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Neuronal Mechanisms Underlying Sex Hormone Dependent Behavior Modulation

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“Any man could, if he were so inclined, be the sculptor of his own brain.”

— Santiago Ramón y Cajal, Advice for a Young Investigator

Preface

This dissertation is written as a completion the Master's degree final project at the Faculty of Sciences, University of Lisbon. All of the work presented henceforth was conducted in the Neuroethology Laboratory at the Champalimaud Research, Champalimaud Center for the Unknown. All procedures were reviewed and performed in accordance with the Champalimaud Centre for the Unknown Ethics Committee guidelines and approved by the Portuguese Veterinary General Board. This study was inserted in the FCT Scientific Research and Technological Development Project PTDC/NEU-SCC/4786/2014

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Thank you all.

Abstract

Social interactions rely on an interplay between external stimuli and internal states to induce specific adaptive behaviors. Depending on its internal condition, an animal might respond differently to the same stimulus. The reproductive cycle is one of the major internal factors capable of changing these behaviors. Sex hormones, e.g. progesterone, might be responsible for modulating behavior by binding to cognate receptors widely expressed across the brain, particular in regions involved in sexual behavior. One such region, the ventrolateral portion of the ventromedial hypothalamus (VMHvl), which plays a major role in sociosexual and aggression behavior in female and male mice, respectively. It is known that, in a sexually receptive state, females have increased neuronal activity in the VMHvl when in the presence of males, however, little is known about the identity of the neurons whose activity is being modulated. To clarify this question, we decided to focus our efforts in one population of VMHvl neurons: neurons that express the progesterone receptor (PgR+ neurons), which we know are male only responsive. In this thesis, we identified the monosynaptic inputs to the PgR+ neurons of the VMHvl using transsynaptic retrograde viral tracing and propose a double-labeling technique based on *in situ* and immunofluorescence to characterize neurons in the VMHvl that are male responsive. This way, we hope to better understand the neuronal populations of this nucleus in terms of gender specific responses. Our labeling approach was not successful but appears to indicate that c-Fos might not be a good neuronal activity reporter in our behavior paradigm. We found reliable input patterns to PgR+ neurons in the VMHvl originating from several regions of the social brain network, some of them already known to be outputs of these same cells, and new regions still to be described.

***Key words**:* PgR+ neurons, VMHvl, c-Fos expression, social interaction, rabies-based-tracing

Resumo

Durante a sua vida, um animal necessita de interagir com o ambiente que o rodeia de forma a aumentar a sua probabilidade de sobrevivência e sucesso reprodutor. Situações favoráveis ou nocivas surgem constantemente e devem ser interpretadas da forma mais eficiente possível. No caso específico de interações sociais estas devem fundamentar-se na interação entre o estado fisiológico interno de um animal e os estímulos externos que recebe; de forma a que o comportamento adaptativo mais benéfico possa ser selecionado. Sabe-se que, conforme uma determinada condição interna, um animal pode responder de forma bastante distinta ao mesmo estímulo, porem, os mecanismos neuronais que medeiam esta seleção são pouco compreendidos. Um dos mais importantes estados fisiológicos capaz de modular respostas comportamentais é a fase do ciclo reprodutivo em que uma fêmea se encontra. Por exemplo, numa fase recetiva uma fêmea pode aceitar um macho que tem como objetivo acasalar, mas cerca de dois dias mais tarde a mesma fêmea, interagindo com o mesmo macho, irá mais provavelmente rejeitá-lo, exibindo normalmente comportamentos agressivos.

Pensa-se que hormonas esteroides como a progesterona ou o estrogénio possam actuar como variáveis internas capazes de mediar estes comportamentos adaptativos. Estas interagirem com receptores específicos expressos em diversas regiões do cérebro envolvidas em comportamento social. Desta forma podem regular a atividade neuronal das células com às quais se ligam. Uma destas regiões de interesse é a porção ventro-lateral do hipotálamo ventro-medial (VMHvl). Este núcleo é importante para um vasto conjunto de interações sociais, tais como agressão ou aceitação de um macho conspecífico como possível parceiro de acasalamento. Apresenta uma população heterogénea de células, sendo uma delas a que expressa receptores para progesterona. Recebe ligações aferentes indiretas e polisinápticas de regiões envolvidas em representar estimulação sensorial relacionada com conspecíficos. Isto induz a ativação de *immediate early genes* (IEGs), marcadores indiretos de atividade neuronal. Outros motivos para nos interessarmos no VMHvl incluem, experiencias de inativação de receptores de progesterona (PgR) que provoca a perda de recetividade sexual em fêmeas e a descoberta recente de que um estado recetivo numa fêmea aumenta a resposta neuronal no VMHvl quando expostas a um macho conspecífico.

No entanto, muito pouco se sabe acerca da natureza molecular e características funcionais da população neuronal que está, de facto, a ser ativada durante estes testes de exposição a machos. Para tentar abordar estas e outras questões este projeto apresenta várias metodologias que pretendem esclarecer certos aspetos da integração neuronal que ocorre durante estas interações sociais e de que forma estes mecanismos podem ser explicados. Para isso efetuou-se um estudo de marcação-dupla para RNA mensageiro de receptores de progesterona (PgR mRNA) e para a expressão proteica de c-Fos, um IEG bastante comum em estudos de representação de atividade neuronal. Este método pode permitir compreender qual é a percentagem de população de neurónios que está a ser ativada em resposta a diferentes estímulos sociais. Também permite saber se essas mesmas células expressam PgR ou se existem neurónios sem PgR (PgR-) capazes de responder especificamente a um ou outro género. Outra questão que queríamos esclarecer implica compreender quais os inputs para células PgR+ do VMHvl e comparar esses inputs com os que se ligam diretamente a todo o VMH, isto ajudaria a compreender até que ponto as células PgR+ são uma sub-população do restante VMH e de que forma estão integradas na rede neuronal mais direta.

A experiência de marcação-dupla foi efetuada num background de testes de comportamento de forma a ser possível comparar a expressão de proteína c-Fos em resposta a um estímulo feminino ou masculino. No entanto não se verificaram diferenças aparentes de c-Fos no VMHvl de fêmeas que foram

sujeitas a estes testes comportamentais. Tal não era esperado porque se sabe que esta região apresenta elevada atividade neuronal mesmo durante as primeiras fases de interação social. No entanto, o núcleo premamilar (PMV) apresenta forte expressão de c-Fos quando a fêmea é exposta a um macho. Isto pode indicar que outros IEGs deveriam ser usados para observar indiretamente de forma mais fidedigna atividade neuronal no VMHvl. No que toca aos marcadores de PgR mRNA, apesar de serem usados regularmente em vários estudos histológicos, ainda não foi possível otimizar o protocolo que permitiria observar simultaneamente atividade neuronal e receptores para progesterona no mesmo cérebro.

O estudo de inputs directos utilizou um método baseado em rabies-viral-tracing para localizar apenas ligações aferentes diretas para os neurónios PgR+ do VMHvl. Obtivemos três cérebros com uma população de células alvo bastante concentrada no VMHvl e padrões idênticos de inputs ao longo do cérebro. Tivemos, portanto, uma base bastante sólida para interpretar e descrever a regiões envolvidas. A maior parte dos inputs vieram de regiões previamente implicadas em comportamento social e algumas já tendo sido descritas como outputs das células PgR+ do VMHvl, havendo, portanto, algum nível de crosstalk. Algumas das localizações de inputs observadas foram inesperadas e é ainda necessário proceder a uma análise mais rigorosa e quantitativa dos dados.

