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Organization of basal ganglia circuits

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"It is not down on any map; true places never are".

— Herman Melville

To my parents.

Thesis advisor: Konstantinos Meletis Daniel Fürth

Organization of basal ganglia circuits

Abstract

Anatomical regions and how we define boundaries between and within structures in the brain put a constraint on our understanding of the brain. More than a century of research on the brain has revealed its fundamental organization to be based on single nerve cells communicating through electrical signals over chemical synapses. The fundamental organization on higher levels than circuits has been harder to unravel and often left to intuitive definitions based on immediate visual appearances like cell density or other cytoarchitectural criteria. In this thesis, I've developed an anatomical method where the entire mouse brain is reconstructed from tissue sections. This method combined with a genetic strategy for retrograde transsynaptic labeling of genetically-defined populations lets us tease apart the connectivity in an interconnected set of subcortical nuclei known as the basal ganglia. The method can further be extended across a large number of cell types to find generic patterns of connectivity across the entire brain. Lastly, the method enables us to utilize recent advances in mRNA sequencing to resolve the entire transcriptome of a single brain. The method, therefore, enables investigators to map out the fundamental organization of the brain both in terms of gene expression and connectivity, thereby providing a novel way to redefine neuroanatomy.

Thesis advisor: Konstantinos Meletis Daniel Fürth

List of papers

This thesis is based on the following papers. Reprints were made with the permission from the respective publishers.

Paper I Pollak Dorocic, I., **Fürth, D.**, Xuan, Y., Johansson, Y., Pozzi, L., Silberberg, G., Carlén, M., and Meletis, K. A Whole-Brain Atlas of Inputs to Serotonergic Neurons of the Dorsal and Median Raphe Nuclei *Neuron*. 2014; 83: 663-678

Paper II **Fürth, D.**, Vaissière, T., Ourania, T., Xuan, Y., Märtin, A., Lazaridis, I., Spigolon, G., Fisone, G., Tomer, R., Deisseroth, K., Carlén, M., Miller, C., Rumbaugh, G., and Meletis, K. An interactive framework for whole-brain maps at cellular resolution *Nature Neuroscience*.(*accepted*)

Paper III **Fürth, D.**, Ourania, T., Xuan, Y., Yoo, E., Lazaridis, I., Carlén, M., and Meletis, K. A monosynaptic scalable architecture revealed by whole-brain transsynaptic rabies tracing (*manuscript*)

Paper IV **Fürth, D.**, Märtin, A., Navarro, J.F., Jurek, A., Frisén, J., Lundeberg, J., and Meletis, K. Atlas of the spatial transcriptome in adult mouse brain (*manuscript*)

Contents

List of abbreviations

AAV: adeno-asscoiated virus
ACB: nucleus accumbens
ACh: acetylcholine
BAC: bacterial artificial chromosome
cDNA: complementary DNA
CMV: cytomegalovirus
CNS: central nervous system
CP: caudateputamen (Allen Reference Atlas term.)
CPu: caudateputamen (Paxinos & Franklin)
D1: dopamine receptor 1
D2: dopamine receptor 2
DIO: double-floxed invertedopen ORF
DNA: deoxyribonucleic acid
Drd1: dopamine receptor D1 (mus musculus gene)
Drd2: dopamine receptor D2 (mus musculus gene)
EF1a: elongation factor 1-alpha
EGFP: enhanced green fluorescent protein
EnvA: avian sarcoma leukosis virus envelope type A
EPSP: excitatory postsynaptic potential
FISSEQ: fluorescent *in situ* sequencing
G: rabiesvirus envelope glycoprotein
ΔG: glycoproetin-deleted
GABA: gamma-Aminobutyric acid
GESTALT: genome editing of synthetic target arrays for lineage tracing
GPe: globus pallidus, external segment
GPi: globus pallidus, internal segment
hSyn1: human synapsin 1
IHC: immunohistochemistry
IPSP: Inhibitorypostsynaptic potential
MEMOIR: memory by engineered mutagenesis with optical *in situ* readout
mRNA: messenger RNA
MSN: medium spiny neuron
NA: noradrenaline
ORF: open reading frame
PNS: peripheral nervous system
PPN: pedunculopontine nucleus
RNA-Seq: RNA sequencing
SADB19: street alabama dufferin field strain B19 of rabies virus vaccine
SC: superior colliculus
scRNA-Seq: single-cell RNA-Seq
smFISH: single-molecule fluorescent *in situ* hybdridization
SNc: substantia nigra pars compacta
SNr: substantia nigra pars reticulata
STN: subthalamic nucleus
TAN: tonically active neurons
TVA: avian tumor virus receptor A
UMI: unique molecular identifier
VTA: ventral tegmental area

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Today several hundreds of regression analysis can be performed per second and you do not pay per analysis. Some things in academia change, other things do not change. By the way, the guy sitting in the back of this lecture hall typing loudly on his Macbook is probably performing some regression analysis as you read this sentence looking for your

own name. His name is Pontus and I would like to thank him for his wit and charm. His best friend is named Niels and couldn't come because he prioritizes bonus air flights to shitty parts of the U.S. A not so shitty place in the U.S. is the skyline of Manhattan seen from Steven's Institute of Technology where my sister is now starting her lab so she beat me to it. Thanks for all the great advice Mirjam and for letting me and Anna stay at your place while we get set up in New York. I believe the domain www.furthlab.org is open.

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"Progress in science depends on new techniques, new discoveries and new ideas, probably in that order."

- Sidney Brenner

1

On Neurons and Spikes

Like most progress in the life sciences the modern study of the brain began with the development of technical innovations. In the case of brain science, the technical innovation came in the form of powerful microscopes developed by the German optical industry at the end of the 1800 century.

Two centuries before the development of the modern microscope Dutch draper and scientist Anton van Leeuwenhoek's (1632-1723) work on microscopes enabled the study on living things at the microscopic level. The advent of the light microscope was the technical innovation that led Robert Hooke (1635 - 1703) to make his seminal observations describing *the cell* as the basic unit of life (Hooke, 1765).

Two hundred years after Hooke's *Micrographia* was published the German engineer Carl Zeiss (1816 - 1888) together with glass specialist Otto Schott

(1851 – 1935) began refining the optical quality of microscopes. Zeiss also hired physicist Ernst Abbe (1840 -1905) who's important theoretical principles on optical quality and light pushed the development of microscopes from a *trail and error* process to become a mature engineering discipline (Abbe, 1875).

Just like van Leeuwenhoek's development of the light microscope enabled Hooke to develop the notion of cells, the German development of high-quality microscopes enabled botanists and physiologists such as Schleiden (1804 – 1881) and Schwann (1810 – 1882) to lay out the conceptual framework of *histology*, and it's associated *cell theory*, in a series of now classical publications (Schleiden, 1838; Schwann, 1847).

For the first time since Aristotle biology had seen a theoretical development shifting it's conceptual focus from the anatomy of organs and bodily fluids to the composition of tissues and cells. *The Cell Theory* suggested that all tissues in both animals and plants are composed of individual microscopic units called cells, where different tissues are made up of different *cell types*.

