 5.3).

5.3.2 Validation of proper transfection following retrograde application of virus

The method of injecting EnvA-ΔG-rabies-eGFP at the site of the terminals, not the cell body, of helper-virus-infected neurons is almost entirely untested in the literature (with the exception of Miyamichi et al., 2011). To ensure that this system of viruses is working as depicted in figure 5.2, the location of double-labeled cells in this aim (table 5.3, pink columns) can be compared with the location of BF cells in previous studies where retrograde tracer was injected in
Figure 5.3 Pseudotyped rabies virus injection sites. Injection sites are shown in grey at one or two anterior-posterior levels per subject. BLA: basolateral amygdala, anterior part; CeC: central nucleus of the amygdala, capsular division; CeM: central nucleus of the amygdala, medial division; Cl: claustrum; DP: dorsal peduncular cortex; DTT: dorsal tenia tecta; IL: infralimbic cortex; M1: primary motor cortex; M2: secondary motor cortex; PrL: prelimbic cortex; VO: ventral orbitofrontal cortex.
Table 5.3 Cytoarchitectonic domain of local BF cell bodies synaptically afferent to target-identified BF cholinergic cells. Injections of EnvA-ΔG-rabies-eGFP in cortex result in retrograde transport to BF ChAT cell bodies previously infected with helper viruses; a sampling of locations of these cell bodies is shown in pink. Local BF cells synaptically afferent to these double-labeled cells are able to be infected with resulting progeny virus and thereby express eGFP themselves; a sampling of locations of these cell bodies is shown in green.
cortex and cells double-labeled with tracer and cholinergic immunohistochemical methods are mapped. For the amygdala case, previous studies detailing cholinergic cells projecting to BLA place cell bodies in VP, SI/EA, and to a lesser extent GP (Woolf and Butcher, 1982; Carlsen et al., 1985). In the case of the PrL and IL, a previous study places cholinergic cells projecting to these areas mainly in the diagonal bands, with a lesser presence in SI/EA and GP/ic (Gritti et al., 1997). For motor cortex, we can compare with the orange bars in table 3.9 from aim 1 above, showing cholinergic projections most in GP, ic, and SI/EA. In the case of OFC, no detailed BF topography appears to be published. Given that each of these patterns of ChAT projection cell distributions in the BF resembles that in table 5.3, and given that no double-labeled cells in this aim were found outside of the BF, it would appear that the virus system results a fair sampling of the known basalocortical cholinergic projections.

5.3.3 Local BF cells afferent to target-identified BF cholinergic cells

When we consider local eGFP-labeled afferent neurons within the BF as they project to cholinergic cells, which themselves project to defined cortical areas, there appears a pattern similar to the results from aim 2. In each BF subregion containing eGFP-labeled afferent cell bodies (table 5.3, green), there are, in almost every case, fewer double-labeled cells to spread the virus, or none at all (table 5.3, pink). However, unlike in aim 2, there are eGFP-labeled cells found in almost all cytoarchitectonic areas across subjects, likely due to the larger distribution of double-labeled virus-spreading cells across the BF. An additional difference between this data and that of aim 2 is that this data is only a
sampling from one-quarter of the sections of the brain, and therefore a different kind of interpretation will follow. The point of local BF afferents to cholinergic cells and what can be concluded from these particular data is revisited in a broader theoretical context in the general discussion chapter.

5.3.4 Global inputs to target-identified BF cholinergic cells

Cell bodies synaptically afferent to target-identified cholinergic cells were mapped and combined in a dataset including areas outside of the BF itself, thereby including long-distance afferents and excluding intrinsic connections. The distribution of afferent neurons was compared in sections at similar anterior-posterior levels from four representative subjects (figures 5.4-5.10). An overview of afferent cell bodies for each subject is shown in figure 5.11.