Palavras-chave: neurónios PgR, VMHvl, expressão c-Fos, interação social, rabies-based-tracing

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1. Introduction

1.1 Background

Throughout their lives, animals need to interact with the environment in a way that increases the chance of survival and reproductive success. Challenging situations, as favorable ones, are constantly arising and need to be addressed in the most efficient way.

During these events, environmental cues and external stimuli are processed by the sensory system and integrated with internal physiological states and a priori experience. These processes should subsequently result in adaptive behavioral actions or decisions, i.e. in accordance to the needs of the animal. Such decisions are often related to locating, defending and allocate resources such as food, mates, offspring and/or shelter, which usually take place in social relevant settings, such as mate choice (Stephens, 2008; Connell and Hofmann, 2011a; O'Connell and Hofmann, 2011b).

Then how do animals decide which adaptive behavior to take when presented with variable internal states combined with sensory stimuli? There is no doubt that neural systems play a very important role in this process and significant progress has been made in understanding the evolution and ecology of social behavior (Krebs & Davies, 1993; Stephens, 2008). Still, much less is known regarding the specific neuronal mechanisms that mediate it. Thus, studying the neural and molecular mechanisms of integrating external stimuli with internal states is a valuable step towards a better understanding of broad ecologic interactions.

1.2 Internal States, Finding the Right Gender and Decision-Making

Animal behavior can be affected by its internal state. A hungry animal will respond more vigorously to the sight of food and will put more effort into getting access to that resource than a fully fed animal. This state driven modulation can affect behavior in many ways, by increasing the contrast of sensory response to a behaviorally relevant stimulus (Niell & Stryker, 2010; Strube-Bloss et al., 2015), or by increasing the motivational drive for exploration, which would allow the animal to encounter desired objects (LeDoux, 2012).

In mammals, most neural networks that incorporate an internal state have been partially described (Wikens et al., 2007 Sokolowski & Corbin, 2012). These circuits allow adjustments in goal-oriented activities and actions to be taken, based on the needs of the animal and its internal physiology. Moreover, these actions, more specifically social encounters, are usually executed in a specific sequence: 1) investigatory phase driven by some motivation for the behavior; 2) consummatory phase.

For the motivational phase, considering a homeostatic mechanism, changes in a critical regulated factor can trigger motivation driven behavior. These factors can be levels of sexual hormones in blood that reach a certain threshold and remain above it for a long period of time. Going through these phases sequentially, regardless of the behavior in question, can be linked to a continuous escalation of a drive triggered by internal states such as hunger, stress or sexual arousal (Berridge, 2004; Kennedy et al., 2016).

Konrad Lorenz created a "hydraulic" allegory to explain how an increasing internal drive might influence several behavioral actions and decision-making processes under external stimuli regulation

(Lorenz & Leyhausen, 1973). Other models propose that these decisions are made in a hierarchical fashion, with more basic choices (e.g. engaging in mating) made before more specific ones (e.g. grooming, biting, mounting). This hierarchical organization is mediated by distinct nodes in the brain, creating a behavioral decision tree (Tinbergen, 1950, 1951).

Although these models are useful for experimental designs they do not address how internal states might modulate sequential decision-making in social interactions, or how these states can be applied in a neuronal mechanistic way. Whichever, the neural mechanisms used to interact with the environment need to be, by necessity, incredibly plastic and capable of dynamically receive, encode, characterize and interpret external stimuli. Lately behavior has been decomposed into more motivational aspects, which allow animals to increase their probability of encountering the resources or conspecifics they need (Ago et al., 2015). Followed by consummatory phases in which the animal decides what action to take towards that stimulus (Jennings et al., 2015).

Their internal state could, in principle, affect both aspects, either the motivational/investigatory (e.g., increasing salience, exploratory drive, decreasing fear) or the consummatory (e.g., decreasing the threshold for starting the behavior). One very important aspect of behavior that we still know little about is the idea that, besides motivation, animals also rely on a filtering capability that allows them to find the correct information or the right stimulus for the behavior while shutting out less important ones, e.g., looking for a male when a female is sexually receptive.

1.3 Animal Model

In mammalian neuroscience research, rodents are the "go-to" model species. This is due to an increasing number of genetic and behavior tools available. One of the most important contributions was the development of transgenic mouse *Mus musculus* (Linnaeus, 1785) lines, allowing scientists to tamper with specific gene modifications and obtain new phenotypes. Compared with other mammalian models mice are relatively easy to obtain and need a relative small amount of resources to maintain in large colonies. Moreover, the ethical questions and technicalities associated with mice based studies are not as constraining as for higher mammals. These characteristics make it fundamentally relevant for neuroscience and biomedical research (Pankevich et al., 2012; Sheena et al., 2012 Huang & Zeng, 2013).

One of the most used inbred mouse strains, and the first to have its genome sequenced, is C57BL/6 (also known as Black 6 or B6). It is studied in a wide range of research areas including, pharmacology, neurobiology and animal behavior. They are commonly used to produce high quality transgenic lines due to their permissive background (Figure 1.1). Being long lived and with a low susceptibility to tumor development B6 mice are also physiologically stable animals (The Jackson Laboratory – Strain 000664).

Considering these characteristics, B6 mice are perceived as a valuable and suitable model to study how animals might interact with their surroundings, from a general behavioral approach to a more specific mechanistic one. Numerous studies have used mice with the aim to better understand how neuronal networks might integrate socially evoked behaviors with internal physiological states. For example, artificial stimulation of certain brain regions induces an associated social behavioral response and, through genetic manipulation, allows to identify possible neuron populations responsible for that behavior modulation (Anderson, 2012; Falkner & Lin, 2014; Yang & Shah, 2014).

Figure 1.1 Adult *Mus musculus* C57BL/6 (The Jackson Laboratory Mouse Strain Datasheet).

1.3.1 Estrous Cycle as an Internal State

It is know that the reproductive state of a female mouse, also referred to as estrous cycle, is one of the major factors that can influence the outcome of social encounters with conspecifics males (Zinck & Lima, 2013). Nonetheless, not much is known about the neuronal mechanisms that mediate this process (Zinck & Lima, 2013). Social behaviors displayed by females can be aggressive, when the animal is non-receptive; or affiliative, when it is in a receptive stage of the cycle. This suggests that the neural mechanisms associated with behavioral decision-making can react to the same stimulus in different manners, depending on the internal state of the female. Unfortunately, little is known about how the reproductive state modulates this socially evoked neural activity (Bobrov et al., 2014; Nomoto & Lima, 2015).