1.1 The Neuron Doctrine

It is in this new scientific environment the son of a medical doctor in northeast Spain, named Santiago Ramòn y Cajal (1852 - 1934), cultivates an interest in drawing and photography (Swanson et al., 2017). His interest in drawing and photography made his father persuade him to help teach human anatomy at the nearby medical school of Zaragosa. At the time microbiology was considered the forefront of biology with the grand achievements of Louis Pasteur (1822 – 1895) making both the first vaccine and contributions to fermentation and germ theory of diseases. However, Santiago Ramòn y Cajal decided to devote his studies to the new field of *histology* where cell types and tissue composition were the essential questions (Swanson et al., 2017).

One of the biggest challenges for *The Cell Theory* at the time was the observation that brain tissue looked to be composed of a single multinucleated reticulum. The reason why this conclusion was drawn is that available staining

methods tended to label with high sensitivity making it difficult to observe cells that didn't look to be fused by their growth processes.

It was during a visit to a friend whom just returned from a scientific meeting in Paris Ramòn y Cajal learned about the most recent advances in histological labeling techniques. It was while looking at a sample prepared with the silver nitrate he first came in contact with a method with low *sensitivity*, i.e. only labeling a subset of cells, and high *efficacy*, i.e. labeling the entire cell membrane including outgrowth processes. Previously, individual cells had been separated using a needle under a microscope, which often resulted in tearing the axons (Deiters, 1865). With silver nitrate staining Ramòn y Cajal realized he could study the structure of individual cells within the brain. Previously Golgi, who had initially developed the silver nitrate stain, discovered that the nerve cell had two different types of cellular outgrowth *processes*: protoplasmic prolongations, now referred to as *dendrites*, and cylindraxil prolongations now called *axons*. Golgi concluded that the role of the dendrites was to provide nutrients to the nerve cell and the other type of outgrowth, the axon, was directly connected to all other cells in the nervous system through a mesh-like reticulum (Golgi, 1906). This theory was referred to as the *Reticular Theory* of the nervous system (Shepherd, 2015).

Using Golgi's technique Ramòn y Cajal would come to the completely different conclusion that the nerve cell, or *neuron*, is both the structural and functional unit of nervous system circuitry. He postulated that neurons are in contact with each other either through direct contact or contiguity and not through a reticulum (an observation that would only be confirmed in the 1950s by the advent of the electron microscope so the small gap between nerve cells, *synapses*, could be visualized (Robertis and Bennett, 1955). This first principle is called *The Neuron Doctrine* (Guillery, 2005; Shepherd, 2015; Ramon y Cajal, 1891).

The second principle Ramòn y Cajal discovered was *The Law of Dynamic Polarization*. This principle was co-discovered with the muscle histologist van Gehuchten when both he and Cajal turned their attention to applying the Golgi method on tissue from the cerebellum, spinal cord and the olfactory bulb (van

Gehuchten, 1891). Here Ramòn y Cajal and van Gehuchten laid out the anatomical work postulating that impulses are conducted *cellulipetally* along dendrites, towards the cell body, and *cellulifugally* along axons, away from the cell body (Ramon y Cajal, 1891; van Gehuchten, 1891). The basis of this was not electrophysiological studies of nerve impulses but rather histological observations on the position and properties of neuronal components. The polarized structure of nerve cells was embraced by the scientific community, it enabled the recognition of synapses as asymmetrical structures specialized for one-way transmission.

The findings of Ramòn y Cajal and others are best formulated in a review by the German anatomist von Waldeyer-Hartz that coined the term *neuron* for the anatomical and functional elements of the nervous system:

I 'Axons ... all arise from nerve cells. There is no connection with a fibrous network, no origin from such a network'. (von Waldeyer-Hartz, 1891; Guillery, 2005)

I 'All of these axons end freely, with terminal arbours with no networks or anastomoses'. (von Waldeyer-Hartz, 1891; Guillery, 2005)

Add to these two principles a third principle in the *The Law of Dynamic Polarization*. Expressed in the words of Ramòn y Cajal:

III 'The transmission of nervous movement occurs from protoplasmic branches [dendrites] and the soma to the nervous expansion [axonal process]' Ramon y Cajal (1891)

The recognition that the fundamental unit of analysis in the brain is the nerve cell and that transmission across synapses is asymmetric enabled the powerful analysis of neuronal circuits in vertebrate brains. As we shall see in the next section this perspective was critical for developing key experiment on synaptic function using electrophysiological techniques that focused on the nature of impulse transmission within the brain.

1.2 All-or-none coding by action potentials

As a histological description of the brain was beginning to take shape, the natural question posed was how the activity of these individual neurons might relate to our perceptions and actions. Already back in 1835, several ideas on this topic were proposed, most notable being the *Law of Specific Nerve Energies* by German physiologist Johannes Peter Müller. *The Law of Specific Nerve Energies* stated that the identity of a sensory stimulus is represented by activity in individual sensory nerves (Müller, 1837). This was based on simple observations like mechanical pressure against the eye would produce senses of light flashes despite the stimuli being mechanical and not optical in nature. It would take until 1885 until Müller's graduate student and founder of *psychophysics* Hermann von Helmholtz provided some observational support purposing the idea that nerve cells at different positions along the cochlear spiral are sensitive to different sound frequencies (Helmholtz, 1912).

In this context it is important to realise that just as *The Neuron Doctrine* was an idea born out of novel observations enabled through a new set of techniques, the ideas of Müller and Helmholtz required the observations of the electrical activity in *individual* sensory neurons, a technique which didn't exist at that moment. Observing the electrical impulses of neurons by measuring in the extracellular space is difficult since the electric potential of these impulses is very small. It would take until the development of the vacuum tube until the scientific community had a new method of low noise amplification. In 1917 Keith Lucas an innovator and scientist at Cambridge University built the first instruments that enabled recording of microvolt signals in bandwidths of several kiloHertz (Rieke et al., 1999). Lucas, unfortunately, died in a plane crash at a young age but the design of his instruments was later used by E.D. Adrian at Cambridge University.

In just about a decade, using the equipment developed by Lucas, Adrian was able to uncover most of what we now take for granted of how the brain represents information about the external world. In 1928 Adrian would publish a monograph entitled *The Basis of Sensation* (Adrian, 1949). In a series of

experiments outlined in his monograph, Adrian described how individual sensory neurons, when stimulated by stimuli, delivered electrical discharge with a fixed energy, now called action potentials or *spikes*. This observation was of an all-or-none nature: either the discharge happened with a fixed energy or it didn't happen at all. This all-or-none nature of the action potential is the first of Adrian's principles.

The second principle discovered by Adrian and Zotterman in 1926 states that a number of spikes within a fixed time interval increases as the magnitude of the stimulus becomes larger (Adrian and Zotterman, 1926). Adrian and Zotterman measured the relation between the forced applied to a muscle and the firing rate in a stretch receptor nerve cell within the muscle. The amount of force applied to the muscle was manipulated by different weights with different masses. The experiment established that the rate of firing was proportional to the stimulus strength. This phenomenon is called *rate coding* (Rieke et al., 1999).