At rostral levels (figure 5.4), there is a high incidence of afferent neurons in nucleus accumbens for the amygdala-injected case, and a larger projection of striatum in the M1 and VO injected cases. This trend continues further posterior (figure 5.5), where there are also many afferent somata in the dorsal part of the lateral septal nucleus in the PrL/IL injected cases. At the level of the crossing of the anterior commissure (figure 5.6), dominance of afferents arising from the striatum in the M1-injected case is apparent, while afferents from the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) are present in all subjects, and a particularly dense source of inputs for the amygdala case arises from the BNST-AL. Further posterior, at rostral amygdala levels (figure 5.7), many afferent cells are evident in the central nucleus of the amygdala and adjoining amygdalo striatal transition area for both BLA/CeC and M1 injected
subjects. Further posterior yet (figure 5.8) can be seen the parafascicular afferent cell bodies common to all cases, as well as afferent cell bodies from the subthalamic nucleus, notably lacking a strong projection in the PrL/IL cases. Notable structures with afferent cell bodies common to all subjects also include the substantia nigra and VTA (figure 5.9) and raphe and reticular brainstem (figure 5.10).

Figure 5.4 Maps of four subjects from Aim III. ac, anterior commissure; Acb, nucleus accumbens; CPu, caudate putamen; LSI, intermediate lateral septum; Pir, piriform cortex; Tu, olfactory tubercle; VP, ventral pallidum.
Figure 5.5 Maps of four subjects from Aim III. ac, anterior commissure; Acb, nucleus accumbens; cc, corpus callosum; CPu, caudate putamen; ec, external capsule; lo, lateral olfactory tract; LSD, dorsal lateral septum; LSI, intermediate lateral septum; Pir, piriform cortex; Tu, olfactory tubercle; VP, ventral pallidum.
Figure 5.6 Maps of four subjects from Aim III. ac, anterior commissure; BNST, bed nucleus of stria terminalis; cc, corpus callosum; CPu, caudate putamen; ec, external capsule; fi, fimbria; HDB, horizontal diagonal band; ic, internal capsule; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; lo, lateral olfactory tract; LSD, dorsal lateral septum; LSI, intermediate lateral septum; LV, lateral ventricle; MPA, medial preoptic area; och, optic chiasm; Pir, piriform cortex; VP, ventral pallidum.
Figure 5.7 Maps of four subjects from Aim III. BLA, basolateral amygdala, anterior part; CeA, central amygdala; CPu, caudate putamen; ec, external capsule; f, fornix; fi, fimbria; GP, globus pallidus; ic, internal capsule; opt, optic tract; Rt, reticular thalamic nucleus; SI/EA, substantia innominata and extended amygdala; sm, stria medullaris; VA, ventral anterior thalamic nucleus; VL, ventrolateral thalamic nucleus.
Figure 5.8 Maps of four subjects from Aim III. 3V, third ventricle; DLG, dorsal lateral geniculate; f, fornix; fr, fasciculus retroflexus; LH, lateral hypothalamus; mt, mammillothalamic tract; PF, parafascicular thalamic nucleus; STh, subthalamic nucleus.
Figure 5.9 Maps of four subjects from Aim III. Aq, cerebral aqueduct; fr, fasciculus retroflexus; MG, medial geniculate nucleus; pc, posterior commissure; Po, posterior thalamic nuclear group; R, raphe nucleus; SN, substantia nigra; VTA, ventral tegmental area.
Figure 5.10 Maps of four subjects from Aim III. Aq, cerebral aqueduct; PAG, periaqueductal gray; R, raphe nucleus; Ret, reticular formation.
Figure 5.11 Origin of afferents from outside the BF synapsing onto target-identified BF ChAT cells. Following EnvA-ΔG-rabies-eGFP injection in either motor cortex, medial prefrontal cortex, orbitofrontal cortex, or the amygdala, those ChAT cells innervating these targets spread ΔG-rabies-eGFP to label their afferents outside the BF. Local BF afferents are not included.
5.3.5 Striatal cells afferent to target-identified BF cholinergic cells

The maps of most cells afferent to these target-identified ChAT cells are best represented by broad summaries, as presented in figure 5.11. However, in the specific case of striatal afferent cell bodies, a striking topography emerged depending on which cortical target received EnvA-ΔG-rabies-eGFP. Motor-injected subjects displayed afferent cells predominately in anterior and ventral parts of the striatum, the amygdala-injected subject had afferent cells almost entirely in the posterior half of the striatum, the ventral orbital-injected cases exhibited diffuse afferent cells through the entire anterior-posterior extent of the striatum, yet tended more medial, and the medial prefrontal-injected animals displayed a small number of afferent cells in the anteromedial and amygdalostratial regions. A summary of this pattern is shown in figure 5.12.

