From Department of Neuroscience
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# MAPPING BRAIN CIRCUITS: ANATOMY, CONNECTIVITY AND FUNCTION

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# Mapping Brain circuits: anatomy, connectivity and function

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To my parents.

献给我的父母。

# ABSTRACT

To decipher neural circuits anatomy is the central basis for all kinds of neurobiological studies. Here in this thesis, I will start with extensive overview of the current methods that have been intensively employed in labeling and mapping neurons and neuronal activities, including tracing techniques, imaging/recording systems and manipulation tools. This will be followed by the introduction of the two model systems that are being studied in this thesis.

Specifically, the ultimate goal of this thesis is to examine the anatomical neural circuits of involving median prefrontal cortex and the serotonergic nuclei, by developing a standardized brain atlas for automatic processing and analyzing anatomical image data, which facilitate functional studies of the serotonergic neurons in rats.

In paper I, we characterized the inputs to two major serotonergic nuclei using a retrograde trans-synaptic virus. We reconstructed a whole brain input map with customized software, and found previously undefined inputs. We also confirmed the existence of the functional connection from basal ganglia, lateral habenula, and prefrontal cortex to the raphe nuclei serotonergic neurons. In paper II, we developed a versatile interactive framework for automatic detection, registration, and analysis. In paper III, we characterized both the local and long-range inputs to four cell types in the mouse medial prefrontal cortex. A new viral strategy for tracing both local and long-range inputs at the same time was developed. We have confirmed the findings by other tracing techniques, and rebuilt our own connectivity map. In paper IV, we explored the roles of serotonergic neurons in impulsive behavior by manipulating and recording the neurons.

In summary, we have developed new methods for neural circuitry study, added our knowledge of the serotonergic and median prefrontal cortex circuitry, and gained deeper insights of functional roles of serotonergic neurons.

# LIST OF SCIENTIFIC PAPERS

I. Iskra Pollak Dorocic, Daniel Fürth, **YANG XUAN**, Yvonne Johansson, Laura Pozzi, Gilad Silberberg, Marie Carlén, Konstantinos Meletis
A whole-brain atlas of inputs to serotonergic neurons of the dorsal and median raphe nuclei.
Neuron, 2014, Volume 83, Issue 3, 663-678

II. Daniel Fürth, Thomas Vaissière, Ourania Tzortzi, **YANG XUAN**, Antje Märtin, Iakovos Lazaridis, Giada Spigolon, Gilberto Fisone, Raju Tomer, Karl Deisseroth, Marie Carlén, Courtney A. Miller, Gavin Rumbaugh & Konstantinos Meletis
An interactive framework for whole-brain maps at cellular resolution
Nature Neuroscience, 2018, 21, 139–149

III. **YANG XUAN***/ Sofie Ährlund-Richter*, Hoseok Kim, Iskra Pollak Dorocic, Felix Wahl, Konstantinos Meletis, Marie Carlén.
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Manuscript

IV. Xinming Wang*/ **YANG XUAN***/ Hoseok Kim*, Iskra Pollak Dorocic, Daniel Kaping, Marc Parent, Konstantinos Meletis#, Marie Carlén#
*) Equal contribution, #) corrsponding author
Manuscript

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-HT | Serotonin, 5-hydroxytryptamine |
| BAC | bacterial artificial chromosome |
| CaM | Calmodulin |
| CAV-2 | Canine adenovirus type 2 |
| CGE | caudal ganglionic eminence |
| ChR2 | Channelrhodosin 2 |
| cpGFP | circular permutated Green Fluorescent Protein |
| CVS | Challenge Virus Standard |
| DRG | Dorsal Root Ganglia |
| DRN | Dorsal Raphe Nucleus |
| EnvA | avian sarcoma and leucosis virus (ASLV-A) envelope protein |
| FIP | Frame-projected Independent-fiber Photometry |
| G | Glycoprotein |
| GABAergic | γ-aminobutyric acid-realeasing |
| GECI | Genetically Encoded Calcium Indicator |
| GRIN | gradient refractive index |
| HSV-1 | Herpes Simplex 1 |
| LH | Lateral Hypothalamus |
| loxP | Locus of crossing (x) over, P1 |
| MGE | medial ganglionic eminence |
| mPFC | Medial prefrontal cortex |
| MRN | Median Raphe Nucleus |
| NAc | Nucleus Accumbens |
| NMDA | N-methyl-D-aspartate |
| NP | Nonpeptidergic |
| oG | optimized G |
| PFC | Prefrontal cortex |
| PV | parvalbumin |
| retro-AAV | retrograde Adeno-Associated Virus |
| RV | Rabies Virus |
| RVdG | G-deleted RV |
| RVdGL | G and L both deleted RV |
| S1 | somatosensory cortex |
| SAD | Street-Alabama-Dufferin |
| SiR | Self-inactivated RV |
| SOM | somatostatin |
| SSRIs | Serotonin Selective Reuptake Inhibitors |
| TET | transactivation-based tetracycline |
| TH | Tyrosine Hydroxylase |
| TPH2 | Tryptophan hydroxylase 2 |
| TRE | TET Response Element |
| tTA2 | tetracycline transactivator |
| V1 or VISp | primary visual cortex |
| VGLUT1 | vesicular glutamate transporter 1 |
| VIP | vasointestinal peptide |
| VTA | Ventral Tegmental Area |

# 1 INTRODUCTION

The ultimate goal for neuroscience study is to understand the principles of the brain. It can be studied at different levels, including subcellular (i.e., intracellular molecular, chemical or electrical signaling), cellular (i.e., synapse and extracellular messengers from glia), circuit (neurons projecting to neurons), and even higher levels. One of the most fascinating and unique features of the nervous system is its ability to recruit billions of intertwined neurons to coordinate together in a sophisticated manner for one single behavior. In this complicated process, information are transferred, processed, and storage by neural circuits. As it is only until the neural circuit level that makes the nervous system particularly special compared with other systems in biology, studying neuroscience at the neural circuits level is of great importance.

## 1.1 HOW TO STUDY NEURAL CIRCUITS?

Two important physiological properties that distinguish the nervous system from other cellular systems are the ability for neurons 1) to generate action potentials (i.e., the electrical signals within the neuron), and 2) to communicate with each other through electrical and chemical synapses. The biological fundament of both properties is anatomy. In detail, the first one depends on the innate biological properties, including the shape, distributions of certain ion channels, and expression level of certain proteins in the neuron. The second is built on how the neurons are connected with each other (i.e., who to whom, and in what way). The study of neural circuits cannot be achieved without either part.

More importantly, the functions of specific neural circuits must be studied *ex vivo* with electrophysiology or *in vivo* under a behavior paradigm.

### 1.1.1 How to approach?

Neurons can be grouped based on numbers of different factors alone or combined, including electrophysiology parameters, morphology, location of the neuron, projection pattern, connecting properties and their molecular identities (expression of different ion channels, neurotransmitters, receptors, or other proteins). The molecular profile of the neuron serves as the physiological basis of most other features, so in theory it should be possible to target any type of neurons based on different combinations of the molecular profile. However, current technique only allows the targeting to be based on a limited numbers of molecular properties. Besides, projection dependent targeting is also frequently used.