In mice, the estrous cycle usually lasts 4 to 5 days and can be divided into four distinct sequential phases: 1) proestrus; 2) estrus; 3) metestrus; and 4) diestrus; with the most receptive stage happening between proestrus and estrus (Byers et al., 2012; Allen, 2012). Functional changes that occur during these four stages depend on the activity of several sex hormones, such as estrogen and progesterone, which are required to induce estrous related physiological receptivity. These two hormones can modulate the activity of distinct brain regions and nuclei involved in sexual behavior, by means of specific estrogen and progesterone receptors (ER and PgR, respectively) expressed in neurons of those regions (Rubin et al., 1983). This expression is fairly prevalent throughout the brain (Kuiper et al., 1997; Brinton et al., 2008).

Such modulation of neuronal function is mostly genomic and takes place during long periods of time. It can increase the tendency of animals to engage in social interaction with opposite sex conspecific, thus encouraging copulation (Blaudstein, 2008). However, little is known about the mechanisms behind this hormonal modulation of social stimulus responses. Addressing this question by relating neuronal activity to different stages of the estrous cycle or studying more specific neuronal networks of social regions would help to shed light on sex hormone dependent behavior variation.

1.4 Hypothalamus and Social Behavior

The brain regions involved in the above-mentioned hormone dependent sexual behavior are usually part of the social brain network (SBN) (Figure 1.2), such as the Medial Amygdala (MeA); Periaqueductal Grey (PAG); Ventral medial hypothalamus (VMH); Ventral premammillary nucleus (PMV); Anterior hypothalamus (AH) and Preoptic area (POA) (Figure 1.2) (Goodson, 2005; Pfaff et al., 2008; Maney et al., 2011). The nodes of this network are reciprocally connected and neurons that express receptors for sex hormones.

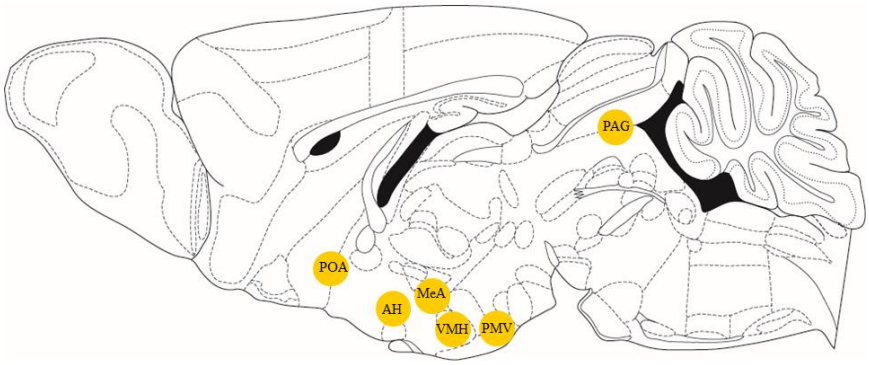


Figure 1.2 Scheme of a sagittal section on a mouse brain (adapted from Paxinos & Franklin, 2014). Yellow circles represent major nuclei of the SBN: Preoptic area (POA); Anterior hypothalamus (AH); Medial amygdala (MeA); Ventral medial hypothalamus (VMH); Ventral premammillary nucleus (PMV) and Periaqueductal Grey (PAG).

1.4.1 Ventrolateral Hypothalamus

One of the regions of interest in this network is the ventrolateral region of ventromedial hypothalamus (VMHvl), which is important for an array of social interactions such as accepting a male conspecific as a possible mating partner (Swanson, 2000). It is located in the caudal hypothalamus, laterally to the third ventricle. The VMH develops as a regular hypothalamic nucleus with expression of SF-1 (steroidogenic factor-1), a specific marker for this nucleus, regulated through development, helping to structure the spatial organization of its neurons. It can be divided in two major parts, the dorsomedial and the ventrolateral (two semi-oval structures) and it is surrounded by a fairly dense fiber plexus (Figure 1.3).

VMH neurons usually present a primary dendrite which varies in length; and a few other secondary ones. These dendrites may be organized in a way that allows for specific contact with afferent axons coming from specific regions, as well as the integration of extrinsic signals and internal activity in a plastic manner (Griffin & Flanagan-Cato, 2009). Considering a more cellular scale, several studies show that this nucleus is not homogenous, but rather comprised of several cell types expressing a diverse range of receptors and neuropeptides (Flanagan-Cato et al., 2001.Simerly, 2002; Flanagan-Cato, 2011). We also know that nearly all VMH neurons, including those in the sexually dimorphic VMHvl sub region, release the excitatory neurotransmitter glutamate and use the vesicular glutamate transporter 2 (Vglut2) (Cheung et al., 2015)

Besides the studies previously mentioned, there are numerous suggestions that the VMHvl, as a region of interest, presents an important role in the socio-sexual behavior of female mice, such as: 1) reception of indirect and polysynaptic inputs from sensory stimuli related with conspecifics, which leads to the activation of immediate early genes (IEG) (Pfaus et al., 1993); 2) expression of a small pool of PgR neurons and a subset of estrogen receptor (ER) neurons (Yang et al., 2013; Lee et al., 2014); 3) existence of projections to several relative rostral and caudal regions of the hypothalamus and the PAG, an area linked to lordosis, a female body posture adopted during copulation (Pfaff & Sakuma, 1979; Canteras et al., 1994; Pfaff et al., 2008); 4) ablation experiments of PgR expressing neurons of the VMHvl lead to a decrease of sexual receptivity in females (Yang et al., 2013); 5) local injection of sex hormones are sufficient for inducing appropriate behavioral responses to males (Pleim et al., 1989); 6) VMHvl regulates male behavior, either aggression towards other males or mating with females, suggesting an important role in encoding motivational drives with internal states and sensorial information (Lin et al., 2011).

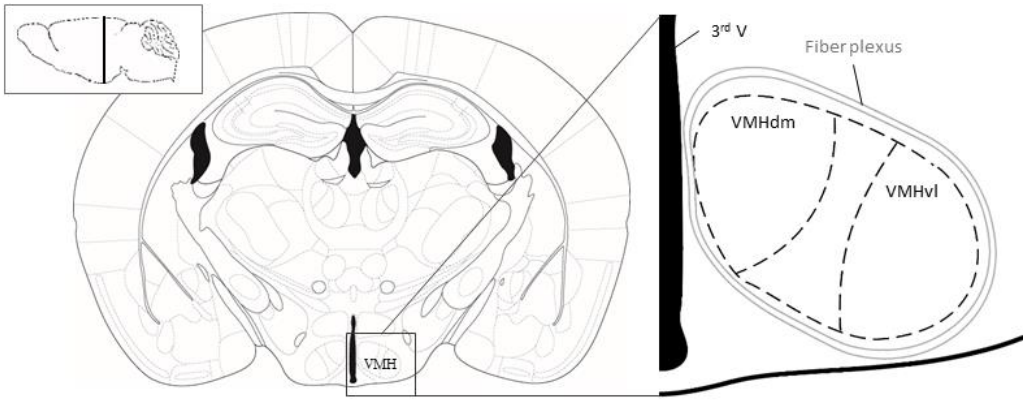


Figure 1.3 Scheme of the VMHvl location and structure details. Scheme of coronal slice with location of VMH nucleus, zoomed region shows both partition of the nucleus (dorsomedial-VMHdm and ventrolateral-VMHvl) surrounded by the fiber plexus along the third ventricle (adapted from Paxinos & Franklin, 2014).