In conclusion, Adrian's work showed that:

I The amplitude of an action potential is independent of the amount of current that produced it.

II Either the stimulus excites the neuron or it doesn't - there is no in-between.

III The spike-to-spike interval is inversely related to the magnitude of the stimulus.

These principles are important. The all-or-none principle highlights that the only way one neuron can represent a signal and transmit this information to another neuron with a mutual set of symbols is through the use of spikes. Just as a message transmits information from one person to another by a text which is a sequence of symbols each taking its value from a finite set of symbols in the alphabet, a sequence of spikes transmit a message with action potentials serving the role of a binary symbol. Hence, Adrian demonstrated that the spike is the fundamental symbol by which information is carried through the brain (Rieke et al., 1999).

1.3 The chemical nature of the synapse

Much of the functional properties of the connections between neurons were initially discovered by the British physiologist Charles Sherrington (1852-1952) (Sherrington, 1906; Pearce, 2004). As stated before the actual structural gap of a synapse would not be revealed until the advent of the electron microscope (Robertis and Bennett, 1955). However, the concept of a gap that conveyed signals between neurons, either electrically or chemically, had long been speculated (Du Bois Reymond, 1849). The observations that supported the notion of a chemical and not electrical synapse were:

I Electrical signals always travel in a assymmetric direction: either from axon to dendrite or axon to soma, not the other way around.

II The existance of excitatory and inhibitory synapses (Brooks and Eccles, 1947).

But the chemical properties of the synapse were discovered from a much more intricate set of discoveries beginning with the work of George Oliver and Edward Schäfer in 1894 at University College London (Oliver and Sharpey-Schafer, 1894). Oliver and Schäfer had found that the administration of a glycerine extract of the adrenals of the sheep produced constriction of the arteries (Oliver and Halliburton, 1916; Schäfer et al., 1908; Oliver and Schäfer, 1895; Oliver and Sharpey-Schafer, 1894).

Six years later the Austrian physician and chemist Otto von Fürth purified the adrenal compound and called it '*suprarenin*' (von Fürth, 1898), the term didn't stick because American chemist Abel partially purified the compound (Abel, 1899) and began marketing it under the tradename '*epinephrin*'. Shortly thereafter the Japanese chemist Jokichi Takamine invented a novel way to purify the compound (Takamine, 1901) and sold the rights to the procedure to Parke-Davis which began market it as "*Adrenalin*". However the name *epinephrine* was already established in the U.S. and *adrenaline* had largely been established as the name in U.K. and the two terms are used to this day.

With the developments in the synthesis of adrenaline made by von Fürth, Abel, and Takamine the compound was readily available for experimentalists to tease apart it's mechanisms (von Fürth, 1898; Abel, 1899; Takamine, 1901). Thomas Renton Elliott (1877–1961) at Cambridge University was the first to suggest that it was the sympathetic nerves that secreted adrenaline. However, Elliott concluded that the postganglionic cells were not capable of producing adrenaline themselves (Elliott, 1904).

The idea that nerves secreted chemical compounds would lay dormant until the 1920s when Dale and Loewi (Valenstein, 2006), who previously had been introduced to each other by Elliot, started performing experiments on the nerve endings of the frog's heart. In 1926 Loewi would identify the inhibitory transmitter released from the vagus nerve as acetylcholine and ten years later he identified the excitatory innervation as adrenaline (Loewi and Navratil).

Loewi's experiments were highly criticised pushing him to respond to his critics in a live demonstration experiment performed in Stockholm in 1926 where he successfully replicated it 18 times (Valenstein, 2006). One of the attendees on these demonstrations was the Swedish pharmacologist Ulf von Euler. It would be von Euler (Von Euler and Hamberg, 1949) who finally determined that the main forms of transmission in the post-synaptic membrane of sympathetic ganglion cells were noradrenaline (NA) and acetylcholine (ACh).

Methods for studying neurotransmission in the central nervous system (CNS) were not yet available and for some time findings from the peripheral nervous system (PNS) were either extrapolated to the CNS or believed to not hold true in the CNS. The major technical achievement that enabled the study of adrenaline and noradrenaline in the CNS came with the Falck-Hillarp method (Hökfelt, 2010; Hökfelt and Ljungdahl, 1972; Carlsson et al., 1961; Falck et al., 1962). This technique made it possible to study biologically active substances called monoamines - e.g. dopamine, noradrenaline, adrenaline and serotonin - on the cellular level using the fluorescence microscope. Initially, these techniques were used to map cell bodies and processes of ACh and NA in the CNS. Later on, the method would be employed to map out the dopaminergic nuclei in the brain

as well as serotonergic nuclei (Hökfelt, 2010).

During the same period, Kuffler (1954) and Florey (1954) described the presence of excitatory and inhibitory control mechanisms in crayfish. Kuffler and Florey made the crayfish stretch receptor preparation a popular assay for detecting the hypothesized chemical substance that would contribute to excitation and inhibition in the brain. Factor I[1], as Florey referred to the inhibitory substance, was extracted from the mammalian brain by Florey and McLennan (1959) and shown to contain gamma-aminobutyric acid (GABA) which the authors suggested might well be the natural neurotransmitter. Krnjevic and Schwartz (1967) would later provide unequivocal evidence for GABA as an inhibitory transmitter.

Surprisingly in the 1950s when it was found that glutamate depolarized and excited individual nerve cells in the spinal cord (Curtis et al., 1959) it was first hoped that this substance was indeed the major excitatory transmitter in the CNS. But due to a preconception that such transmitter would be present in the brain at relatively low concentrations and have only a single function the idea of glutamate being the major excitatory neurotransmitter was only accepted 20 years later. Again the conceptual shift was not done by theoretical argumentation but rather by technical improvements in electrophysiological recordings that established the similarity in ionic conductance change induced in the neuronal membrane by glutamate to that induced by endogenously released transmitters (Mayer and Westbrook, 1987). The major establishment of glutamate as the excitatory transmitter came from the discovery and synthesis of specific glutamate antagonists that were able to block the actions of endogenous glutamate (Watkins and Evans, 1981). The discovery of neurotransmitters revolutionized the way we think about the brain and opened up the field of psychiatry to slowly adopt a biological framework.

[1] Here *I* represented inhibitory action on neuronal activity

1.4 Conclusions

In this chapter we have examined how neuroscience has developed into a mature field sharing a set of fundamental concepts on the structural and functional units of observation: *neurons*, *action potentials* and *synapses*. I've tried to highlight the major conceptual revolutions that established the single neuron as the fundamental unit of analysis as well as the action potential as the fundamental information carrying event. Lastly, I've focused on the not so straightforward history of how the chemical properties of the synapse, the major structural unit mediating transmission between neurons, was outlined.

The historical dispute of whether communication between neurons is chemical or electrical is known in the history of neuroscience as the *The War of the Soups and the Sparks* (Valenstein, 2006). Neuroscience, like other scientific fields, can sometimes experience disputes about its major structural and functional elements. Neuroscience is also an interdisciplinary field and the opinions of individual investigators often depend on the scientific training and background of individual investigators. This can be seen both in the disputes regarding the neuron doctrine as well as the nature of the synapse.