Figure 5.12 Topography of striatal cell bodies synapsing onto target-identified ChAT cells. Representative levels through the caudate-putamen show overlap and segregation of afferent somata in each case. Cells afferent to M1/M2-targeted ChAT are found in the diagonal cross-hatched area, cells afferent to PrL/IL/DP/DTT-targeted ChAT are found in the area with curvy lines, cells afferent to VO-targeted ChAT are found in the dotted area, and cells afferent to BLA/CeC-targeted ChAT are found in the area with the vertical-horizontal gridlines. Templates based on Paxinos and Watson, 2007.
5.4 Summary of the main findings

a) Retrograde labeling of ΔG-rabies-eGFP following cortical injection reproduces the BF topography of ChAT cells projecting to cortical areas shown in previous conventional retrograde tracer studies, indicating monosynaptic spread originating from a representative sample of each target-identified cholinergic projection population.

b) The origin of somata afferent to BF ChAT cells is heavily dependent on the cortical target of the ChAT cells’ axon terminals.

c) The origin of cells afferent to target-identified BF ChAT cells is reproducible across subjects when the same cortical target is under study.

d) There appears to be a differential topography of striatal projection to the cholinergic BF depending on the cortical target of the ChAT projection.
6.1 Topography of BF ChAT cells projecting to M1 and V1/V2 (Aim 1)

Utilizing retrograde tracing, this study defined in each subject the codistribution of cells projecting to $M_{1_{\text{FL}}}$ and V1/V2, categorizing these projection cells as cholinergic or noncholinergic with immunohistochemistry, and classifying them into cytoarchitectonic zones. The original rationale for this study was to identify any region of overlap between cholinergic projections to both cortices, and thereby generate stereotaxic coordinates for further experiments probing the physiological parameters and behavioral utility of this co-projection. However, the results raised much more immediate concerns regarding the current concepts about the anatomy of the BF.

6.1.1 Limitations and strengths

Studies using retrograde tracing have a number of limitations. First, they are taken up non-selectively by terminals in cortex, while it would be more informative to label cells retrogradely from genetically-targeted cortical cell types (e.g. to probe the difference between projections to pyramidal cells versus interneurons). Second, in order to use multiple fluorophores emitting different wavelengths of light in a single animal, one must use retrograde tracers of different chemical character, which do not all achieve the same efficacy.

Third, there is another limitation that arises when one considers those cells double-labeled with both retrograde tracers, whether cholinergic or noncholinergic. There were so few detected that no conclusion can be made about the BF subregion they might regularly appear in. Such cells may be extremely important for the animal, but given that retrograde tracing deals with
populations of cells best, those cells projecting to both motor and visual cortices might be better studied individually, e.g. via juxtacellular methods or genetic targeting.

A further limitation of this study affects the interpretation of results. From this dataset one can understand the topography and identity of BF cell bodies projecting to motor and visual cortex, but no conclusion can be made about the meaning of overlap of these projections for the behaving animal. Cells of both populations within the overlapping region might share inputs, or might not. This particular limitation proved to be a hidden strength, as it led to the development of the experimental questions of aims 2 and 3, which do address this question, even if not addressing the visual projection in particular.

6.1.2 Potential further studies

While it is known that acetylcholine modulates cortical interneurons as well as pyramidal cells (Disney et al., 2006), it is not known whether different innervations among cortical cell types might be reflected in a cell topography at the level of the BF, in a manner similar to the differential topography of BF cell bodies projecting to different cortical regions. Similarly, there is no controlling for which cortical layer receives retrograde tracer: by necessity injections are made in a bolus large enough to traverse multiple cortical layers. This would be a worthwhile area of study, as it is known that ACh has layer-specific effects (Poorthuis et al., 2013). Fortunately, it is now feasible to perform layer-specific tracing experiments using novel transgenic tools (Rowland et al., 2013).
6.2 Distribution of local somata afferent to cholinergic cells (Aim 2)

This study utilized viral tracing to label local cells afferent to cholinergic cells in several small subregions of the BF. One reason to investigate this topic was to identify the direction of information flowing through the BF. If there were local somata afferent to any given cholinergic cells arising from all parts of the BF, one would expect a mixing of inputs prior to basalocortical output. However, this same mixing might be achieved on a local scale all the same, since many cholinergic projection populations overlap to some extent in the distribution of their cell bodies in the BF, and local afferent cells could contact both projections within this zone of overlap.