It has been recently discovered that a sexually receptive state in females is correlated with an enhancement in VMHvl neuronal response to males, associated with investigatory behavior (Nomoto & Lima., 2015). Nonetheless, there are still several unknowns.

1.4.2 Neuronal Activity

Knowing the architectural organization and genetic makeup of VMHvl neurons is important but not enough to understand its role in social behavior modulation. One should also take into consideration the assortments of neuronal activity that occur during the behavior of interest, as they might help identify which sub-population of cells is of actual significance. Localized measurements of neuronal activity can be made through several well described methods, such as electrode based recording of electrical neuronal activity or fluorescence calcium reporters that are limited to small subsets of neurons or superficial regions of the brain (Buzsáki, 2004; Cogan, 2008; Grewe & Helmchen, 2009).

A more spatially relevant technique, that nonetheless lacks the temporal resolution of the prior ones, is the use immediate-early genes (IEGs) as indirect markers for recent neuronal activity (Bullitt, 1990; Cruz et al., 2013). It is known that external stimuli, such as premating social interaction, can cause activity in neuronal networks and rapid neuronal responses. However, these signals can also elicit slow and long term responses, which can sometimes be correlated with the induction of new gene expression programs. These genes that respond to transsynaptic activation are known as IEGs and reflect an integration of activity-dependent calcium influx of strong and consistent activity over a time frame of seconds to minutes. They also encode several regulatory proteins that control the expression of late response genes. One commonly used IEG in neuronal activity studies is c-f*os*, a proto-oncogene that encodes a DNA binding protein and has a time-locked relationship to the stimulus that drove its expression, making it an ideal indirect marker of neuronal activity. More specifically, c-Fos expression is rapidly induced at a transcriptional level within minutes of extracellular stimulation. (Sheng & Greenberg, 1990).

The transcribed messenger RNAs (mRNAs) have a half-life of approximately ten to fifteen minutes and quantity of translated proteins in the neurons tends to reach a peak at one hour and a half after the stimulus (Morgan & Curran., 1988; Sheng & Greenberg, 1990). Moreover, there is the possibility of double labeling brain slices for c-Fos and other known neuronal molecular markers. This scenario presents the opportunity of characterizing neurons involved in the circuitry that responded to a specific external stimulus and better understanding specific neuronal networks. (Wisden et al., 1990; Lin et al., 2011; Lee et al., 2014).

1.4.3 Place in the Network

The nervous system is the most important mediator of biological information in the vast majority of animals. Hence, understanding the proprieties and strategies of its organization into complex circuits could help to clarify the mechanisms of biological information processing (Lerner & Deisseroth, 2016). For example, neuronal circuits, such as the ones present in the VMHvl, are complex and very specific due to distinct neuron types involved in sub circuits and their different connectivity and functions. One important step to start simplifying these circuits is to understand the inputs to, and outputs from, a given type of genetically defined neuronal population part of an intact network (Yoshimura et al., 2005; Griffin & Flanagan-Cato, 2009; Watabe-Uchida, 2012).

The first steps to the anatomical study of the nervous system were taken by Ramon y Cajal and his colleagues. Through the Golgi stain technique, they were able to start describing several types of neurons and respective structural organizations (Ramon y Cajal, 1995; Garcia-Lopez et al., 2010). Nowadays, researches possess several well established techniques based on injectable markers or dyes that allow to signal and analyze short and long ranged whole-brain projection (Katz & Iarovici, 1990; Reiner et al., 2000), while other approaches have adapted proteins for neuronal tracing (Conte et al., 2009). Although the protein tracers are not exclusively retrograde (from axon terminals to cell body) or anterograde (from dendrites/cell body to axon terminal), some of them have the capability of trans-synaptic labeling, i.e. the cell bodies of cells that synaptically input to a population of starter cells (the ones that have the marker) are labeled.

Unfortunately, this more sophisticated technique cannot guarantee that only monosynaptic connections are labeled since it can spread to second or third order ones, which makes it exponentially to comprehend a specific network organization. The recent development of engineered viral vectors has improved the progress of circuit tracing, with one of the most used being adeno-associated viruses (AAVs). They are safe, easily obtainable and can generally infect any type of neuron and brain region with genes encoding protein markers (de Backer et al., 2010).

As a standalone technique, it is only specific to cell-body location in the brain region of interest; but, used together with other methods, it can also target genetically defined neurons. Still, the previous problems of distinguishing inputs from outputs while, at the same time, restricting the labeling to monosynaptic connections remains. A recent method of monosynaptic circuit tracing has been developed to tackle these limitations (Wickersham et al., 2007a & 2007b). It describes a process in which the aim is to infect a neuronal population of interest with a deletion-mutant tracing virus that lacks a gene necessary for transsynaptic spread, while the missing genes are provided in *trans* in the initially infected cells only.

With the necessary genes the virus can spread to monosynaptic connections in a retrograde fashion but, because these connections now lack those genes, it cannot spread beyond them. If we combine this technique with a Cre/loxP FLEX gene expression system that discerns which neurons the virus will infect then we can finally obtain a genetically defined population of starter cells and comprehensively identify regions that monosynaptically input to our localized cells of interest (Figure 1.4). This ultimately enables us to obtain input defined elements of the VMHvl, in this case, the PgR^{+} neurons.

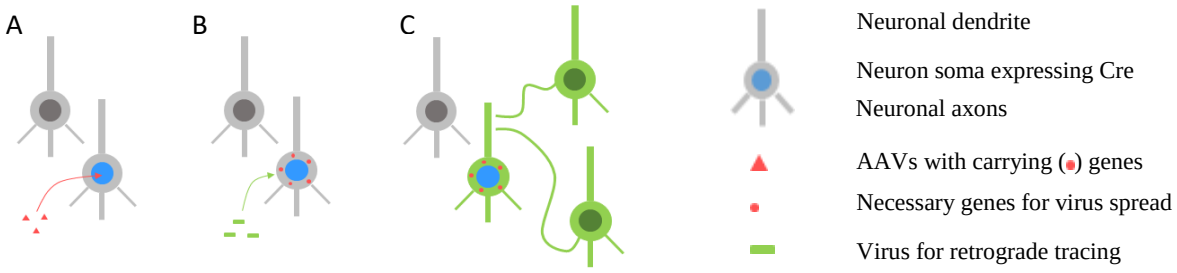


Figure 1.4 Simplified schematic representation of virus based circuit tracing. (A) Using the Cre/loxP system AAVs carrying necessary genes for the spread of the tracing virus infect solely neurons that express promoter-dependent Cre recombinase enzyme; (B) The neurons of interest (starter cells) now *trans* express genes that allow rabies to infect them and also spread retrogradely; (C) The virus infects starter cells and respective monosynaptic inputs, allowing to visualize a more specific sub-circuit.

1.5 Aims & Hypotheses

The VMHvl plays a very important role in mice socio-sexual behavior. However, we currently have very little insight into how the coordinated activity of the VMHvl can underlie female receptivity changes across the cycle. Therefore, we feel it is necessary to understand better the functional organization and connectivity of inputs into the VMHvl. Currently, we know that:

1- Neurons within the VMHvl respond to male and female stimuli (Nomoto and Lima, 2016). 2- The activity of VMHvl neurons towards males is enhanced when females are sexually receptive (Nomoto and Lima, 2016). However, we do not know the identity of neurons whose activity is modulated. 3- In contrast, when we only record from its PgR+ expressing population (a population that is necessary for female receptivity) we only observe activity in response to male stimuli (Nomoto and Lima, unpublished). This suggests that PgR+ expressing neurons receive a set of inputs that are male specific, while the whole VMHvl receives inputs related to both sexes.