Before a technological advance has been done there is often a myriad of more or less well-grounded theories about a certain topic, e.g. *Law of Specific Nerve Energies* (Müller, 1837). It is first when a novel technology that can be used to examine claims made is established conceptual change happens (e.g. silver nitrate staining or ability to record microvolt signals). From this we can reach two conclusions:

I New techniques that enable observations on a scale or precision that couldn't be made before are essential.

II Conceptual change often involves questions targeted at identifying the fundamental functional or structural unit of organization.

Or as Sidney Brenner (2002) described it in the quote at the beginning of this

chapter: “Progress in science depends on new techniques, new discoveries and new ideas, probably in that order.”

$$\text{New technique} \mapsto \text{Novel discovery} \mapsto \text{New ideas}$$

*"A map **is not** the territory it represents, but, if correct, it has a **similar structure** to the territory, which accounts for its usefulness."*

- Alfred Korzybski, *Science and Sanity* (1933, p. 58)

2

On Transcriptomes and Connectomes

The brain is structured at multiple scales: subcellular, cellular, circuits, networks, brain regions all the way up to the level of analysis of the entire organ and its embodiment manifested through behavior and cognition. Given the painstaking efforts to find the fundamental structural and functional units of organization described in the last chapter (i.e. the neuron, the action potential and the synapse) one is left to wonder if the field of neuroscience might have stopped asking these fundamental questions with respect to how the brain is functionally and structurally organized on the spatial scale of hundreds of micrometers: the *mesoscale* level.

It is not surprising that the initial efforts to find structure in the brain at levels higher than microcircuits largely were based on completely arbitrary criteria to define cytoarchitecture. Most famously the German neurologist Korbinian

Brodmann (1868–1918) identified forty-three distinct regions in the human cerebral cortex based on observation using Nissl staining (Brodmann, 1909). Brodmann, together with Constantin von Economo (1876-1931) and others, mapped out most of the basic neuroanatomical regions based on visual inspection of cyto- and myeloarchitectural criteria defined on only a few properties such as cell density, cell shape etc. (Economo et al., 1925; Brodmann, 1909).

Early studies on cytoarchitecture used some sort of staining to label parts of the tissue. Seldom these early investigators reflected on what those dyes actually might label on the molecular level. We might, therefore, start by examining exactly what class of molecules provide the fundamental structural unit.

2.1 The importance of proteins

Neurons are composed of water, inorganic ions, and carbon-containing molecules. It is the carbon-containing, organic, molecules that are the unique constituents of cells. Most of the organic molecules can be classified either as lipids, carbohydrates, nucleic acids, or proteins.

Although lipids are abundant in the brain and provide much of the structural support for the brain it is not lipids that provide the main structural components classifying one type of neuron from the other. Likewise in most animals proteins are used for structural purposes, but this is not their main function, indeed in plants, the polysaccharides usually have this role. As emphasized by Francis Crick: *"The main function of proteins is to act as enzymes"* (Crick, 1958). It is essential to grasp the unique importance of proteins in biology as a majority of chemical reactions inside cells are catalyzed by enzymes.

2.1.1 Limitations in multiplexing protein detection

Given the importance of proteins, it might at first seems like a good idea to focus on measuring and visualizing proteins in tissue. Indeed, visualization of specific proteins by means of immunohistochemical (IHC) staining is one of the most widely used contemporary methods in neuroanatomy. Nevertheless, protein

detection methods are currently limited by their ability to simultaneously measure multiple proteins (*multiplexing*). Highly multiplexed mass spectrometry methods of cells can be done by staining with antibodies carrying isotopically pure metal reporters via nebulized single-cell droplets before sequential analysis by inductively coupled plasma time-of-flight mass spectrometry (Bendall et al., 2009). This method is limited to dissociated single-cell analysis of up to 100 different antibodies without spectral overlap between channels. Subsequently, ion mass spectrometry has been used to image metal isotope-carrying antibodies directly in the tissue (Angelo et al., 2014). But the ion beam scanning is a point-scanning process with high exposure time resulting in imaging times on several hours and days for a single field of view.

Other alternatives to multiplexed IHC include SWITCH (system-wide control of interaction time and kinetics of chemicals) which is a hydrogel-based clearing method that enables multiple rounds of staining but limits the number of channels that can be used in a single round to one so the method is limited to 22 rounds with 22 proteins that can be targeted (Murray et al., 2015).

Even so, all of these methods rely on immunohistochemistry which is ultimately based on the availability of highly specific antibodies that can detect antigens at necessary concentrations. For polyclonal antibodies, the manufacturing process of immunization, serum-collection and titer analysis cannot be easily multiplexed thereby setting an absolute cost and time ceiling on each antigen that can be produced and detected.

2.1.2 The size of the proteome

There are about 22032 mouse protein-coding genes. Out of these genes about 12934 identified proteins have been detected in the mouse brain corresponding to 11690 ENSEMBL genes, which represents 50% of all protein-coding genes in the mouse genome (Sharma et al., 2015)

The difficulties with identifying unique proteins also stem from regulation of some proteins by covalent modification of proteins (e.g. non-phosphorylated,

phosphorylated, dephosphorylated) resulting in genuine different versions of proteins. Other genes are alternatively spliced giving rise to proteins with different internal amino acid sequences. These are also genuinely different proteins from the same gene. In the most extreme case in *Drosophila Melanogaster* the cell adhesion molecule *Dscam* gene, which encodes an axon guidance receptor, contains 95 alternative exons that could potentially produce 38016 splice variants from the same gene (Wojtowicz et al., 2004). Any given scientist's estimate of how large the proteome is can vary with a range of tens of thousands to more than 100000 depending on how many protein-coding genes the scientist believe are alternatively spliced to yield several different proteins.

2.2 Protein synthesis and the transcriptome

Given the unknown size of the proteome and the limitations of multiplexing immunohistochemistry we either need a completely novel *in situ* protein labeling technique or that polyclonal antibody manufacturing scales considerably better than what it does at the moment until we will have cost-effective methods to detect the entire proteome *in situ*.

The construction of reference genomes and the development of short-read massively parallel DNA sequencing launched a "next-generation" in genomic sciences where sequencing output has outpaced Moore's law and more than doubled each year (Heather and Chain, 2016). This wast expansion of DNA sequencing enabled the reverse transcription of RNA and construction of cDNA libraries to sequence not only DNA but also RNA (RNA-Seq). RNA-Seq provides comprehensive information on mRNA abundance and alternative splicing (Wang et al., 2009). The development of both single-cell RNA-Seq (scRNA-Seq) (Tang et al., 2009; Shapiro et al., 2013) as well as fluorescent *in situ* sequencing (FISSEQ) (Lee et al., 2014) further pushed the next-generation sequencing approach to the single cell and subcellular tissue level.