6.2.1 Local synaptically-afferent cells in relation to previous findings

The distribution of afferent cells arising from one part of the BF to another was described, at the scale of querying the whole BF, only via classical tracing methods, with results difficult to interpret due to the uptake of these tracers by the pervasive fibers of passage in the BF. Using classical tracers in the BF, it seemed that afferents could arise from many parts of the BF, as it was reported that MS/VDB, HDB, and VP projected to subpallidal regions (SI), and that SI and HDB projected to peripallidal regions (GP/ic) (Carnes et al., 1990). However, using viral tracing to monosynaptically identify somata afferent to cholinergic cells, the experiment from the current aim 2 found more spatially limited afferent somata, and of those previously traced by Carnes and colleagues, only VP projecting to SI/EA and SI/EA projecting to GP/ic were verified. A direct
comparison is perhaps premature though, as aim 2 does not address afferents to noncholinergic cells, which may be more widespread.

6.2.2 Limitations and strengths

Subject 14111 had an injection centered in the BNST, immediately proximal to the medial VP/SI just caudal to the crossing of the anterior commissure. However, what one might presume to be grounds to remove this subject from the dataset was actually a non-issue. Given that every section from the basal forebrain was mounted and mapped, the locations of all double-labeled cells (capable of spreading virus monosynaptically) are known, and in the case of this subject, there was only one double-labeled cell, found not in the BNST, but in the SI/EA. Given that there was only one cell spreading virus, this turns out to be a strength of this experiment. The single-labeled eGFP for subject 14111, found in a discrete volume of VP and SI/EA along with one cell in the HDB, is all synaptically connected to a single cell in SI/EA. Thus, in figure 4.6, the pink cell bodies are a sampling of cells synaptically afferent to the single red cell.

In the other three subjects, as there are multiple double-labeled cells, it is impossible to know which single-labeled eGFP cells are connected to which double-labeled cells; rather, we can describe the local BF such that each eGFP cell is connected to at least one of the double-labeled cells in the same subject.

One additional concern is lack of a positive control. If the progeny monosynaptic virus does not easily cross synapses of certain types (as in Wall et al., 2013), and such difficulty is not labeling local BF afferents from distal parts of the BF, there may indeed be inputs to ChAT cells from all over the BF, as is
reported in classical tracing studies. There was no opportunity to make a positive control for this, for instance by introducing a nonselective nonreplicating retrograde viral tracer in the same BF locations and viewing the spatial extent of labeling, (as in Wall et al., 2013).

6.2.3 Conclusions, anatomical implications, and potential further studies

For the subregions of the BF under study in aim 2, it can be said that cholinergic cells enjoy a local source of input from within the BF that is spatially limited in volume, rather than spread out over the entire BF from medial septum to posterior pallidum. The spatial limit of the distribution of afferent cells is reproducible across subjects with different injection sites. Additionally, the two subjects with similar injection sites in the ic (14110 and 14112) display remarkably similar pools of afferent cells, demonstrating reproducibility of this result within a particular BF subregion.

These results could potentially mean one of two things. If hypothetically this experiment was repeated for every point in the BF ad nauseam, it could be predicted that either (a), each injection results in a slightly different center of mass of afferent cell bodies, meaning that hundreds of injections at slightly different points in the BF have overlapping pools of inputs, or (b), each injection falls within one of several defined networks which overlap minimally in space, giving an entirely different, modular network connectivity. With the prospect of one of these interpretations within reach, a follow-up experiment adding more subjects may yield a more decisive theoretical leap as a result.
6.3 Topography of afferent cells synaptically connected to target-identified cholinergic BF projection neurons (Aim 3)

This experiment utilized a monosynaptic viral tracing system to create maps of cell bodies afferent to cortical-target-identified BF ChAT neurons. The results show that each cortical area receives a BF ChAT projection that is itself innervated by a specialized pool of afferent cells, and that this pool of afferents is reproducible per each cortical area under study. It is easily recognizable that motor, medial prefrontal, and ventral orbital subjects have different combinations of BF afferent cells between groups while they are each similar within-group, as can be seen in figure 5.11. This section offers some limited speculation as to the functional relevance of this data.