To move forward and understand which population within the VMHvl is modulated by sex hormones and the function of activity enhancement, we must first further functionally characterize the VMHvl. In particular, we would like to address the following questions:

Aim 1: are male responsive neurons a homogenous population (are they all PgR expressing neurons) or are there PgR-negative neurons that also respond to male stimuli? Aim 2: How do the inputs to PgR positive neurons in the VMHvl relate to the rest of the VMHvl? We know that PgR+ neurons are only responsive to male stimuli, suggesting that they must receive a subset of inputs that are different from the rest of the VMHvl.

To address these questions, we first performed double-labeling experiments using *in situ* based markers for subsets of PgR+ neurons in the VMHvl and IEGs immunofluorescence as readout of neuronal activity to characterize neurons in the VMHvl that are male and/or female responsive. Second, using a virus based mono and transsynaptic tracing technique, we identified the regions with direct afferent connections to the genetically defined PgR+ neuronal population of the VMHvl. It is expected that the observed inputs will mostly originate from other SBN nuclei. This will help understand the mechanisms that regulate sensory integration upstream of the VMHvl.

2. Methodology

2.1 Animals

As representative of the *Mus musculus domesticus* Schwarz and Schwarz, 1943 subspecies, we used the classical laboratory inbreed strain C57BL/6. Animals were purchased from the vivarium colony of the Champalimaud Foundation, which was established by C57BL/6J animals from The Jackson Laboratory (stock number 000664). For the anatomical tracing studies PgR-Ires-Cre C57BL6 and Vglut2-Ires-Cre C57BL6 animals were used. Both lines where obtained from The Jackson Laboratory. Animals were kept under controlled temperature (23 ± 1 °C) and photoperiod (12:12 h light:dark cycle, with light available from 20:00 to 08:00) conditions in standard cages (1284L, Techniplast, 365 x 207 x 140 mm and 1145T, Techniplast, 369 x 165 x 132 mm). Water and food (Global Diet 2914, Mucedola s.r.l) chow were provided *ad libitum*. Cotton (cocoon cylinders, LBS) and paper houses (GLP Des Res Mice Dome Home, LBS) were provided for environmental enrichment. Cage maintenance was performed once per week to provide new bedding, food and water. Animals were weaned at 20-21 days of age and housed in same-sex cages with three to five animals.

2.2 Characterization of Neuronal Activity in the Hypothalamus

Experiments in our lab already demonstrated that neuronal electrical activity in VMHvl fluctuates in social behavior dependent manner (Nomoto & Lima, 2015), but little is known about the molecular and genetic nature of these conspecific responding cells. To do so, we adapted a preexisting exposure test developed in our lab that allowed different social stimuli to be presented. Immunofluorescent IEG reported neuronal activity was later visualized in combination with neuronal molecular markers already known to be present in the VMHvl. The first approach to the tests was of optimization and served to first access the reliability of the proposed IEG labeling and *in situ* protocol for progesterone receptors mRNA.

2.2.1 Experimental Animals

We used twenty-two adult females (8-12 weeks old) and ten males (8 to 16 weeks old) for all the described experiments. Behavior testing was performed during the dark period, approximately 6h after dark conditions onset (3pm)

2.2.2 Female Social Exposure Tests

We established two experimental tests, both consisted of exposing females to social stimuli. For the first experiment 18 females were separated in 3 groups: Control, Female stimulus and Male Stimulus. Each group had six animals. Every animal was habituated to an acrylic box (arena) 3 days prior to the experiment, 15 minutes per day. On the day of the experiment the females were placed in the arena for 15 minutes, next,

depending on the female's group, the correct control or stimulus animal was annexed to the arena. Nose-to-nose interaction was allowed and recorded for 5 minutes. After the interaction, the female was left in the arena for an extra 5 minutes (buffer time). These experiments were conducted over a time period of 3 weeks. Each week 6 females were habituated, tested and subjected to tissue processing. Of these 6 females, 2 belong to control group, 2 to female and 3 to male. This arrangement allowed to account and control for variables across the 3 weeks.

For the second experiment females were single-housed in small cages (1145T). From three females available we selected one to be exposed to male stimuli (T3), another to female stimuli (T2) and the remaining one to no social stimuli (T1), constituting a control for specific neuronal social evoked responses. To characterize neurons that were active during socio-sexual behavior we decided to use a previously constructed acrylic box (200 x 150 x 150 mm) to serve as an arena for female mice to freely behave and interact with presented stimulus. In a more detailed description, the test consisted in placing females, one at a time, in the center of the acrylic box pre-prepared with fresh bedding.

They were allowed to explore the new environment. After 15 min of free behavior the social stimulus was presented by placing a conspecific, female or male, in the center of the box. For the control female (T1) the animal placement movement was simulated so there is still some non-social related activity being accounted for. Animals were left to interact for 5 min and then the stimulus was removed. Females remained in the box for an extra 5 min before being taken back to their home cage.

After the behavior tests were performed we prepared the perfusion hood for brain harvest (Gage et al., 2012) and the anesthesia (20 µl/g of Ketamine + Xylasine mixture) for sacrificing the animals exactly 90 min after social stimuli presentation, in order to have maximum c-Fos protein expression.

2.3 Tracing of Monosynaptic Inputs to VMHvl

To study the regions with direct connections to a specific genetic population of neurons, in a given brain area, an experimental approach identical to the one developed by Watabe-Uchida, 2012 was used. In this study, a Cre/loxP FLEX gene expression system (Gong et al., 2007) was combined with rabies-virus-based transsynaptic retrograde tracing technique (Wickersham et al., 2007b) to efficiently find monosynaptic inputs to a genetically defined neuronal population of PgR+ in the VMHvl of female mice.

2.3.1 Experimental Animals

Animals used in the anatomy studies where obtained from in lab crosses of B6.129S(Cg)-$Pgr^{tm1.1(cre)Shah}$ (PgR-Ires-Cre) C57BL6 obtained from The Jackson Laboratory with C57BL6 mice. Six adult females (8 to 12 weeks old) were used for the study of monosynaptic inputs to progesterone receptor positive neurons of VMHvl. They were identified from p01 to p06. For some control experiments, three C57BL6 mice were used: c01, c02 and c03.

2.3.2 Virus description

This procedure used a rabies virus SADDG-GFP(EnvA) with two major modifications to determine the specificity of the initial infection and transsynaptic spread (Wickersham et al., 2007b). Firstly, the virus

was pseudotyped with an avian virus envelope protein (EnvA), which made it theoretically impossible to infect regular mammalian cells. In mammalian brains, the initial localized infection can only occur if neurons are planned to express a cognate receptor (e.g., TVA receptor). Secondly, a gene necessary for the production of rabies virus envelope glycoprotein (RG), fundamental to transsynaptic spread, was replaced by a gene for a fluorescent marker (EGFP; enhanced green fluorescent protein) (Figure 2.1). Thus, transsynaptic spread only occurs if the target neurons are engineered to express RG protein. The result should be a virus that has the capacity to infect initially contacted cells, replicate only its core, express high levels of EGFP, but be unable to spread beyond these initially infected cells.