### 1.1.1.1 Genetic targeting (molecular identity)

Modern genetic tools allow scientists to approach neurons based on their known molecular identities, and it is mostly done with the help of Cre recombinase (Sternberg & Hamilton, 1981). Cre recombinase is a protein that recognizes the loxP (Locus of crossing (x) over, P1) sites, and causes the recombination of it. Cre is usually inserted inside or close to certain regulatory elements (i.e., promoter region) of the gene of interest, so that Cre can be driven when that gene is expressed. For example, in the cortex, the principal pyramidal neurons are the glutamatergic neurons, and they all use glutamate as their neurotransmitter. For targeting pyramidal neurons in the cortex, one can utilize the genes involved in glutamate synthesis, transport (vesicular glutamate transporter 1(VGLUT1)), or re-uptaking. Taken the VGLUT1-Cre mouse as an example here, it can be used for knock-out, or over-expressing of genes of interest in pyramidal neurons by breeding with another transgenic animal (**Figure 1a, b**). For local manipulations in the brain, viruses containing loxP sites is mostly used under a similar mechanism. One thing has to be kept in mind is that some of the genes may only be transiently expressed during development which would lead to an ectopic recombination of the loxP sites, and using virus would solve this problem.

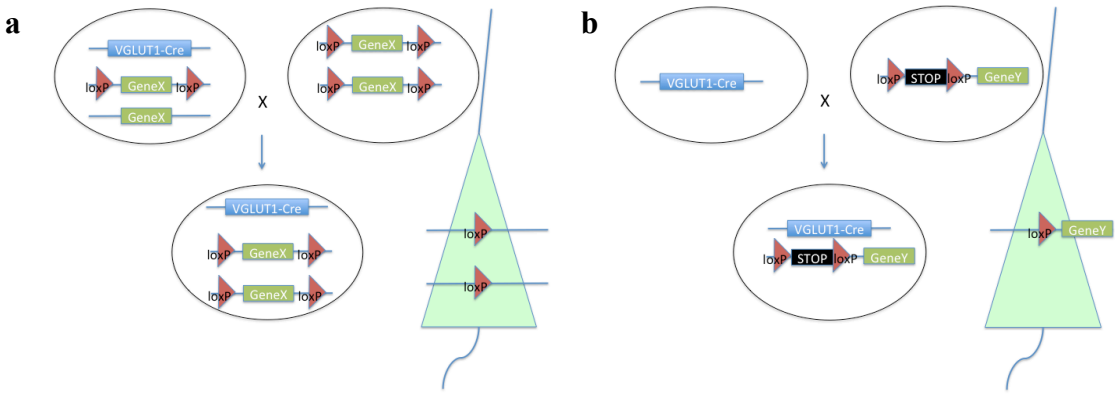


**Figure 1a VGLUT1-CRE breeding with an animal flanking with loxP sites for Gene X, resulting in loss of Gene X expression in offspring cortical pyramidal neurons.**
**Figure 1b VGLUT1-CRE breeding with an animal that has a stop cassette flanking with loxP sites before Gene Y, resulting in over-expressing of Gene Y in offspring cortical pyramidal neurons.**

### 1.1.1.2 Projection dependent targeting

The recent development of a series of genetically engineered viruses including retrograde Adeno-Associated Virus (retro-AAV) (Tervo *et al.*, 2016), Herpes Simplex 1 (HSV-1), Canine adenovirus type 2 (CAV-2) (Soudais *et al.*, 2001), and Rabies Virus (RV)

(Wickersham *et al.*, 2007a) have expanded the field to target neurons in a projection-specific manner. For instance, neurons sending their exon projections to area A can be targeted by injecting a retrograde spreading virus in that area.

RV is a retrograde neurotropic virus. It is a rod shaped, enveloped, single stranded, negative-sense RNA virus, with an encoded genome of 12 kb carrying five genes. The virus infects neurons through axonal terminals, replicates in the hosting neurons, buds out from dendrites, and spreads to presynaptic neurons though the synapse (Baer, 1991). The five encoded genes are nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the RNA polymerase (L) (Finke & Conzelmann, 2005).

Because of its neurotropic feature, RV has become widely utilized in neuroscience for characterization of anatomical connections. In the beginning, when RV's pathogen (i.e., how they enter and spread in the neurons) was not well identified, non-modified, wild type RV was used for circuit studies. Early studies controlled the spread of RV in a time-dependent manner (Ugolini, 1995). Unmodified RV is able to infect presynaptic axons widely, as it has a wide tropism and does not require a specific receptor to be taken up. This feature can be useful for revealing series of synaptically linked neurons. As the transsynaptic passes is not precisely controlled, it was hard to know exactly how many synapses the RV had crossed.

### 1.1.2 How to study connectivity?

Neural connectivity can be divided into the architectural connectivity (i.e., neuroanatomy), the functional connectivity (i.e., connections through synapses), and effective connectivity. Different approaches for studying neural circuits can sometimes cover two aspects. Because the spread of the RV is through synapses, the labeling does not only represent neuroanatomy, it also shows functional connectivity. However, the effective connectivity, meaning which connectivity has how much influence on what behavior in what way at which time point has to be studied under certain behavior paradigm in combined with methods that have fine special and temporal resolutions such as electrophysiology or calcium imaging.

#### 1.1.2.1 G-deleted RV (RVdG)

Engineering the RV for monosynaptic spread takes advantage of the RV glycoprotein (G), a protein expressed in the envelope of the virus which mediates the budding of viral particles from the host cell. With the knowledge that the protein G is responsible for the propagation

of RV (Finke & Conzelmann, 2005), researchers started to engineer RVdG for retrograde circuit tracing in a controllable manner. Deletion of the G protein from the RV genome makes the virus unable to cross synapses (Mebatsion *et al.*, 1996; Etessami *et al.*, 2000). Trans-complementation with G, introduced separately, allows for monosynaptic spread of the RVdG. For visualization of both the presynaptic input population and the starting neurons, the deleted G is replaced in the RV genome by a fluorescent maker (e.g., GFP or mCherry). The most recent development of RV as a retrograde tracer includes a system which limits the transsynaptic spread to a single synapse passing (Wickersham *et al.*, 2007b).

Compared to conventional tracers, the RVdG system has a lot of advantages. The most prominent advantageous feature is the ability to target and trace the input populations to specific cell types, rather than roughly mapping connectivity between brain regions in a broader spectrum (Kobbert *et al.*, 2000). Non-viral tracers, such as Phytohaemagglutinin-Leucoagglutinin (Gerfen & Sawchenko, 1984), biocytin (King *et al.*, 1989), Horseradish Peroxidase (Kristensson & Olsson, 1971), and a few others, lack the ability for cell type specific targeting, and they also label axons passing through the injection regions. Other tracers, such as Tetanus Toxin C fragment (Schwab & Agid, 1979), Wheat Germ Agglutinin (WGA; (Gonatas, 1979)) can be used in a Cre-dependent manner and thereby allow for transneuronal tracing to/from specific neuronal types. However, these methods do not have very strong signals, and therefore make it difficult to detect and to quantify weak connectivity. Most importantly, unlike the rabies virus, which passes over synaptic connections between neurons, these tracers cannot distinguish between directly connected neurons and passing through projections. In summary, the development of RVdG marks a significant advancement in methodology and genetically modified rabies viruses have proven to be powerful biological tools for neural mapping, and manipulation.