The transcriptome is the complete set of transcripts in a cell for a specific developmental stage or physiological condition. At the quantitative level, mRNA

and protein levels are only modestly correlated (Fournier et al., 2010; Maier et al., 2009; Gygi et al., 1999), suggesting post-transcriptional regulation in gene expression control. Nevertheless, the key to understanding protein synthesis, posttranslational modifications and transcriptional regulation in the brain lies in the transcriptome and the generation of transcriptomic maps of the brain (Lein et al., 2007).

2.3 Connectivity and computation in the brain

Traditionally the brain has not only been divided into anatomical regions based on cytoarchitecture and gene expression. Connectivity, how the brain is wired together, has also been a large contribution to what we define as a brain region (*brain ontology*). Within a single transcriptionally homogenous area of the brain, there can be subregions based on efferent and afferent connections Oh et al. (2014). It has therefore been suggested that anatomy should be defined by connectivity (Thompson and Swanson, 2010; Swanson et al., 2016; Swanson and Bota, 2010). The rationale for this is based on taking connectivity as the main computational unit in the brain. Until now we haven't discussed the role of computation in the brain but it is important to understand the rationale behind seeing computation and connectivity as the fundamental functional unit in the brain.

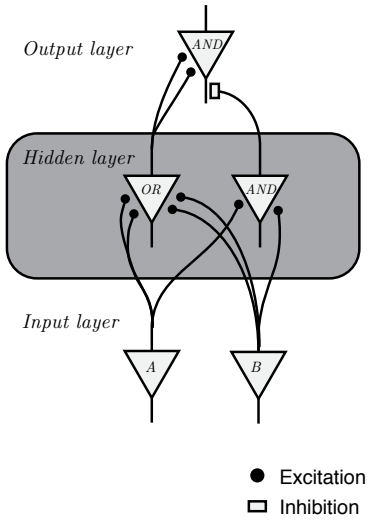

Figure 2.3.1: Exclusive or gate. Representing an exclusive or gate, `XOR`, by a feedforward network

Almost all physical implementation of computation we know of use the *logic gate* as the basic computational unit (e.g. `AND`, `OR`, `NOT`, `NAND`, `NOR` etc.). Each gate takes a set of symbols and computes some Boolean function. These gates can then be combined into circuits, or networks, by connecting the outputs of some gates to the circuit inputs of others.

Adrian's work on the all-or-none nature of the action potential demonstrated that the action potential provides the basic symbol of information transmission in the brain. It is therefore commonly accepted that synapses can take on certain properties of logic gates (`AND`, `OR`, `NOT`), (McCulloch and Pitts, 1943). When the action potential arrives at the presynaptic terminal it releases neurotransmitter into the cleft and through binding of the neurotransmitter to either ionotropic or metabotropic receptors will lead to alterations in ion-channel conductances which may either transiently depolarize the postsynaptic neuron (an Excitatory Post-Synaptic Potential, EPSP), or transiently hyperpolarize it (an Inhibitory Post-Synaptic Potential, IPSP). The IPSP will cancel any excitatory changes within the postsynaptic neuron thereby effectively implementing the Boolean `NOT` function. The `AND` function is achieved by summation of two EPSPs which neither alone is sufficient to trigger an action potential. The `OR` function is naturally achieved the same way through the summation of EPSPs where either one of the EPSPs are sufficient for initiation of an action potential. Of course in this view, the conceptual important element is the threshold for initiation of action potential which may not even occur at the axon hillock but can have causal antecedents upstream in single dendrites.

Other functions such as `XOR`, meaning exclusive or (i.e. either A or B, but not both), can be achieved by connecting a set of neurons into a circuit. More specifically, by connecting two input units A and B into a hidden layer which contains a `AND` gate and a `OR` gate before fan-out into the output neuron one can achieve the logical operation `XOR` $(A \vee B) \wedge \neg(A \wedge B) = A \oplus B$, which is seen in **Figure 2.3.1** (Minsky and Papert, 1972).

The idea that certain information is stored in an array of network activity is an old idea in neuroscience (connectionism/empiricism) but got revitalized with

the advent of Elman/Jordan types of simple *recurrent networks* (**Figure 2.3.2**) where a *context layer* receives input from either the hidden layer (Elman type, Elman (1990)), or the output layer (Jordan type, Jordan (1997)), and then propagates this back to the hidden layer. The advantage of this is that the network instead of just computing on single symbols now can distinguish sequences (Jordan, 1997), e.g. ***before*** $(a \prec b)$, ***after*** $(b \succ a)$, ***between*** $(a|b|c)$, and therefore operating on strings of symbols (messages).

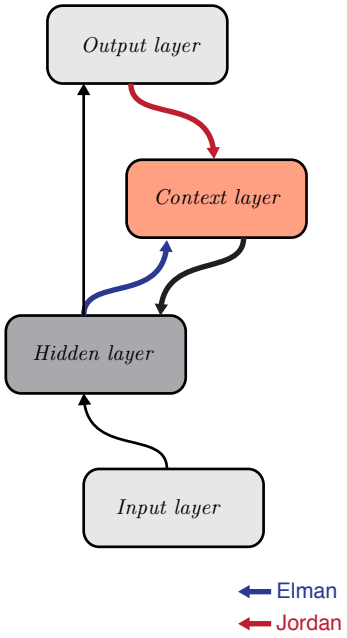

Figure 2.3.2: Simple recurrent network. can distinguish sequences of symbols

The implications that computations can be performed in networks of neurons is that the connectivity might provide the essential structure of the brain. Any serious attempt at finding structure in the brain must, therefore, evaluate if and how connectivity might provide the basis for how the brain is organized. In this regard, the basal ganglia, an interconnected set of subcortical nuclei provide the prototypical set of anatomical regions grouped together into a structure based on functional connectivity alone. A central aim of this thesis is therefore to examine both the circuit structure as well as the transcriptomic structure of the brain regions involved in the basal ganglia. A connectome is a comprehensive map of connections in the brain. Hence, out approach must be able to handle both connectomic and transcriptomic data.

2.4 Rosetta brains

As we have seen there are both convincing arguments for that the basis of the organization of the brain is either gene expression or connectivity. Instead of theorizing, we might try to think about what the ideal dataset of the brain would look like and what technologies are needed to be developed in order to get to that dataset. As the American geneticist George Church (2015) recently pondered:

"...regardless of apparent feasibility, what dataset would we ideally like to have to help understand how the brain's structural and functional biological levels interlink to form an integrated system?" - George Church (2015)

George Church (2015) claims that what we ideally would like to observe, simultaneously - within a single brain - is information about all the multiple interrelated phenomenological levels of the brain's biology and allow these levels to be directly compared to one another with single-cell precision. He calls such a brain sample a *Rosetta Brain* and ideally such a brain would have a data format reporting on the following:

- Cell types
- Connections
- Connections strengths and types
- Developmental lineages
- Histories of electrical activity patterns over time
- Histories of molecular changes over time

It is important to distinguish George Church effort from that of other large-scale brain projects in that what he wants to develop is a method for collecting multivariate data; there is no aim of understanding computation. This is in contrast with other large-scale brain projects that aim to collect univariate data and then pool this together to feed as input to computational models already defined. The uniqueness of George Church's idea is his appreciation for the need of collecting multivariate data inorder to map between one set of observations to the other (Oh et al., 2014; Lein et al., 2007). Just like the archeological *Rosetta*

stone with its exact same statement in three different languages, two of which were known (Ancient Greek and Egyptian demotic hieroglyphs) and the third unknown (ancient Egyptian hieroglyphs) provided a key resource for cracking the then-unknown code of the hieroglyphics. In essence, George Church (2015) says, all of the structural observations we wish to make across the different levels of a *Rosetta Brain* come down to operations of *labeling* and *counting*. It is therefore natural that the procedure we need to develop is that of which enables brain-wide labeling and counting of the features.