6.3.1 Confirmation of historically described BF afferents

When the neurons synapsing onto cholinergic BF neurons from aim 3 are considered as a sample of general afferent cells, without respect to the cortical target of the cholinergic cells, one can compare these afferents with what was previously described in the literature. In this way, afferents synapsing onto BF cells as confirmed via EM can be reiterated, and described afferents that were not confirmed (putative contacts described with simple retrograde or anterograde tracers) can be confirmed as a synaptic contact onto cholinergic cells in certain cases. Without respect to the method of previous identification or level of confirmation of the synapse, comparisons between the current results and those previously described in the literature are summarized in table 6.1. In addition to confirming previously putative synapses, data from aim 3 adds another level of
information, analyzing input streams separately according to cholinergic target, and identifying the comparative strengths of inputs from various afferent areas according to cholinergic target.
Table 6.1 Areas synaptically afferent to cholinergic BF confirming historical reports. Afferents historically described, with or without synaptic confirmation by electron microscopy, reported afferents to the BF arising from areas in the left column, all of which are confirmed as sources of synaptic inputs to cholinergic BF cells in aim 3.

6.3.2 Theory of prefrontal control of cholinergic BF efflux

The results from aim 3 indicate that differential control of cholinergic BF projections to different cortical areas is likely achieved by the large differences in overall afferent cells, mostly subcortical in origin, impinging on each of these cholinergic projection neurons. The importance of the mPFC in controlling the cholinergic BF outputs is well known: as electrical stimulation studies predict (Golmayo et al., 2003), muscarinic and glutamatergic agonists in the PFC.
increase ACh efflux in the posterior parietal cortex (Nelson et al., 2005), while the GABA-A agonist muscimol in the PFC decreases ACh efflux in the auditory and somatosensory cortex (Rasmusson et al., 2007). However, the results from aim 3 indicate that, of the inputs from outside the BF synapsing onto ChAT cells, on average 2% arise from the mPFC, whereas an average of 10% arise from other cortical areas. Thus, the possibility arises that other afferents to the BF are at least as potent in modulating cortical ACh efflux as is the mPFC, although this remains untested.

In understanding the role of the mPFC in influencing the BF cholinergic output, it should be noted that this 2% of afferent cells synapsing onto cholinergic cells is not the only component at play. Medial prefrontal cells are known to synapse onto noncholinergic cells of the BF (Záborszky et al., 1997), some of which may affect cholinergic projections indirectly via local connections. Further studies regarding the cortical influence on cholinergic BF cells might make use of this information by considering not only the inputs to noncholinergic BF cells, but also the afferents from a range of cortical regions beyond the medial prefrontal alone.

6.3.3 Amygdala, extended amygdala, bed nucleus and related structures

The amygdala and associated structures form synapses on different target-identified BF cholinergic cell groups in differing proportions (figure 6.1). The central nucleus innervates cholinergic projections to cortical areas as seen in the six subjects with frontal cortical pseudotyped rabies injections, although this
Figure 6.1 Afferents from amygdala and extended amygdala. Locations of eGFP+ cells afferent to target-identified ChAT cells are shown, including local afferents from within the BF itself. Note that the SI/EA falls in both categories of the cholinergic BF and the extended amygdala, thus here it is shown independently of the rest of the BF, while in other parts of this document it is grouped with the local BF rather than with extra-BF inputs.
is a relatively minor proportion of total afferent somata, with the exception of the larger CeC input in both motor cases. The most prominent individual difference is the larger proportional influence of amygdalar afferents in the case of 13134, in which the viral-spreading cells send axons back to the amygdala (BLA and CeC, although due to the lack of ChAT in CeC, it can be assumed that this is mainly reflective of a projection to BLA). With this reciprocal circuit, central amygdala exerts direct synaptic influence over the cholinergic efflux of the BLA, while comparatively little (or no) influence exerted by other areas within the amygdala.

This is a potentially critical detail given the known functions of components in this circuit. It is known that BF ChAT cells exhibit a variety of effects on BLA interneurons and principle cells, the net effect of which is proposed to be an increase of signal-to-noise ratio for BLA outputs (Ünal et al., 2015). The BLA impinges directly and indirectly on the output cells of the central amygdala. Signals carrying diverse information from other amygdalar nuclei innervate different central nuclei regions topographically (for review see Cassell et al., 1999; Olucha-Bordonau et al., 2015). The projection of all subregions of the central nucleus back to BF cholinergic cells which themselves project to the BLA could provide a type of teaching signal when it comes to associating cues of various types with unconditioned stimuli of some affective valence, potentially an important and quick mechanism for the amygdala to update its state, especially since the central nucleus for the most part sends few reciprocal connections back to other amygdaloid nuclei directly. Additionally, this feedback to the cholinergic projection may be a candidate for controlling short-term increases in ACh efflux,
such as is seen in the BLA during spontaneous alternation training and recall, a BLA-involved task (McIntyre et al., 2003).