A further modification to restrict the initial transduction of RVdG to a specific population is to pseudotype the virus with an envelope protein from a separate avian virus, the avian sarcoma and leucosis virus (ASLV-A) envelope protein EnvA. This protein can only bind to the complementary TVA receptor and therefore leads to the taken-up of the virus by the TVA-expressing cells. Mammalian cells lack the TVA receptor, and therefore only target cell populations engineered to express this receptor will be susceptible to RV transduction, leaving neighboring cells unaffected (Wickersham *et al.*, 2007b) (**Figure 2**).

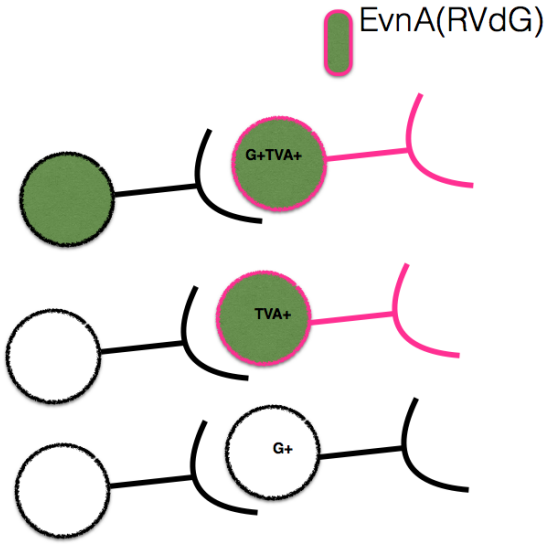


**Figure 2 Illustration of the RVdG system for restricted, monosynaptic transduction**

Several approaches have been developed to target specific cell types, including viral and transgenic strategies. A commonly used approach to deliver TVA and G into desired neural populations takes advantage of the Cre-loxP system and can be achieved by both viral and transgenic approaches. The first step is to define the primary (starter) population, most often a specific neuron by cell type in a specific brain region, and identify a specific Cre-driver mouse line targeting the primary population. TVA and G are delivered virally using an AAV with expression under the control of Cre. TVA and G can be carried into the cell by either one single virus (Haubensak *et al.*, 2010; Wall *et al.*, 2010) or by several viruses (Watabe-Uchida *et al.*, 2012; Miyamichi *et al.*, 2013). After a period of approximately 14-21 days, necessary for sufficient TVA and G expression, RVdG is injected into the same location. The Cre starter cells are now expressing TVA, allowing binding and entry of the RVdG, and the expression of G in the starter neurons allows for monosynaptic retrograde spread of the RVdG to neurons giving input to the starter neurons.

It is possible to use the transactivation-based tetracycline (TET)-system combined with the Cre-loxP system to label the primary population in a temporal dependent manner. Expression of the tetracycline transactivator tTA2 can be engineered to be CreER-dependent, and when the ligand for CreER (i.e., tamoxifen) is administered, tTA2 is

expressed and binds to the TET Response Element (TRE) to initiate the expression of TVA and G (Miyamichi *et al.*, 2011).

Other recombination-based systems (e.g., Flp-frt or Dre-rox) can also be introduced in combination with the Cre-loxP system for more precise targeting, as for most neurons, they are identified best by combining two or more features (i.e., active promoters or enhancer elements) together. For example, in the Flp-frt combined with Cre-loxP intersection approach, TVA and G expression are only expressed when both Flp and Cre are present (Fenno *et al.*, 2014).

TVA can also be delivered by crossing of a Cre-dependent TVA mouse line (Seidler *et al.*, 2008) with a cell-type specific Cre line, followed by Cre-dependent viral G delivery. This strategy has been used in the hippocampus (Sun *et al.*, 2014) and somatosensory brainstem (Bechara *et al.*, 2015). However, as the RVdG travels in a retrograde manner, long-range TVA/Cre-expressing neurons projecting to the injection area holding the 'true' TVA/Cre-expressing starter cells can also be transduced by the RVdG, and function as starter neurons. Inputs to these neurons can thereafter be retrogradely labeled, rendering this strategy susceptible to false positive labeling.

To limit the amount of spread of pseudotyped RV outside of targeted area, as well as to spatially restrict the neurons transduced, at least one element (either TVA or G) is delivered virally in all of the studies mentioned above.

Engineering the tropism of rabies virus can also limit the expression pattern. A recent study screened different avian-derived receptors and avian sarcoma leukosis virus (ASLV) envelopes pairs with pseudotyped lentiviruses *in vivo*. Three pairs (EnvA-TVA950, EnvB-TVBS3, and EnvE-DR-46TVB) have shown high infectious efficacy with no observed cross-activity between each other *in vivo.* In combination with any of the systems mentioned above, this approach could also be applied to a rabies virus system to simultaneously trace the inputs to diverse neuronal circuits independently (Matsuyama *et al.*, 2015).

Like other neurotropic viruses, RV eventually causes neurotoxicity and leads to the death of infected cells. Several studies have attempted to determine viability of RVdG transduced cells. Normal electrophysiological responses for up to 12 days post-infection are seen (Wickersham *et al.*, 2007a), and normal neural activity measured by calcium-imagining *in vivo* up to 11 days post-infection (Osakada & Cui, 2011) are seen. After 16 days, the

number of fluorescent cells decreases and morphological changes related to cytotoxicity appear (Wickersham *et al.*, 2007a).

For short-term use (up to two weeks) and anatomical studies, first generation RVdG (both G coated and EnvA coated) is more than enough because of its high efficiency of labeling presynaptic neurons and its ability to selectivity target certain neuron types (EnvA coated system) or even in a projection-specific manner. But to be able to manipulate neuronal pathways in a transsynaptic-based manner, it is necessary to develop a non-toxic rabies system. Several modifications and new approaches have been developed to reduce or overcome the toxicity problem.

The first approach is to modify the existing RV system (using the same Street-Alabama-Dufferin (SAD)B19 strain) by deleting or modulate the "toxic" components. The deletion of both the viral RNA polymerase L and the Glycoprotein in the genome of the RV made the virus to survive for at least 4 months with non-detectable morphological changes (Chatterjee *et al.*, 2018). *Ex vivo* electrophysiological recordings of the infected neurons showed normal electrical properties up to 8 weeks, and *in vivo* two-photon calcium imaging experiments showed stable visual responses after 4 months. It also has a wider tropism compared to other retrograde viruses such as CAV2 and retro-AAV. However, it has a reduced viral expression level, and this feature allows the second-generation SADB19 G-RVdGL only for delivering genes that do not need much expression levels to be functional, such as cre and flpase. It is not compatible with transsynaptic tracing system yet, and this property made the virus more like other retrograde viruses with a less bias of infection (Chatterjee *et al.*, 2018). Future direction would be to make the virus compatible for transsynaptic labeling.

The second strategy is to go back to nature and use another RV strain or even other viruses. Screening from other laboratory RV strains apart from the commonly used SADB19 strain, the glycoprotein-deficient Challenge Virus Standard (CVS)N2c was shown to have reduced cytotoxicity, showing optically induced neural activity of cortical cells *in vitro* for up to 28 days, and calcium responses *in vivo* for up to 17 days post-infection (Reardon *et al.*, 2016). This improved viability comes at a cost of reduced virus expression and lower fluorescent signal (in part due to lower titer), perhaps making the strain better suited for neuronal modulation such as optogenetic manipulation rather than detailed anatomical tracing.