3

The Basal ganglia

The basal ganglia are a large, interconnected set of subcortical nuclei that have been functionally implicated in motor control, as exemplified by the difficulties in control of movement that occur when these nuclei are affected by diseases such as Huntington's chorea or Parkinson's disease. Historically these nuclei have been recognized all the way back to antiquity by Claudius Galenus (129-201) who called them *glutia* (buttocks), possibly referring to the round shape of the caudate-putamen in both hemispheres. Andreas Vesalius (1514-1564) was the first anatomist to delineate these structures in the human brain but he never named them. The British surgeon Thomas Willis (1621-1675) would be the first one to define the largest structure of the basal ganglia as the *corpus striatum* and it was also Willis who first suggested that the *corpus striatum* was involved in the control of movement (*Cerebri anatome*, 1664). Willis was

even so specific that he attributed *corpus striatum* with voluntary motion, the involuntary movement was controlled by the cerebellum.

Much of the subcortical nomenclature that is still with us today was defined by the German physician Karl Friedrich Burdach (1776-1847) in a three-volume treatise entitled *Vom Baue und Leben des Gehirns* (Of Structure and Life of the Brain) published between 1819-1826. Burdach gave the names to subcortical regions of the brain we still use today: the pulvinar, amygdaloid complex, red nucleus, cingulum, subiculum, pallium, alveus, cuneus, lamina terminalis, precuneus, and the fasciculus cuneatus among others. It would be Burdach who for the first time carefully delineated the structures of the basal ganglia, but he referred to them as *Hirnstammganglien* (brainstem ganglion) and not basal ganglia.

Although Ramon y Cajal described some neurons in the striatum the study of basal ganglia as an interconnected set of nuclei were not of concern for the histologist since they primarily studied the cellular composition of tissues. The practice of studying the interconnected structures in the brain on the mesoscale would come from scientists such as James Wenceslas Papez (1883–1958) and Samuel Alexander Kinnier Wilson (1878-1937). And the notion of basal ganglia would slowly develop by scientists working from the perspective of functionally connected structures. Here the work of David Ferrier (1874, 1873) stands out as the first to experimentally induce lesions in the corticostriatal pathway and observing motor dysfunction, thereby cementing the view of the basal ganglia as a regulator in involuntary movement

The current definition of basal ganglia came by the studies of projections from the *lentiform nucleus* (putamen and globus pallidus) in monkey (Nauta and Mehler, 1966). Nauta and Mehler (1966) combined a relatively novel tracing method, Nauta-Gygax technique, that stained degenerating myelin and combined it with focal lesions to identify the efferent projections within and from the putamen and globus pallidus. The results of these investigations can be seen in **Figure 4.0.1 a**.

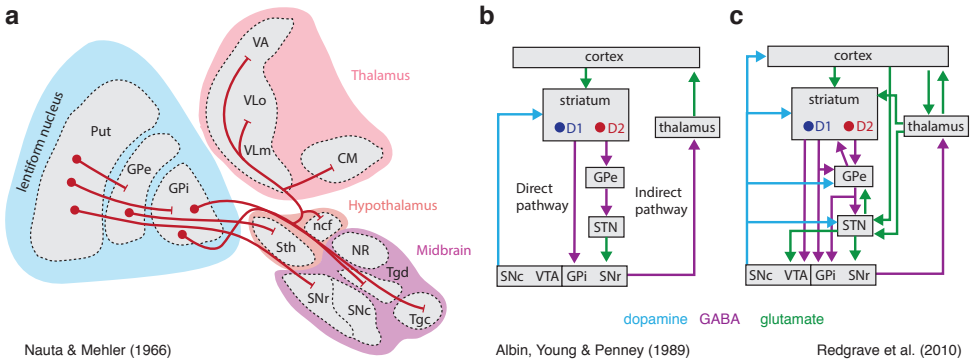

Figure 3.0.1: Models of the Basal Ganglia (a) Conclusions from Nauta and Mehler (1966) study on efferent projections from putamen and globus pallidus. **(a)** The direct-indirect pathway model of the basal ganglia by Albin and colleagues is the most influential model of the basal ganglia. **(c)** Updated model based on additional connections between the basal ganglia suggested by Redgrave et al. (2010) and colleagues rendering the direct-indirect pathway not as straight forward.

3.1 Gross anatomy of the basal ganglia

This basic description of accepted definitions of basal ganglia follows that of (Steiner and Tseng, 2010). Briefly, cortical layer V pyramidal neurons from most cortical areas provide major glutamatergic input onto the striatum which is composed of the caudate-putamen (CPu or CP) nucleus accumbens (ACB). The main output hubs of the basal ganglia are the GABAergic neurons in globus pallidus internal segment (GPi) as well as the substantia nigra pars reticulata (SNr). The GABAergic neurons of the GPi and SNr provide input to several thalamic nuclei, the pedunculopontine nucleus (PPN), as well as superior colliculus (SC). Within the striatum, the two major classes of GABAergic projection neurons either express the G-protein coupled receptor dopamine receptor 1 (Drd1, D1), or the dopamine receptor 2 (Drd2, D2). The D2 striatal projection neurons mainly send indirect output to the GPi and SNr via the globus pallidus external segment (GPe) and the subthalamic nuclei (STN). The D1 striatal projection neurons mainly send direct input to GPi and the SNr while

also sending collaterals to the GPe. Feedback into the striatum is mainly achieved through the nigrostriatal dopamine pathway from the substantia nigra pars compacta (SNc), and the ventral tegmental area (VTA). Thalamostriatal feedback is mainly coming from the parafascicular thalamic complex.

3.2 Cell types in the basal ganglia

3.2.1 Striatal medium spiny projection neurons

The first cell type characterized in the striatum was the medium spiny neurons (MSN). These neurons are GABAergic and as stated previously they either express D1 or D2 receptors (Beaulieu and Gainetdinov, 2011). The Drd2 gene is alternatively spliced into three isoforms one that is a long form which functions as a postsynaptic receptor in striatal MSNs and a second shorter form which is presynaptic on dopamine neurons and functions as an autoreceptor regulating dopamine levels in the synaptic cleft (Beaulieu and Gainetdinov, 2011). D1 MSNs also coexpress Substance-P (Bolam et al., 1983) and D2 MSNs coexpress enkephalin (Pickel et al., 1980).