The main strategy of the study is then make only a genetically defined cell population express both TVA receptors and RG protein. To make such strategy possible, AAVs that expressed either TVA (AAV5-EF1a-FLEX-TVA-mCherry) or RG (AAV8-CA-FLEX-RG) were used (Figure 2.1). The transmembrane type of the TVA protein (TVA950) was fused with a red fluorescent protein to help visualize the injection site and injection spread of these AAVs (Figure 2.1). The particular characteristics of these virus were selected to optimize the rate and extent of diffusion within the injection area, in this case a spread of 0.5 to 2 mm from the tip of the injection needle. Both proteins can only be expressed under the control of Cre/loxP FLEX switch recombination system that should restrict the expression of these AAVs to only PgR+ neurons. SADDG-GFP(EnvA) can then infect these neurons, use RG to generate viral envelopes and spread transsynaptically to only direct inputs that lack the TVA and RG proteins.

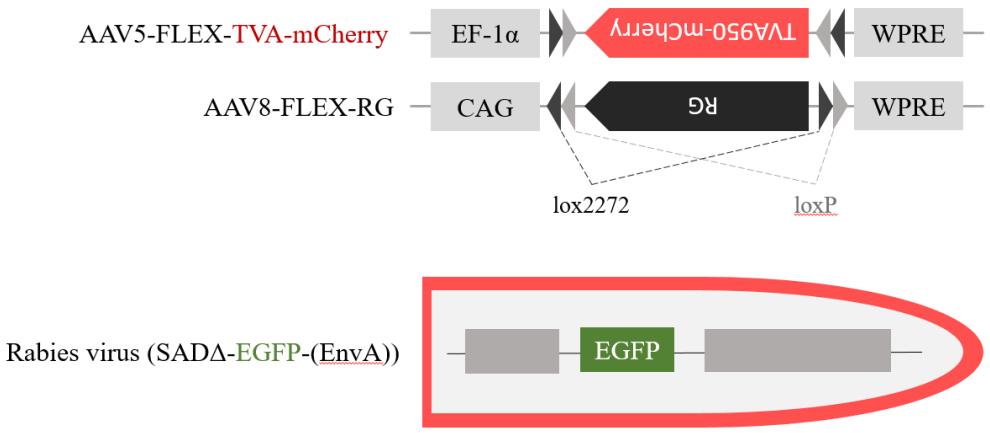


Figure 2.1 Recombinant AAV strains and modified rabies virus

2.3.2 Intracranial Injection

Injection procedures were performed using a stereotaxic device that allowed precise viral delivery to atlas defined coordinates (Appendix 5.1). Fine-tuned volumes were injected with a Nanoject II Auto-Nanoliter Injector Drummond Scientific (Appendix 5.1). To visualize monosynaptic inputs to the PgR+ cells of the VMHvl we injected AAV5-FLEX-TVA-mCherry and AAV8-CA-FLEX-RG in the VMHvl of six transgenic female mice expressing Cre in PgR neurons. VMHvl mouse atlas-based coordinates were adjusted based on my personal technique of injection: the drilling was at ±AP -1.5; ML -0.75 and the injection adjusted from DV -5.4 to DV -5.8. After 14 days of viral spreading and infection SADDG-

GFP(EnvA) was injected in the same coordinates and the brain was analyzed after seven days as described in section 2.4 of this chapter (Figure 2.3A).

The tissue should contain three distinct types of signal: red fluorescent PgR cells, expressing only Cre dependent TVA and RG (Figure 2.3B); yellow (red + green fluorescence) starter cells, coexpressing TVA-RG and EGFP (Figure 2.3C); and monosynaptic inputs expressing only rabies EGFP (only green fluorescence) (Figure 2.3D). For tracing specificity analysis, replicate injections where performed in three C57BL6 mice with no Cre expression.

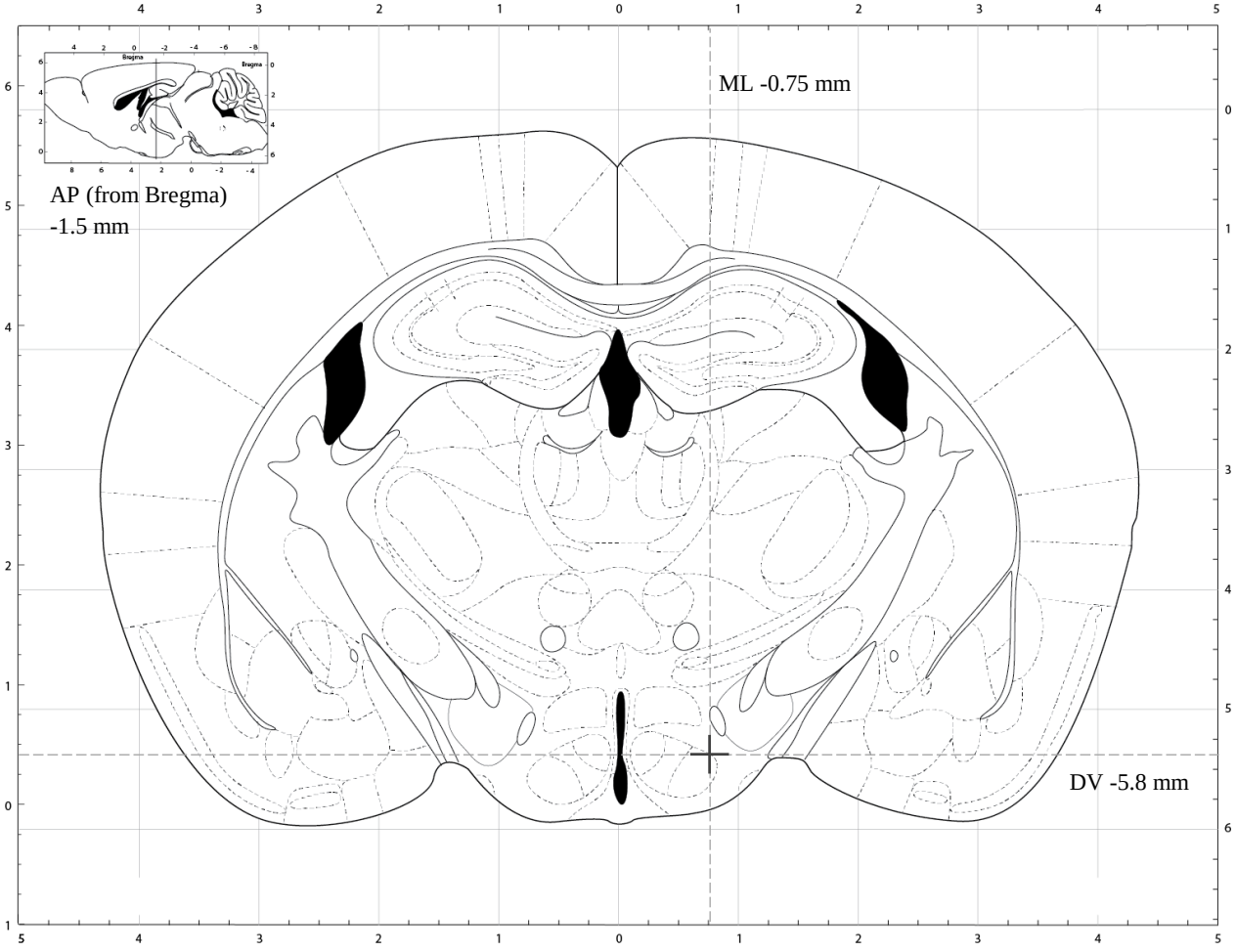


Figure 2.2 Stereotaxic coordinates for injections on VMHvl (adapted from Franklin and Paxinos, 2008). The injection point his shown as a grey cross. DV coordinates were adjusted from -5.35 to -5.8 from optimization experiments.