The third one is to conditionally inactivate the virus after infection, creating a manageable time window before the virus terms to uncontrollable toxicity. As the transient expression

of recombinase (e.g., cre or flpase) carried by the modified rabies is sufficient to drive certain gene expressions, it would leave a marker of infection, which also shows the labeling of the synaptical-based connection (Ciabatti *et al.*, 2017).

#### 1.1.2.2 Visualization of connectivity

Several projects including the Allen Mouse Brain Connectivity Atlas (Oh *et al.*, 2014), Mouse Brain Architecture project (Bohland *et al.*, 2009) and Mouse Connectome project (Zingg *et al.*, 2014) are using several different multidisciplinary methods to understand the brain connectivity. One crucial step to put all those massive efforts together and to start comprehending the complicity of the brain is to visualize the data in a computational and standardized way. That is, we need tools to automatically detect the object (e.g., neurons, processes, different markers labeled by antibody staining, ect…), to register it onto the brain atlas, to annotate the position, to represent it digitally and to analyze the whole brain datasets.

A lot of individual labs have also initiated whole-brain mapping projects with semi-automated or automated pipelines for transforming images obtained from different imaging methods to standardized brain datasets. For example, ClearMap is used for intact cleared tissue with light-sheet microscope data (Renier *et al.*, 2016). Other brain mapping approaches using different algorithms have been invented for processing the images from serial two-photon microscope (Kim *et al.*, 2015; Vousden *et al.*, 2015) or wide-field microscopes (Hunnicutt *et al.*, 2014). A detailed comparison of recent developed pipelines for brain atlases can be seen in **Paper II, supplementary Table 1**.

### 1.1.3 Effective connectivity of circuits

Two fundamental issues to study the function of circuits are how to observe the circuits and how to manipulate it. For the first one, intracellular (e.g., patch clamp, voltage clamp, ect…) and extracellular (e.g., silicon probes) electrophysiological recordings remain the mainstay in the neuroscience field. In this thesis, we used a calcium indicator that can be visualized by microscope in vivo and therefore will discuss about it in this chapter.

Like the general strategy for studying other biological questions, one can use gain-of-function (i.e., activation of neurons) and loss-of-function (i.e., inhibition of neurons) to study neural circuits. This can be achieved by the injections of various reagents including pharmacological drugs (e.g., ion channel agonists or antagonists), chemicals, and virus systematically through the circulation system or locally in the brain. However, they do not

have a cell type specific resolution and cannot control the neurons in a millisecond time scale. In this thesis, we used optogenetics to manipulate the firing of different types of neurons.

#### 1.1.3.1 Visualization of neuron activity

GCaMP protein is a single fluorophore based Genetically Encoded Calcium Indicator (GECI). It consists of a circular permutated Green Fluorescent Protein (cpGFP) in the middle, and is flanked by Calmodulin (CaM) on the C terminal and myosin light chain kinase peptide (M13) on the N terminal (**Figure 3a** (Nakai *et al.*, 2001)). Without calcium, the cpGFP in GCaMP is in a poorly fluorescent status. In the presence of calcium, CaM changes its conformation and is able to bind to the CaM binding peptide, M13. This results in bringing the cpGFP to a brighter fluorescent status (**Figure 3b** (Nakai *et al.*, 2001)). Therefore, by monitoring the fluorescent level change, one can measure the intracellular calcium signal change.

As neuronal activity changes result in dramatic changes in intracellular calcium level, GCaMP serves as a very good candidate for measuring neuronal activities. Compared with other calcium indicators such as small synthetic chemical dyes, GCaMP is less toxic and can be delivered into desired neuronal populations. Both features make it possible to do less invasive and chronic imaging recordings combined with more complicated behavior experiments. However, it is not as sensitive and dynamic as chemical dyes. Since the invention of GCaMP (Nakai *et al.*, 2001), it has been genetically engineered to be adapted to *in vivo* neuroscience studies by increasing the sensitivity, stability, and kinetics (Nakai *et al.*, 2001; Tian *et al.*, 2009; Akerboom *et al.*, 2012; Chen *et al.*, 2013). The widely used GCaMP6 series have significant increased the sensitivity of detecting, and the accuracy of following action potentials. Furthermore, their fine spatial resolutions made it possible for multiple applications usages, ranging from synaptic compartments recordings to population-based neuronal activity recordings (Chen *et al.*, 2013).

To monitor calcium signal changes (indicated by GCaMP fluorescent signal changes here) in neurons of active animals, different imaging recording systems with resolutions at subcellular, individual neuron, and population neuron levels have been developed accordingly with the development of calcium indicators.

At the subcellular level (i.e., for visualizing dendritic spines or axonal boutons), the laser scanning based two-photon microscope is very widely used (Dombeck *et al.*, 2007; Petreanu *et al.*, 2012). The conventional desktop two-photon microscope is huge in size and needs a

heavy air table to reduce vibration-induced artifacts. Consequently, all the applications with this system are built in a head-fixed format. Although it may increase the stress level of the animal under such setups, it also gives a unique opportunity for using other behavior paradigms such as virtual reality based sensory manipulations, which are difficult to approach in non-head-restrained animals. However, the two-photon microscope based system can only be used for superficial brain areas (approximately 800-900 μm from the skull) such as the primary visual cortex (Andermann *et al.*, 2013) or folded brain structures such as the entorhinal cortex with the help of an angled microprism (Low *et al.*, 2014), mainly due to light scattering through the brain tissue. Moreover, the drawbacks of head-fixed animal experiments include elevated stress level of the animal due to constrained physical activity and the incompatibility for complicated natural behavior setups such as social behavior study (Jercog *et al.*, 2016). A recent break through innovation is a high-resolution, miniature two-photon microscope that can be used in freely moving and behaving mouse with a comparable performance level to a normal two-photon microscope (Zong *et al.*, 2017). Future directions of the two-photon system would be to find non-invasive ways for deep brain structure imaging, perhaps by using less scattered red-shifted calcium indicators (i.e., mRuby based jRCaMP1a, b, and mApple based jRGECO1a (Dana *et al.*, 2016)) or combining the system with the miniature gradient refractive index (GRIN) lens based microendoscopic lenses (Jung *et al.*, 2004).

At individual neuron level, the head-mounted miniature single-photon wide field microscope is commonly used. As it is light in weight (1.9 grams), it is often used for imaging in freely moving and behaving animals (Ghosh *et al.*, 2011). This system covers a large brain area (approximately 0.5 $mm^2$), making the observation of hundreds of neurons at the same time possible. It is fast in acquisition and does not have many artifacts from animal movement. By combining the miniature microscope with GRIN lens based microendoscope probes (Jung *et al.*, 2004), deeper brain structures such as lateral hypothalamus are also approachable even for chronic recordings (Jennings *et al.*, 2015).