3.2.2 Cholinergic striatal interneurons

Out of the striatal interneurons the cholinergic interneuron has been extensively studied due inpart to its large size (Bolam et al., 1984; Wainer et al., 1984; Kawaguchi, 1993; Chang et al., 1982). *In vivo* electrophysiological recordings have observed tonically active neurons (TAN) (Kimura et al., 1984) and these cells have been shown to be cholinergic (Bennett and Wilson, 1999).

3.2.3 GABAergic striatal interneurons

GABAergic interneurons of the striatum are mainly divided into fast-spiking parvalbumin-expressing interneurons or somatostatin-expressing interneurons with a low-threshold-spiking electrophysiological profile. Most somatostatin

interneurons also express neuropeptide-Y and neuronal nitric oxide synthase (Tepper et al., 2010).

3.2.4 Patch versus matrix compartments

Graybiel and Ragsdale (1978) were the first to observe patch-like structures within the striatum when doing immunohistochemical staining of the striatum targeting the acetylcholinesterase (Graybiel, 1984; Graybiel and Ragsdale, 1978). These patches or striosomes were later identified to express opioid receptors and are contrasted against nonstriosomes that are localized to the striatal matrix (Gerfen, 1992). These differences also reflect differential afferent and efferent connectivity (Gerfen, 1992).

3.2.5 The indirect and direct pathways

A very influential model of the basal ganglia in hypokinetic disorders, such as Parkinson's disease, was suggested by Albin et al. (1989) (**Figure 4.0.1 b**). Briefly, the two populations of MSNs: D1 and D2, are segregated into two separate pathways. One pathway, direct pathway, involves D1 MSNs and promotes movement directly via projections in the striatonigral pathway, whereas the indirect pathway suppresses movement and mainly involves D2 MSNs and relays on the globus pallidus external segment and the subthalamic nucleus. The disease model assumes that the dopaminergic neurons in SNc and the VTA regulates the balance between these two pathways by differentially acting on D1 and D2 dopamine receptors.

Although the model of Albin et al. (1989) provides a useful heuristic it has been acknowledged that the model might be too much simplified and ignoring several connections and collaterals (Redgrave et al., 2010) (**Figure 4.0.1 c**).

4

Methods

Although this thesis largely aims at developing a systematic method for examining structure across the whole brain in both gene expression and connectivity the method itself is used in conjunction with two other methods: one for examining connectivity and another one for spatially mapping the transcriptome. The approach to mapping connectivity across the entire brain is monosynaptic cell type-specific retrograde glycoprotein-deleted rabies tracing. The method to spatially map the entire transcriptome across the whole brain is based on arrayed mRNA capturing oligonucleotides with positional barcodes on microscope slides, a method more comonly known as spatial transcriptomics.

4.1 Cell type-specific transsynaptic circuit tracing

Rabies virus is a highly neurotropic enveloped virus known to spread within the CNS by means of axonal transport (Wickersham et al., 2007b,a). It has been shown that rabies virus exclusively travels in the retrograde direction, transsynaptically infecting presynaptic neurons (Tang et al., 1999; Ugolini, 1995). The envelope spike glycoprotein (G) is required for virion attachement to neuronal membrane receptors and for viral entry as well as transsynaptic spread (Etessami et al., 2000; Anilionis et al., 1981).

Here we use a genetically modified rabies virus that enables monosynaptic circuit tracing in specific cell types (Wickersham et al., 2007b). First the a attenuated strain of rabies virus (SAD B19 vaccine) is made replication incompetent by deleting the gene for the envelope spike glycoprotein and replacing it with the enhanced green fluorescent protein (EGFP) as a reporter. Specificity is achieved by pseudotyping the rabies virus with the envelope protein from avian sarcoma leukosis virus type A (EnvA) and then expressing its cognate receptor (TVA) in a cell type-specific manner so that the resulting vector, (EnvaA)SADB19ΔG-EGFP, is only transduced in cells expressing the TVA receptor.

The cell type-specificity of TVA expression is achieved by means of transgenic mice. Adeno-associated viruses (AAVs) introduce the rabies envelope spike glycoprotein *in trans* as well as the TVA receptor in a Cre-dependent manner. This system enables (EnvaA)SADB19ΔG-EGFP to infect only cells expressing the TVA receptor and if G is also expressed in the same cell the rabies virus will be replication competent and can spread transsynaptically only one synapse, thereby labeling the pre-postsynaptic pair with the fluorescent protein (EGFP).

A specific cell type is targeted by injecting in transgenic mice that are generated either by knock-in strategies or bacterial artificial chromosomes (BAC) to express Cre recombinase under control of an endogenous promotor of interest (e.g. Drd1, Drd2 etc.) by placing the transgene downstream of the promotor. Site specific recombination is then achieved by the Cre enzyme in the presence of two

recognition sites (Lox sites). The AAV vector is then designed to achieve cell type-specific expression by Cre dependency due to the presence of a double-floxed inverted open-reading-frame (DIO) in the viral vector. The gene of interest, e.g. G or TVA, is situated between two incompatible Cre recombinase recognition sequences and therefore depends on Cre-Lox recombination. If the cell expresses Cre the open-reading-frame (ORF) is irreversibly inverted and then allows the TVA or G gene to be expressed under the promotor used in the AAV, e.g. elongation factor 1-alpha (EF1a), cytomegalovirus (CMV), synthetic promotors such as CAG, or human synapsin 1 (hSyn1), depending on user-specific criteria and packaging size of the viral vector. Details of the cell type-specific transsynaptic rabies tracing strategy can be found in Wickersham et al. (2007b) and Wickersham et al. (2007a).

4.2 Spatially resolved transcriptomics

Spatial transcriptomics is a method where RNA-Seq data can be spatially resolved by mounting histological sections obtained from flash frozen tissue on microscope slides with arrayed reverse-transcription oligo(dT) primers with unique positional barcodes (Ståhl et al., 2016). Cryosectioned tissue is mounted onto the array and fixed, stained with hematoxylin and eosin and imaged with a microscope. After tissue permeabilization the Poly-T tail of the capture probe can bind the Poly-A tail of the RNA molecules. cDNA synthesis with Cy3-labeled nucleotides reveals fluorescent cDNA and each arrayed spot of positional barcodes is then imaged. The position of Cy3-labeled nucleotides are then superimposed on the image of the tissue staining to determine the location of each barcode in the tissue section.

The 33×35 spot array is composed of $\sim$200 million oligonucleotides in each spot with a diameter of 100 μm and a center-to-center distance of 200 μm limiting us to position only half a mouse brain hemisphere cut at the coronal plane.

After capturing and reverse-transcribing mRNA, the cDNA-RNA-hybrids are cleaved off the chip and library preparation is performed. The created libraries are

sequenced using ordinary RNA-Seq. Each capture probe contains a cleavage site, a T7 amplification and sequencing handle, a spatial barcode, a unique molecular identifier (UMI), and an oligo(dT) capture region. See Ståhl et al. (2016) for further details on spatial transcriptomics.