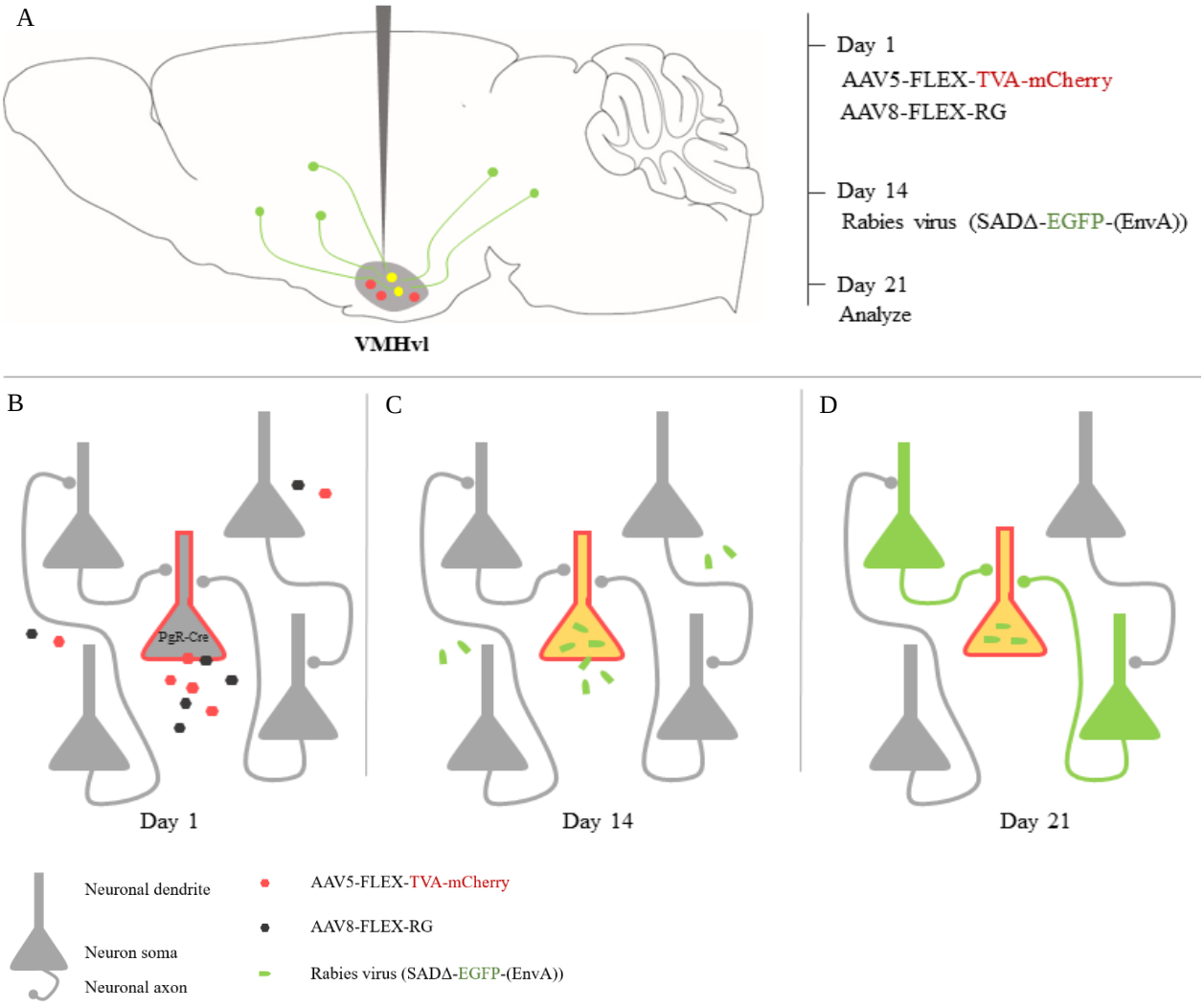


Figure 2.3 Schematic summary of injection strategy. A) Monosynaptic input tracing experimental design. B) First injection of Cre dependent TVA and RG with PgR neuron expressing TVA receptors. C) Second injection of pseudotyped rabies virus infecting TVA expressing neuron (now identified as starter cell). D) Rabies transsynaptic spreading to direct inputs.

2.4 Tissue processing

Experimental animals were subjected to transcranial perfusion fixation, as described by Gage et al. (2012), in order to harvest brain tissue. Mice used in the exposure test were perfused 90 min after being exposed to the stimulus conspecific; while animals used in the anatomy study were fixed 1 week after pseudo-rabies injection. A 10ul per 10g dose of (Ketamine–Xylazine mixture – 30ul) was administered before every procedure to induce stable anesthesia, which consisted in removing the blood from the circulatory system using phosphate-buffered saline (PBS) solution 1x and replace it with the fixation reagent paraformaldehyde (PFA) 4%.

Afterwards, brains were carefully harvested and stored overnight in PFA 4% at 4°C, and then switched to a 30% sucrose-PBS solution where they remain to cryoprotect the tissue by extracting water. After at least three days of sucrose saturation, coronal brain slices at 40 µm thickness were prepared using

a Sliding Microtome SM 2000 R. The brain slices obtained from the exposure tests were arranged in three series and stored in PBS1x-azide 0.1% using 12-Well Plates (NEST® Cell Culture) at 4°C for further processing. Brain slices from the anatomy study were directly and sequentially mounted in Superfrost® plus microscope slides (Thermo Scientific) *in toto*, counterstained with DAPI-PBS 1x (1:1000) for 15 min, washed with PBS 1x three times, and allowed to dry. Then, using Mowiol mounting medium and cover glass (VWR International) the slides were coverslipped, sealed with nail polish, and stored at 4°C for later image acquisition.

2.4.1 Immunofluorescence staining

For accessing social evoked neuronal activity in the VMHvl, we implemented an anti-c-Fos indirect immunofluorescence procedure previously optimized in our lab by Silvana Araújo. The protocol was applied in pre-selected brain slices containing the regions of interest, in this case the VMH and some antero-posterior buffer regions. It was performed using anti-c-Fos polyclonal rabbit primary antibody (Synaptic Systems, Germany) at 1:7500 and Alexa Fluor® 488 goat anti-rabbit IgG H&L secondary antibody (Abcam) at 1:1000. Slices were permeabilized, incubated with antibodies and washed with 0.05% Triton X-100. The final steps where to counterstain the slices with DAPI and mount them in Superfrost® slides for later image acquisition (Appendix 5.2).