At population neuron activity level, Frame-projected Independent-fiber Photometry (FIP) (**Figure 3c, 3d**) (Kim *et al.*, 2016a) for recordings from several brain areas is a recent development of the fiber photometry system (Lutcke *et al.*, 2010; Schulz *et al.*, 2012; Lerner *et al.*, 2015). The system has a fast cMOS camera mounted directly to a single-photon wide field microscope with a 20x/0.75NA objective. A series of dichroic mirrors are also integrated into the platform for allowing the excitation from different wavelengths (470nm blue light for excitation of GCaMPs and 410nm purple light for baseline

reference). The front of the objective is connected to a patchcord at a working distance for imaging. The other end of the patchcord terminates with several individual metal ferrules, which can be connected with brain-implanted optic fibers via metal sleeves. Unlike the miniature one-photon or two-photon microscope setups that need docking platforms for the camera to be mounted, it is much more practical in this system to image from multiple brain areas simultaneously as the optic fibers implanted in the animals are only 400μm in diameter. Moreover, the capability for imaging from deep brain structures in a freely moving animal manner is also a plus. Although it is also possible to do dual cortical recordings in mice with a dual-axis two-photon microscope (Lecoq *et al.*, 2014), FIP still serves as one of the most popular neuron ensemble activity imaging system due to its simplicity. In **paper IV**, we also used FIP to image from the Tryptophan hydroxylase 2 (TPH2) population in Dorsal Raphe Nucleus (DRN) in the rats. However, based on comparison between FIP and electrophysiological recordings, whether calcium signal from FIP recordings represent population neuronal activity are still debatable (London *et al.*, 2018).

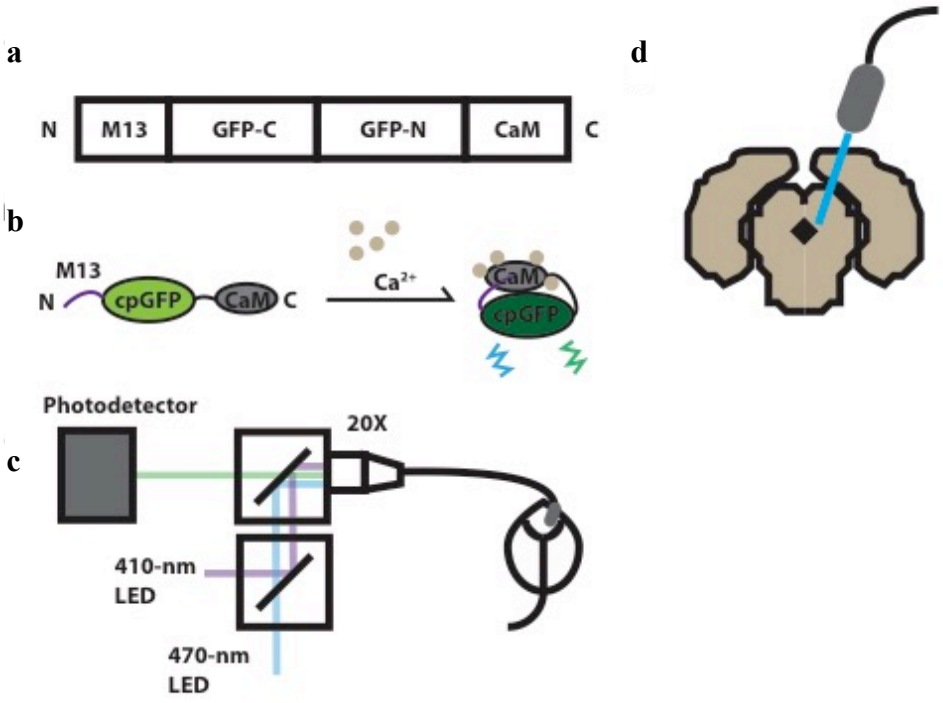


**Figure 3a Schematic representation showing GCaMP protein structure.**
**Figure 3b Illustration showing GCaMP functions as a calcium indicator**
**Figure 3c Fiber photometry setup**
**Figure 3d Schematic drawing showing fiber photometry implant in dorsal raphe nucleus in rats for populational neuron activity measurement**

#### 1.1.3.2 Manipulation of neuron activity

Optogenetics is the technique to control neuron activities by utilizing light-activated microbial opsins. The opsins are usually delivered to the neurons by viruses or by genetically engineered into the genome. Combining with recombinase dependent viral vectors or mouse lines, the opsins can be delivered in a cell type specific manner. Depending on the viral tropism, they can also be delivered in a projection specific manner.

The most widely used opsin is Channelrhodosin 2 (ChR2), which is a light activated non-selective positively charged ion channel taken from the algae *Chlamydomonas reinhardtii* (Nagel *et al.*, 2002). It is the first microbial opsin used in mammalian cells (Boyden *et al.*, 2005). In neurons expressing ChR2, when ChR2 is activated by blue light, the channel opens which leads cation influx and triggers neuron depolarization. By controlling the blue light pulses, neurons can be controlled for firing in a millisecond timescale manner. Early inhibitory opsins (e.g., Halorhodopsin or Bacteriorhodopsin) are chloride or proton pumps. The progress in high-resolution crystal structure of ChR also helped for engineering the pore to get chloride channels (i.e., iC1C2) (Berndt *et al.*, 2014). The red-shifted opsin such as ChrimsonR (activated by 590nm light) (Klapoetke *et al.*, 2014) can be used in combining with GCaMP based brain imaging.

## 1.2 PREFRONTAL CORTEX-ANATOMY

The prefrontal cortex (PFC) is located in the forefront of the brain. It is involved in various aspects of cognition, including attention, memory and decision-making. Based on a lot of functional brain imagine studies in human, PFC can be divided into dorsolateral, dorsomedial, ventromedial and orbital frontal. In mouse, it consists of secondary motor, anterior cingulate, prelimbic, infralimbic, orbital, and agranular insular area (Carlen, 2017).

### 1.2.1 Afferent projections to mPFC

Anatomically, the mPFC is defined as the projection area of mediodorsal nucleus of the thalamus from early anatomical studies (Rose & Woolsey, 1948). Beside the thalamus, the mPFC is known to also get massive afferent inputs, most often identified with conventional retrograde tracer methodology, from diverse brain areas that are involved in cognitive functions (Hoover & Vertes, 2007). A recent study using the RVdG system, which limits the spread of the virus in a monosynaptic and cell type specific manner, showed that mPFC Layer 5 (L5) receives inputs mainly from other prefrontal areas, including the contralateral

mPFC, the agranular insula, and the motor cortex (DeNardo *et al.*, 2015a). Other brain areas, such as hippocampus, claustrum, nucleus of the diagonal band, medial septum, and basolateral amygdala also project to mPFC L5.

### 1.2.2 Efferent projections of mPFC

Given that mPFC receives abundant local afferent input, it is interesting to note that the mPFC also reciprocally project back. The mPFC also send reciprocal projections to the horizontal diagonal band, substantia innominate, and the basolateral amygdala. The prelimbic subarea of the mPFC projects to the opiate receptor rich striatum striosomes, while the dorsomedial PFC terminates more diffusely throughout the striatum, preferably in the striatum matrix (Donoghue & Herkenham, 1986; Sesack *et al.*, 1989). In the brainstem, mPFC sends massive efferent projections to the superior colliculus, ventral tegmental area, periaqueductal gray, and the mesencephalic reticular formation (Sesack *et al.*, 1989).