With regards to the other six subjects, all reporting afferent cells from the central nucleus contacting target-identified ChAT cells, this projection may serve as an update for the entire cholinergic ascending system, such that general cortical arousal can be quickly changed via the BF in the event of sudden increase in central nucleus output. The extended amygdala / SI, IPAC, and BNST may be serving a similar function via the cholinergic BF, although further study, both physiological and behavioral, is needed to tease apart the differential functions of these projections, if any.

6.3.4 Afferents from septal nuclei

The large between-group differences in afferent cells from septum underscores an important function of the cholinergic projection to mPFC. As seen in figure 5.11, afferent somata from outside the BF contacting target-identified cholinergic cells arise from the septal nuclei (excluding medial septum) in varying proportions: for cholinergic cells targeting the mPFC, this proportion is 19-20% of extra-BF afferent cells, yet for all other BF targets under study, septal areas comprise just 2% or less of extrinsic inputs. Within septal nuclei, the area with the largest proportion of afferent cells was consistently the lateral septum (LS), with a particularly high density in the dorsal lateral septum (for an example of this topography, see figure 5.5).

Lateral septal lesions have been described alternately as anxiogenic (Yadin et al., 1993) and anxiolytic in rats (Menard and Treit, 1996). Electrical
stimulation of the LS inhibits cells of the central nucleus, while stimulation of the central nucleus excites the LS (Thomas et al., 2012). The neural pathway by which this occurs remains unclear, as there are only very sparse labeling of LS axons in central and medial amygdala (Risold and Swanson, 1997). One potential route for this information could be through the mPFC, via the cholinergic BF. This could potentially explain the paradoxical anxiogenic and anxiolytic roles of the LS, as the PrL and IL have been shown to have a similar duality of effect on the central nucleus (Vidal-Gonzalez et al., 2006). However, further study would be needed to either reject or confirm this candidate pathway.

6.3.5 Afferents from caudate putamen and the amygdalostratial transition zone

Cholinergic cells projecting to different cortical areas receive different monosynaptic inputs from the caudate putamen (CPu) in a topographic manner (figure 5.12). There is a previously described topography of the CPu: that of its inputs. Anterograde tracers placed in various orbital cortical and thalamic areas result in a topography of labeled fibers within the CPu (Berendse and Groenewegen, 1990; Schilman et al., 2008), and retrograde tracers placed in varying parts of the CPu result in a varied pattern of labeled cell bodies in particular cortical areas (Kelley et al., 1982; McGeorge and Faull, 1989; Deniau et al., 1996; Reep et al., 2003). Interestingly, the topography of striatal inputs (as separately described by Kelley, Deniau and Reep) appears to map onto the topography of striatal outputs directed towards target-identified cholinergic cells of the basal forebrain, such that it may be possible for all four cortical areas under study in this aim to participate in their own three-node striatal-cholinergic-
corticostriatal loop, partially segregated from the others, as postulated in figure 6.2. However, this schema may merely represent a small portion of circuits linking the regions involved, as individual cortical areas appear to send axonal arbors to more vast regions of the striatum (Kelley et al., 1982).

It is worth noting that the overall striatal counts for aim 3 include the amygdalo striatal transition area (ASt), here grouped with the caudate putamen. Given the lack of obvious cytoarchitectonic boundary between the CPu and ASt under Nissl stain, the two structures were not delineated separately, due to the inherent variability across subjects it would create. Qualitatively speaking, ASt labeling was present in M1/M2, PrL/IL/DP/DTT, VO, and BLA/CeC cases.

6.3.6 Afferents from nucleus accumbens

The accumbens was found to project to target-identified cholinergic cells in all subjects but one, # 14068 (figure 5.11). However, the lack of reported
afferent cells in this subject could be due to a sampling error, as this animal displays far less eGFP labeling than the other ventral orbital subject, and indeed the lowest of all subjects, at just 156 afferent cells total outside the BF, and the overall pattern of areas projecting in both ventral orbital subjects is similar. The most striking difference is seen for subject 13134 with pseudotyped rabies injection in the amygdala: of mapped afferent cells outside the BF, accumbens makes up 36% of these for this BLA/CeC injected animal, whereas for other subjects the average proportion of afferent cells from accumbens is just 5.6%.