5
Aims and results

The overall aim of this thesis is to build a comprehensive and systematic way to map both connectivity and gene expression on the single-cell level across the entire brain. The basal ganglia as a set of interconnected nuclei are suited for this purpose because of its functional definition based on connectivity that sometimes, but not always, reflects underlying differences in gene expression.

- In **Paper I** we mapped the monosynaptic inputs to the serotonergic neurons in dorsal and median raphe using cell type-specific retrograde rabies tracing. To verify the validity in our whole-brain mapping approach connections of particular interest such as prefrontal cortex $\longmapsto$ dorsal raphe, lateral habenula $\longmapsto$ dorsal raphe, and striatum $\longmapsto$ dorsal raphe were all verified by slice electrophysiology combined with optogenetics.

We found that the striatal $\longmapsto$ dorsal raphe projection was largely provided by D1 striatal GABAergic projection neurons.

- The aim for **Paper II** was to extend the framework to handle not only transsynaptic genetically defined tracing data but also handle immunohistochemistry of cell type markers, relating immediate early gene activity across the brain to behavior, as well as cell type characterization by single-molecule fluorescent *in situ* hybridization (smFISH). In addition to this we demonstrated the utility of our approach in pooling retrograde monosynaptic tracing data over several cell types and injection targets. With a special emphasis on the impact of intracortical communication on striatal circuits we were able to identify the orbital cortex as providing a contextual layer to both motor cortex as well as striatum. In **Paper II** we also verified the role of the orbital cortex by modulation of cocaine-induced motor activity. This was done using the immediate early gene *c*-fos as a readout of neuronal activity.

- The aim in paper **Paper III** was to scale our approach of whole brain rabies circuits tracing to include multiple cell types and brain regions in order to search for common patterns between the number of pre- and postsynaptic cell counts. We found that the number of presynaptic neurons scale supralinearly to the number of labeled postsynaptic neurons.

- **Paper IV** was done to extend the abilities of our whole-brain anatomical framework to also include spatially resolved next-generation sequencing approaches to map the entire transcriptome at the resolution of 100 m across an entire mouse brain. We found that a relatively small set of genes could describe much of the anatomical variability in gene expression. With about 50% of spatial variance explained by a subset of 270 genes and 95% of the spatial variance could be explained by a subset of 1899 genes. Thereby providing the possibility to reduce neuroanatomy to gene expression alone.

6

Conclusions and Future Perspectives

Given the observed results in **Paper I** such as a hyperdirect pathway directly from the striatum to the serotonergic raphe nucleus (which also sends reciprocal connections to striatum), one might wonder what criteria one should employ in what regions belong to the basal ganglia and which regions fall outside of these interconnected nuclei. Clearly, connectivity might not be the best choice in selecting membership and family resemblance between regions.

In **Paper III** we found a scale-invariant principle of neuronal wiring across the brain. More specifically a pair of pre- and postsynaptic neurons might be seen as the Cartesian product $N_{\text{pre}} \times N_{\text{post}}$. The interest of **Paper III** was to examine if there exist a numerical scale ψ_i on N_i such that $\psi = \psi_{\text{pre}}\psi_{\text{post}}$ preserves the ordering $\succeq$ of how many postsynaptic neurons we have labeled in the network to start with. In plain English; what we are asking is if the ratio $\psi_{\text{pre}}(N_{\text{pre}})/\psi_{\text{post}}(N_{\text{post}})$ could be treated as a given constant for a population of

neurons. This is similar as in physics where the ratio of mass and volume, m/V, gives a constant for a given material; its *density* ρ, or the applied force F and the length l is a constant for a given spring (Hook's law). I leave it up to the reader to realize the importance of such a qualitative result, but in essence, it would provide us with the blue print of how connectivity in the brain scales; an *Archimedes' principle of neuroanatomy*. At the same time, we have to be skeptical about this finding since a lot of factors are unknown with respect to the mechanisms of transsynaptic spread of rabies virus (Etessami et al., 2000). At the same time, the mathematical formalism will allow us to test strong predictions derived from theory.

As an example, log-interval measurement scales, like density in physics, are scale invariant and this is simply a result of its representational theorem (we refer to p. 487 in Krantz et al. (2006) for the full theorem) that states if a law of similitude between two measures (e.g. mass and volume, or number of pre- and postsynaptic neurons in out case) exist then there should be a set of constants $a, a_{\text{post}}, \gamma, \gamma_{\text{post}}$, such that the following three axioms hold:

(i) $\psi_{\text{post}}\psi_{\text{pre}} = \gamma\varphi^{a} \quad \text{and} \quad \psi_{\text{post}} = \gamma_{\text{post}}\varphi_{\text{post}}^{a_{\text{post}}}$

(ii) $a > 0 \quad \text{and} \quad a_{\text{post}} > 0$

(iii) $\frac{a}{a_{\text{post}}} = \frac{n}{m}$

Where m and n are positive integers, and ψ has an additive representation $\log\psi_{post} + \log\psi_{pre}$ and φ is additive. If we compare two injections a and u, from **(i)** and **(iii)** we then have:

$$\psi_{\text{pre}}(u) = \left(\frac{\gamma}{\gamma_{\text{post}}}\right)\left[\frac{\varphi(a,u)^{am}}{\varphi_{\text{post}}(a)^{a_{\text{post}}n}}\right]^{\beta}, \tag{6.1}$$

We call β an allometric scaling parameter, $\beta = a/m = a_{\text{post}}/n > 0$. In physics, its common to define the measurements (e.g. mass and volume) such that the greatest common divisor of m and n is one and then set $\beta = 1$ this is why density,

ρ, is related to mass, m, and volume, V, through $\rho = (m/V)^1$, but this is quite arbitrary we might as well construct measurements where $\rho = (m/V)^{3.83}$. $\beta = 1$ is just more convenient when we calculate stuff, if the quantity $(m/V)^2$ would occur a lot in physics one would have settled for $\beta = 2$. In neuroscience we are not as lucky since the procedure of counting cells is not arbitrary the same way as selecting units for measuring mass or volume is. Our finding in **Paper III** that β approximates $3/2$ across a range of cell types and brain regions strongly suggest that complex wiring diagrams might be reduced to a handful of heuristic rules.

In **Paper IV** we extended our whole-brain reconstruction method to also handle spatially resolved RNA-Seq methods (Ståhl et al., 2016). With this technique, an entire brain can now be mapped entirely with its transcriptome detecting the expression of more than 20000 different genes.

All in all, our framework shows that it is a flexible method able to handle multiple sorts of anatomical data ranging from: detection of cell bodies and processes labeled by fluorescent proteins, immunohistochemistry, *in situ* hybridization and spatially resolved transcriptomics. Future studies might use these techniques combining them with several of the recently developed barcoding schemes for either detecting and sequence individual synapses (Kebschull et al., 2016) within the tissue or to reconstruct cell lineages in development using genome editing of synthetic target arrays for lineage tracing (GESTALT) (McKenna et al., 2016), or other methods such as memory by engineered mutagenesis with optical *in situ* readout (MEMOIR) (Frieda et al., 2017). Either way, development and lineage tracing is certainly the next step for being able to arrive at a reference atlas with meaningful anatomical parcellations.

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