### 1.2.3 Layers and connectivity within mPFC

Unlike other neocortical areas, the rodent mPFC does not have the granular layer IV. Layer IV is usually also the input-receiving layer. Nevertheless, all other layers in mPFC gets long-range afferent projections from other brain areas, including both cortical and subcortical areas (Hoover & Vertes, 2007). The mPFC consists of excitatory principle pyramidal neurons and intertwined interneurons - fast spiking parvalbumin (PV), somatostatin (SOM) and vasointestinal peptide (VIP) being three major interneuron types. The perisomatic targeting PV neurons governs the outputs of the principle pyramidal neuron, as they employ rapid, dominant and uniformly suppression on principal pyramidal cell firing ( Sparta *et al.*, 2014). The dendritic targeting SOM modulates the input the principle pyramidal neuron get, and the influence is weaker yet longer (Kvitsiani *et al.*, 2013).

## 1.3 INTERNEURONS

Interneurons are the inhibitory γ-aminobutyric acid-realeasing (GABAergic) neurons. In cortex, it consists of about 20% of the whole neuron population. It coordinates together with the principal excitatory neurons and forms the highly organized neural network.

Different approaches have been applied with the attempt to identify interneuron cell types in different brain areas. Using single cell RNA sequencing data, 16 types of interneurons have been clustered base on their molecular profile in adult mouse somatosensory cortex (S1) and hippocampus (Zeisel *et al.*, 2015). In the primary visual cortex (VISp or V1), 23 different types of interneurons were identified, of which 18 belongs to the three major types

(PV, SOM, VIP) (Tasic *et al.*, 2016). Using simultaneous *ex vivo* octuple whole-cell electrophysiology recordings, 15 morphologically defined types of interneurons were characterized in V1 (Jiang *et al.*, 2015).

Developmentally, interneurons originate from the medial (MGE) and caudal (CGE) ganglionic eminence. It is also believed that these two areas give rise to different interneurons: VIP neurons are from CGE (Lee *et al.*, 2010), while PV- (Butt *et al.*, 2005) and SOM- (Fogarty *et al.*, 2007) expressing interneurons derive from MGE. Single cell RNA sequencing analysis using E12.5 and E14.5 dissected mouse MGE and CGE samples identified 13 and 11 different interneuron progenitor types (Mi *et al.*, 2018). By comparing the embryonic data with the adult data (Tasic *et al.*, 2016), the analysis shows that the interneurons have a determined fate long time before they migrate into the right position (Mi *et al.*, 2018).

## 1.4 THE SEROTONERGIC SYSTEM AND IMPULSIVITY

Serotonin (5-hydroxytryptamine, 5-HT) is a neurontransmitter first discovered in 1937 and later re-identified (Erspamer & Asero, 1952). 5-HT-expressing neurons are located in the caudal part of the brain, mainly in the midbrain and brainstem regions. The serotonin nuclei are divided into two groups, with one group having ascending projections and the other group projecting to the spinal cord (Dahlstrom & Fuxe, 1964). The serotonergic neurons belonging to ascending serotonergic group target mPFC, striatum, and the thalamus, which are also areas involved in decision-making.

### 1.4.1 Anatomy of the raphe nuclei

The major location of 5-HT neurons in the rostral raphe nuclei can be divided into the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN).

Serotonin also moderates the activity of a lot of different neurotransmitters, including the dopamine system. The interactions between the serotonin system and the reward system is believed to be important in different types of impulsivity (Dalley & Roiser, 2012).

### 1.4.2 Impulsivity

Impulsivity can be grouped grossly into impulsive action and impulsive choice. The impulsive action is characterized as the failure to inhibit undesired action, and the impulsive choice means the preference of choosing small instant rewards over bigger postponed rewards. A large number of studies have implied that 5-HT is involved in

impulsivity. Pharmacological depletion of 5-HT in projecting forebrains results in the chossing of a small immediate reward (increased impulsive choice). Moreover, administration of Serotonin Selective Reuptake Inhibitors (SSRIs), which increases 5-HT intracellular level, showed a declined impulsive choice (Bizot *et al.*, 1999). (Also see review (Miyazaki *et al.*, 2012))

# 2 AIMS

The general aim of this thesis is to study long-range and local circuits involving mPFC, the 5-HT system in a neuron type specific manner, and the role of 5-HT in impulsive behavior. The specific aims are:

1) To characterize the monosynaptic inputs to the serotonergic system in the raphe nuclei of the mice. **(Paper I)**

2) To generate a framework for automatic registration, annotation, visualization of whole-brain data on a standardized mouse brain atlas. **(Paper II)**

3) To understand long-range and local circuitry in mPFC in mice by using a genetically modified rabies tracing system. **(Paper III)**

4) To comprehend the roles of serotonergic neurons in impulsive behaviors (impulsive action and impulsive choice) in rats by using optogenetic manipulation and recordings with imaging. **(Paper IV)**

# 3 METHODS

## 3.1 VIRUS

### 3.1.1 AAV

The AAV viruses used in this thesis were produced by Vector Core of University of North Carolina, Gene vector and virus core of Stanford University or Virovek. The titeration of ther virus is $10^{12}$-$10^{13}$.

### 3.1.2 RVdG

The rabies virus used for Paper II, III, and I were produced under a modified protocol based on (Wickersham *et al.*, 2010) paper. The main modification is at the pseudotying step to get rid of the contamination of G-RVdG-EGFP. Here is the detailed protocol. G-RVdG-EGFP virus was placed onto BHK-EnvA2 cells with one infection unit per cell on Day 1. After 24 hours of infection, cells were treated with 15 mL trypsin (Sigma-Aldrich, USA) for at least 10 minutes at 37 °C. Once the cells were all detached from a 175-$cm^2$ flask (VWR, USA), they were collected into 50 mL tubes and dissociated by pipetting up and down. The cells were collected at 150*G for 10 minutes. Supernatants were discarded and replaced with 50 mL DPBS (Thermo, USA). The cells were resuspended and dissociated by pipetting up and down for several times. The washing step was repeated for 3 times. At the end of the procedure, the cells were replated with DMEM (GE Healthcare, UK) onto a new 175-$cm^2$ flasks. Such procedures were repeated for two more days, and the supernatants were collected, filtered by 45μm filter (VWR, USA), and concentrated by ultracentrifugation on the final day.

## 3.2 TRANSGENIC ANIMALS

In this thesis, both mice and rats are used for anatomical or behavior studies. Compared with other model organisms, rodents are closer to human beings in evolution. In neuroscience studies, this closeness in evolution feature/ similarity is especially important, because researchers could implement complicated behavior paradigms that are derived from human studies. Rodents as a model organism has other benefits: 1) they are small and easy to handle; 2) they are prolific breeders with short life expectancies, so it takes shorter time to generate new rodent lines; 3) there are many well-characterized inbred and outbred rodent lines available, and their stable genetic status makes the experiment results unwavering and liable.

Genetically modified mouse models (knock-in and knock-out lines) are known to be well developed and widely used. As targeting certain neuronal population is essential for neuronal circuitry studies, several projects (Gong *et al.*, 2003; Madisen *et al.*, 2010) using Bacterial Artificial Chromosome (BAC) and knock-in technique have been initiated to generate CRE lines. In this thesis, both BAC and knock-in cre mouse lines were used (also see methods in **Paper I**, **II**, **III**).