As above, it can reasonably be assumed that in this subject, the portion of the amygdala receiving most labeled afferent cells would be the BLA, as the CeC has a near-total lack of ChAT, and the BLA displays one of the highest ChAT densities in the brain (Carlsen et al., 1985). Given this assumption, along with the knowledge that a major input to the accumbens arises from the BLA, it would appear that a loop can be defined. Those accumbens cells that synapse onto BF cholinergic cells projecting directly to the BLA are likely themselves innervated by the BLA. Both BLA lesions and scopolamine infusion cause deficits in conditioned place preference acquisition (White and McDonald, 1993; McIntyre et al., 1998). The cholinergic-mediated lengthening of BLA principle cell afterdepolarization, proposed to be a mechanism by which stimuli are associated by the structure (Ünal et al., 2015), may allow a window of time for the BLA to update itself with a fresh signal regarding motivational salience of current stimuli (in the accumbens), via this back projection. Further studies would be useful in
either confirming or rejecting this pathway as a viable updater of signals processed in the BLA.

6.3.7 Afferents from brainstem

A variety of inputs arise from brainstem structures to innervate target-identified BF cholinergic cell groups. Figure 6.3 shows the proportion of brainstem afferent cell bodies as compared to the entirety of other eGFP-labeled cells in each subject, including local afferent cells from the BF. Dopaminergic centers, Raphe nuclei, and the reticular formation account for the origin of most commonly labeled cells. Afferent cells in each subject arise mostly from the same brainstem centers, although the proportion of all afferent cells in each subject arising from the brainstem in particular varies according to location of rabies injection. The brainstem accounts for 13-16% of inputs to ventral orbital-targeted ChAT cells, while this proportion is less in the cases of mPFC (10%), motor (5-7%), and especially BLA/CeC (1.5%).

Brainstem inputs to the telencephalon are notoriously diffuse, and supply information regarding the behavioral state across medium to long timescales. The between-group difference in afferents arising from brainstem could reflect the strength of behavioral state information required for temporally appropriate cholinergic efflux of specific cortical structures. On the other hand, since the chemical identity of these afferent cell bodies is not part of the current dataset, a projection from the same brainstem structure in two different groups could be supplying different
Figure 6.3 Afferents from brainstem. Locations of eGFP+ cells afferent to target-identified ChAT cells, including local afferents from within the BF itself (included in 'other'). VTA_SN_RRF combines ventral tegmental, nigral and retorubral dopaminergic centers. LC_PB combines locus coeruleus and parabrachial nuclei. DMTg, CI, DK combines various oculomotor nuclei.
neurotransmitters in each case, as some brainstem centers house heterogeneous projection cell types in a single cytoarchitectonic domain. Finally, brainstem cells afferent to cholinergic cells may have less to do with the particular cortical target innervated by acetylcholine and more to do with the regulation of sleep-wake states instead. Whichever case is most likely, the functional implication of these results could be clarified by further investigation of this monosynaptic pathway.

6.3.8 Limitations and strengths

Helper virus injections in the BF were made across the BF, resulting in similar regions of expression in every animal. However, medial septum was not targeted for helper injections. Medial septum efferents are mainly directed at the hippocampus and entorhinal cortex, and is not a major component of efferent pathways to motor cortex (Baskerville et al., 1993), prefrontal cortex (Bigl et al., 1982), or the amygdala (Nagai et al., 1982), therefore it was omitted.

One peculiarity of working with multiple viruses in a transgenic animal is that the resulting transfected cells are only a subsample of the cells under study. This is a result of a number of compounding factors. Firstly, the cells transfected by both helper viruses are not 100% of available ChAT-cre cells, as viruses can fail to deliver their genome to the nucleus or even fail to endocytose. Secondly, supposing a number of cells have both helper viruses and also project to the cortical site of pseudotyped rabies, some rabies will not succeed in traversing the many millimeters of axon stretching between the terminals and nucleus of the cell. And finally, when the progeny virus is budding off to synapses contacting the
starter cell, some synapses will fail to take up the virus for reasons unknown (Wall et al., 2013). The resultant eGFP-labeled afferent cells are but a "sample of a sample" of the population of afferent cells of interest to this study. This is the main reason for including multiple subjects per cortical injection site. Given that afferent cells are reproducible between subjects with the same cortical injection, it would appear that results from this dataset are robust enough that this repeated sampling does not impede interpretation, although this limited sample means that it can not be claimed that afferent cells do not arise from certain areas, but merely that they were not detected. This limitation may be leading to an underrepresentation of afferents from certain areas, such as the prefrontal cortex and locus coeruleus, which were reported as much more prominent afferent sources in classical tracing publications. Further study is needed in order to confirm this reported smaller proportion of inputs from these areas.

6.3.9 Conclusions and functional implications

The current results demonstrate that the various somata afferent to the cholinergic basal forebrain are mostly segregated on the basis of where the cholinergic cells are projecting to in addition to where the cholinergic cells are located along the medio-lateral-rostro-caudal axis of the BF. Some key structures are represented to some extent in every subject (brainstem, striatum, septal nuclei, amygdala, prefrontal cortex, IPAC, thalamus, and hypothalamus), yet the proportions of the total afferent cells coming from each region varies widely depending on which region of cortex the infected cholinergic cells target (figure
Furthermore, the topography of cells within each of these areas across subjects varies as well, as is seen in the case of the striatum (figure 5.12).

This monosynaptic input-output relationship speaks to the heterogeneity of functions the various cortical cholinergic projections subtend, which would not likely be controlled in such a temporally independent manner were the afferents to the cholinergic BF all diffuse. When comparing between basalocortical projection streams, these inputs fall somewhere between a diffuse architecture and an orthogonal one, and given the heterogeneity of inputs reported here, the architecture may lean towards the orthogonal side (figure 6.4).

Figure 6.4 Schematic of potential theoretical outcomes of Aim 3. Given the many unknowns regarding afferents to the BF, this diagram presents two extreme possibilities: completely convergent inputs (left) or completely orthogonal inputs (right), with green cells indicating the hypothetical viral spread of eGFP from the monosynaptic viral injection site (green dashed circle). Given the results of Aim 3, a diagram reflecting the true network architecture may be somewhere between these two, with some convergence, but to a large degree orthogonalized.
6.4 General conclusions

The experiments detailed in this document underscore the utility of employing methods beyond classical retrograde tracers when attempting to determine BF organization. Local innervation of ChAT cells from afferents originating within the BF is limited to smaller pockets, a surprising finding due to the few studies published previously on the intrinsic BF network, which relied on classical tracers and predicted connections across large distances in the BF, at the time fueling the argument for a diffuse system. Still, these previous studies might in fact describe a component of the BF, as in the current experiments the intrinsic afferents of noncholinergic BF cells were not investigated, and it could potentially be the case that noncholinergic BF projection neurons are more promiscuous in contacts they receive from within the BF. Further studies would undoubtedly be useful to determine what, if any, the difference is between afferents to cholinergic and noncholinergic BF cell groups.

Another surprise comes with the discovery of a topography of dorsal striatal inputs as dependent on the target of BFc outputs. It was long since known that the caudate putamen was not a unitary structure in its anatomy or function, and multiple differential connections are seen between striatum and cortex as well as subcortical structures, however, such topography was never described for striatal inputs to the BF, much less according to the targets of cholinergic cells upon which synapses arrive. As the literature regarding the function of the caudate putamen evolves, these results will likely prove useful to investigators for whom elucidating the intricate functions of the striatum is a fundamental goal.
There is utility in verifying the synaptic contact of mPFC afferents onto cholinergic BF cells, which was only previously described at the physiological and light-microscopic levels, and which was thought to be a polysynaptic interaction by those who looked for this synapse via electron microscopy. The mPFC, while occupying a central role in modulating BF cholinergic output to cortex, emerges here as a smaller proportion of the total inputs to ChAT cells than its functional notoriety might lead one to believe.

Generally, this work marks the first evidence for an anatomical organization by which spatiotemporally discrete ACh efflux might occur in different cortical regions. The potential hopefully exists for the present results to give rise to a number of future studies examining the functional influence of information throughput via parallel cholinergic input-output streams.
CHAPTER VII: BIBLIOGRAPHY