Genetically engineered rat models are poorly developed, because the successful derivation of rat embryonic stem (ES) cells is only available until quite recently (Buehr *et al.*, 2008; Li *et al.*, 2008) and techniques that did not necessarily require ES cells were not available back then. For a very long period of time, disease models using rats are driven by phenotype based breedings and selections. It has only been until recently that researchers are able to generate genetically modified rat lines using BAC (Gong *et al.*, 2007; Witten *et al.*, 2011) for multi-copy insertion, and using Zinc Finger Nucleases (ZFNs) (Geurts *et al.*, 2009; Cui *et al.*, 2011), Transcription Activator-Like Effector Nucleases (TALENs) (Tesson *et al.*, 2011), CRISPR-Cas (Ma *et al.*, 2014) mediated approaches for site-specific knock-out and knock-in. Except for early technique barriers, rats have their own advantages over mice in neuroscience studies. First of all, they are bigger than mice in size, which makes it much more convenient to do microsurgeries and put implants on them. For example, it is more practical to implant multiple fibers or electrophysiological recording probes in rats if one needs to record from multiple brain areas simultaneously. Second, rats are known to be more capable of performing complicated cognitive behavior experiments than mice. In this thesis (Paper IV), the outbred strain Long-Evans rats were chosen over other rat strains as they are faster in learning tasks (Turner & Burne, 2014). As Tryptophan hydroxylase (TPH) serves as the enzyme for the rate-determining step of 5-HT synthesis, we chose BAC CH230-51G14 that carries TPH2 for targeting 5-HT neurons specifically.

## 3.3 GCAMP AND FIP RECORDING

In **Paper IV**, a total volume of 0.7μL AAV DJ/DIO-GCaMP6s virus is injected into each well-trained TPH2-CRE rats (also see methods in **Paper IV**), and fibers were implanted just above the Dorsal Raphe Nucleus with an angle of 20°. After 7-14 days of recovery, we used FIP to measure population neuron activity.

# 4 RESULTS & DISCUSSION

## 4.1 PAPER I

### A WHOLE-BRAIN ATLAS OF INPUTS TO SEROTONERGIC NEURONS OF THE DORSAL AND MEDIAN RAPHE NUCLEI

This project aims to characterize the circuitry controlling the serotonergic system in the raphe nuclei of the mice. To employ this, a genetically modified rabies virus system was used for tracing the monosynaptic inputs to DRN and MRN 5-HT neurons in mice. From the methodological aspect, we generated two AAVs (pAAV-DIO-TVA-mCherry and pAAV-DIO-Rabies Glycoprotein). One is to deliver the receptor for rabies virus infection (TVA) and the other is to bring the glycoprotein that is necessary for rabies virus transsynaptical labeling. Those two genes were cloned into a CRE dependent AAV vector so that their expression could be controlled in a CRE-dependent manner by using transgenic CRE animals.

We then created a whole brain diagram of the inputs by using customized software developed in the lab. We found inputs onto 5-HT from cortical areas, basal ganglia, midbrain, and hypothalamus. We found similar long-range inputs to 5-HT neurons in DR and MR on a gross anatomical level. We also functionally confirmed some of the monosynaptic inputs by in vitro slice recordings (i.e., inputs from the frontal cortical areas and the lateral habenula). Based on our tracing results, we were able to modify the conventional raphe nuclei 5-HT afferent model by adding directly monosynaptic inputs from the frontal cortical areas, striatum, globuspallidus, lateral hypothalamus, and substantial nigra, reticular part.

## 4.2 PAPER II

### AN INTERACTIVE FRAMEWORK FOR WHOLE-BRAIN MAPS AT CELLULAR RESOLUTION

We developed an open source interactive platform for processing brain images obtained from different light microscope (i.e., light-sheet, confocal, wide-field) using different labelling techniques (i.e., fluorescent protein, in situ hybridization, immunohistochemistry) and mapping the marked neurons to a standardized brain atlas. This also allows researchers all

over the world to present, compare, and share neuroanatomical data under different experimental setups by standardizing and registering data onto the same brain atlas.

We first created the reference atlas based on nonuniform rational B-splines (NURBS), and this feature also enables the images/data to be transformed into arbitrary angles other than coronal or sagittal sections, which is a huge advantage for visualizing certain projection pathways or brain areas as they might been observed better with other angles. For automated marking the features of interest (e.g., process, cell body, nuclei, staining of proteins) from the image, we applied a set of wavelet filters onto the image, and every output is stored. By playing with the intensity of fluorescence with the best stored outputs, we can extract the feature of interest (i.e., cell bodies). By grouping connected pixels and marking their borders, we easily labelled the feature of interest. The whole process is called segmentation. We also used the auto-fluorescent of the brain slice to locate the brain outline, and aligned the brain image with our standardized reference atlas by a set of reference points. Once this is done, the marked feature of interest from segmentation can be registered to the standardized atlas. Based on this basic feature, we further developed functions to reduce incorrect registration by removing and changing reference points manually.

We applied the framework to different experimental setups for different needs as a proof of concept. We first tested it for detecting fibre tracts. We then tested our framework with innate EGFP-expression brain sections stained with other neuron markers (in other fluorescent channels) and used for mapping co-labelling. It is also compatible with RNA in situ hybridization data. We also applied our framework to monosynaptic rabies tracing images, which usually labels heavily both the cell bodies and the processes. We checked the inputs to different types of neurons in motor cortex (CamKIIa- and Gad2-expressing neurons) and striatum (D1R-, D2R- and ChAT-expressing neurons), and found the software was able to segment the cell bodies from the rest. We finally segmented the nuclei-located c-fos ( a marker for immediate early gene activity) expression (labelled by immunohistochemistry) and compared the expression levels before and after cocaine administration.

## 4.3 PAPER III

### A WHOLE BRAIN ATLAS OF THE MONOSYNAPTIC INPUT TARGETING FOUR DIFFERENT CELL-TYPES IN THE MEDIAL PREFRONTAL CORTEX OF THE MOUSE

In this project, we aim to understand the circuitry within the median prefrontal cortex (mPFC) and the key players from other brain areas that controls it. We traced the monsynaptic inputs to three of the inhibitory (PV, SOM, VIP) and the excitatory neurons (Pyramidal neurons) in the median prefrontal cortex (mPFC) by using a modified rabies system. In order to look at the long-range and local inputs at the same time, we developed a single viral system by putting TVA and Glycoprotein into one CRE-dependent AAV vector (pAAV-DIO-TVA-V5-RG). By staining the V5 tag after TVA, the real primary infected populations can be distinguished from the input neurons.

Based on our results, we have found that all of the four different types of neurons receive strikingly similar long-range inputs. The main input to both excitatory and inhibitory neurons in mPFC is from local, but all four types receive extensive long-range inputs from the rest of cortex. Basal forebrain and thalamus provide the most prominent inputs from subcortical areas. Within mPFC, SOM receive lots of inputs from PV and VIP, but they provide little inputs back. VIP and PV send reciprocal efforts to each other.

## 4.4 PAPER IV

### SEROTONERGIC NEURONS DIRECTLY CONTROL IMPULSIVE BEHAVIORS IN RATS

This study aims to comprehend the roles of serotonergic neurons in impulsive behaviors (impulsive action and impulsive choice) in rats. To target specifically the 5-HT neurons, we developed for the first time a transgenic rat strain. The CRE-recombinase gene is introduced right after the start codon (ATG) of the first exon in the TPH2 gene in the bacterial artificial chromosome (BAC), and this is to control CRE under the TPH2 promotor. As TPH2 expresses in 5-HT neurons in the raphe nucleus, offsprings of this BAC inserted founders can be used for manipulating 5-HT neurons.

The animals were trained using a modified behavioral paradigm(Staddon & Cerutti, 2003) to study the impulsive action. They were firstly trained to associate visual cues with reward delivery. The reward would be delivered in a fixed delay (FI) after the animal has poked to the reward port. Fully trained animals showed boosted nose-poking rates (NPR) at the end of the delay. Later, unrewarded probe trials (Peak Interval, PI) with longer delays were randomly introduced in between FIs. In FI trials, activation of 5-HT by optogenetic manipulations resulted in a decrease in impulsive actions in longer delays, while deactivation of 5-HT neurons showed an increase of impulsive actions in all different delay

length. In PI trials, activation of 5-HT neurons showed a decrease in impulsive action, while deactivation of 5-HT showed an increase of impulsive actions. With the same behavioral paradigm, in vivo fiber photometry recordings revealed an upregulation of the innate calcium signals in DR 5-HT neurons during reward consumption.

To study the impulsive choice, the animals were trained first to associate two different visual cues with either a short delay small reward (SS) or a long delay large reward (LL). Deactivation of 5-HT neurons by optogenetic modulations in DR showed an increased rate of correctness in different LL trial delay times.

Taken results together, we have shown the direct involvement of DR 5-HT neurons in both impulsive action and impulsive choice.

# 5 DISSCUSSION & PERSPECTIVES

## 5.1 RABIES VIRUS- CONTROVERSIAL ISSUES

### 5.1.1 Is RV infection biased?

This issue can be discussed under two situations: 1) direct and 2) trans-synaptic labeling. When injected in Anterior Cingulate Cortex in a reporter line, RVdGL-cre showed a labeling of all layers in V1, whereas CAV2-cre (Soudais *et al.*, 2001) labeled mostly layer 5 neurons. Retro-AAV (Tervo *et al.*, 2016) had labelings in other layers but very few in layer 5 (Chatterjee *et al.*, 2018). In another direct labeling by RV study, Self-inactivated RV (SiR) and retro-AAV were both injected in Ventral Tegmental Area (VTA). SiR showed a better labeling of subcortical structures such as Lateral Hypothalamus (LH) and Nucleus Accumbens (NAc), whereas retro-AAV is better in labeling cortical areas such as mPFC (Ciabatti *et al.*, 2017). In Dorsal Root Ganglia (DRG), Tyrosine Hydroxylase (TH)+ and nonpeptidergic (NP) neurons are highly resistant to direct infections from either SAD-G coated RVdG or CVS-G coated RVdG. When EnvA coated RVdG were injection into TVA-expressing mouse, NP neurons has a strong increase of labeling, but not TH+ neurons (Albisetti *et al.*, 2017).

Using the monosynaptic RVdG tracing system in the same study, TH+ neurons showed no trans-synaptic labeling with either SAD-G or optimized G (oG) (Kim *et al.*, 2016b) coated RVdG, whereas NP showed slightly increase of trans-synaptic labeling in oG coated RVdG (Albisetti *et al.*, 2017). In **Paper III** of this thesis, our RVdG system also failed to label the presynaptic but not the postsynaptic SOM+ neurons. Those results have indicated that the mechanisms of rabies virus initial infections and the trans-synaptic infection may be different.

### 5.1.2 What is it actually labeling?

One study for tracing monosynaptic inputs to Layer 2/3, 5 or 6 GluN1 (the essential subunit of N-methyl-D-aspartate (NMDA) receptors) deleted neurons with RVdG showed an increased fraction of labeling of Layer 5 to Layer 6 neurons and a decreased labeling of Layer 6 to Layer 6 neurons (DeNardo *et al.*, 2015b). As lacking the functional NMDA receptors in the post-synaptic population results in drastically decreased GluN-mediated EPSCs at 40mV, it indicates the trans-synaptic labeling could be postsynaptic-population dependent.

Another study using RVdG for tracing monosynaptic inputs to dopaminergic VTA neurons after addictive cocaine administration have found an increased proportion of inputs from Globus Pallidus externus (GPe). Following experiments did not found any distinguishable changes in the number of synapses or the strength of the synapses. However, there was an increased spontaneous activity and excitability of the PV neurons in GPe. Activation of the Gpe showed an increased labeling with RVdG monosynaptic tracing in VTA, and inhibition showed the opposite (Beier *et al.*, 2017). This study suggested that the trans-synaptic labeling could be presynaptic-population activity dependent.

### 5.1.3 The need of a better understanding of RV pathogenisis

Since the day when RVdG system was invented (Wickersham *et al.*, 2007a), it has been widely used in almost every field by the neuroscience community. It is the first time allowing tracing almost every input to one single neuron at the same time in a controlled manner. Nevertheless, we are still using the system without fully understanding it. For instance, we still don't know much about the mechanisms of the initial infection, the transportation in the neuron, RV life cycle, its reassembly, budding out, trans-synaptic spreading, ect…

The attempt for understanding RV pathogenisis has been progressed a lot (Sissoeff *et al.*, 2005; Albertini *et al.*, 2012) and several proteins such as p75NTR/nerve growth factor receptor (Ngfr) (Tuffereau *et al.*, 1998), neural cell adhesion molecule (NCAM) (Thoulouze *et al.*, 1998), and nicotinic acetylcholine receptor (nAChR) (Lentz *et al.*, 1982) are identified as the glycoprotein ligands for initial entry. Yet, it is still limited. For instance, none of the known ligand showed negative in RV-resistant TH+ or NP DRG neurons (Albisetti *et al.*, 2017) when being analyzed with the single cell transcriptomic data from sensory neurons (Usoskin *et al.*, 2015).

The crystal structure of glycoprotein pre- and post- fusion is known over a decade (Roche *et al.*, 2006; Roche *et al.*, 2007), and our understanding of the RV pathogenesis is still limited. It fails to answer many questions. There is a strong need for a better collaboration between virologists, biochemists, molecular biologists and neuroscientists to engineer and generate a more powerful RV based tool.

## 5.2 THE NEED OF OTHER VIRAL TRACERS

Given the fact that every virus has its own preference of infection, the results and conclusions drawn from studies using different viruses or tracers might differ even if they are using the same experiment setups. In fact, each method can serve as a complementary to one another.

There are always attempts to find the 'perfect' virus for different applications, even though it is sometimes strongly against the natural feature of the virus. For example, there are attempts to generate an anterograde trans-synaptic tracer using the glycoprotein deleted vesicular stomatitis virus (VSV) pseudotyped with lymphocytic choriomeningitis (LCMV) glycoprotein, and it turned out to be contaminated with a wild type VSV glycoprotein (Beier *et al.*, 2011). However, such attempts should always be encouraged.

## 5.3 THE NEED OF COMPUTATIONAL METHODS

The study of neuroanatomy involves highly labor-intensive work, including sectioning, staining, imagine, and data analysis. With the emergence of global collaborative brain projects such as the BRAIN Initiative, more and more high-throughput data have been produced. Therefore the urge of powerful computational tools been implemented into every aspect of neuroscience studies is in strong need. Our **second paper** is trying to make such an attempt. Moreover, better computational methods are needed for combining high-throughput neuroanatomy data with other high-throughput techniques (e.g., single cell sequencing).

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