2.4.2 *In situ* for PgR mRNA

Given that progesterone regulates female reproduction, it is important to have a reliable tool to identify neurons that express its cognate receptor. Literature shows these receptors are widely distributed across several brain regions, nevertheless PgR+ neurons, which regulate sexual receptivity, are still to be explicitly identified (Cohen & Pfaff, 1992, Blaustein, 2008, Quadros et al., 2008). To help visualize PgR+ neurons and assess their role in socio-sexual behavior by means of IEG activity, we adapted a protocol developed by Jianbo Xiu (2014) named tyramide-amplified IHC-FISH (TAI-FISH).

The original process improves a double-labelling technique that allows to differentiate mRNA and protein signals of IEG *c-fos* following neural induced activation (Chaudhuri et al., 1997). So, when presenting two stimuli at a fitting interval, activated neural circuits can be characterized by the c-Fos protein and mRNA signals, which in turn can be detected by double labeling with fluorescence immunohistochemistry (FIHC) and fluorescence *in situ* hybridization (FISH), correspondingly (Farivar et al., 2004). Based on this double-labeling possibility, the modified protocol used in this project strived to successfully represent PgR mRNA and c-Fos protein expression on the same tissue samples. In order to study of gender and social dependent neuronal activation on neurons modulated by progesterone.

2.5 Image analysis

Whole section mosaics of high magnification images were taken semiautomatically with Axio Scan Z1 slidescaner microscope (Leica) and analyzed using Zen lite 2.3 software (Zeiss). The locations of c-Fos labeled neurons, monosynaptic inputs and starter cells were manually registered using scalable vector graphics from Franklin and Paxinos mouse brain atlas, 2008.

3. Results

3.1 Characterization of Neuronal Activity in the Hypothalamus

To test if the neuronal activity in the VMHvl fluctuates during male oriented investigative behavior IEG activity was observed from females exposed to social stimuli. The first set of exposure tests produce results very similar to the second one (data not shown), putting into question the reliability of c-Fos protein as an IEG indirect reporter of neuronal activity for this behavioral setup of stimuli exposure. Three females were identified as T1 (– exposed to only handling stimulus), T2 (– exposed to female stimulus) and T3 (– exposed to male stimulus). The protocol for c-Fos immunofluorescence produced clearly labeled neurons in all brain samples, appearing as green oval structures (Figure 3.1).

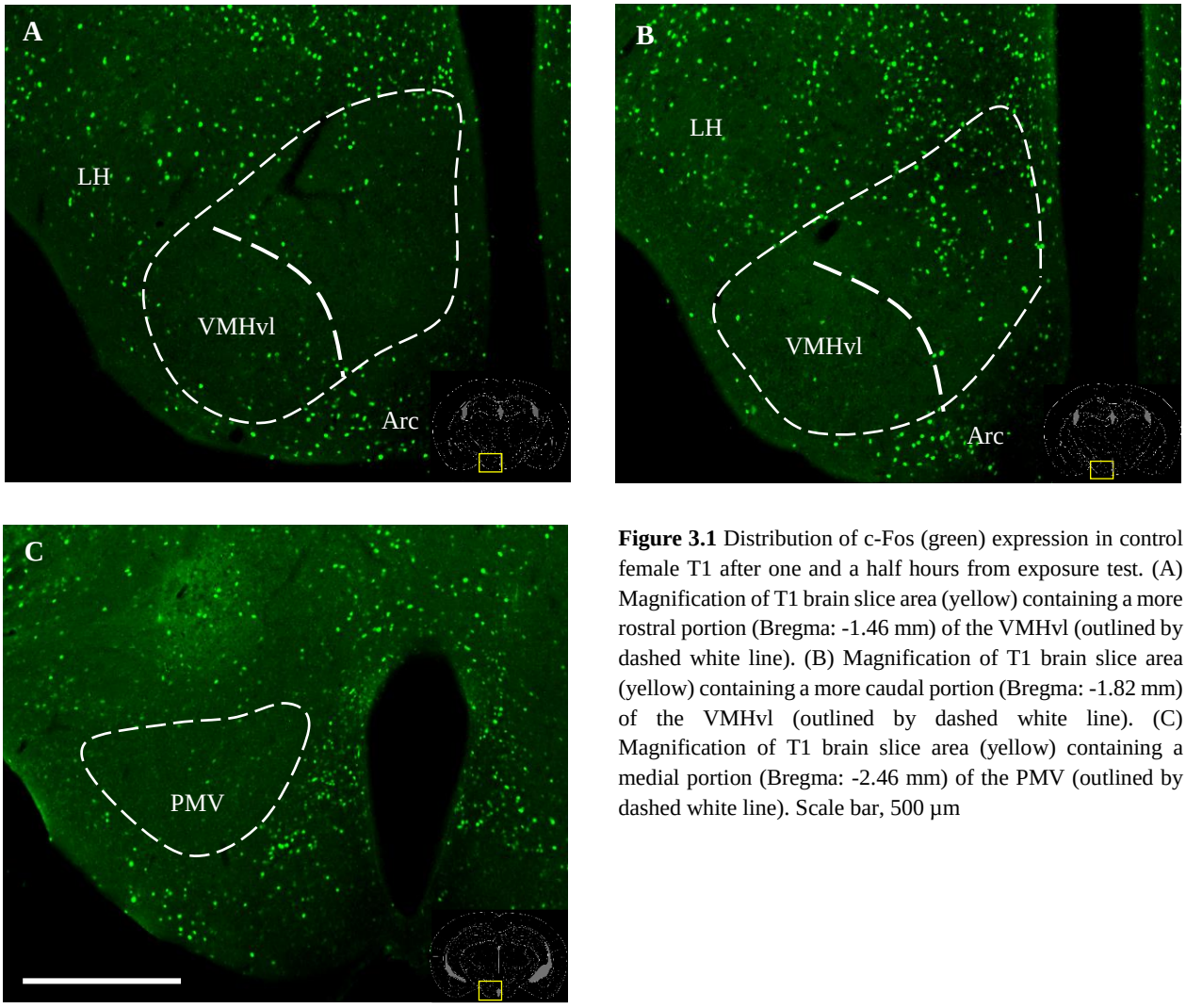


Figure 3.1 Distribution of c-Fos (green) expression in control female T1 after one and a half hours from exposure test. (A) Magnification of T1 brain slice area (yellow) containing a more rostral portion (Bregma: -1.46 mm) of the VMHvl (outlined by dashed white line). (B) Magnification of T1 brain slice area (yellow) containing a more caudal portion (Bregma: -1.82 mm) of the VMHvl (outlined by dashed white line). (C) Magnification of T1 brain slice area (yellow) containing a medial portion (Bregma: -2.46 mm) of the PMV (outlined by dashed white line). Scale bar, 500 µm

Control experiments for anti-c-Fos immunofluorescence show that no signal resembling c-Fos expression can be seen using solely secondary antibody with Alexa Fluor 488 dye (data not shown). This indicates that all images obtained from these immunofluorescence essays present a positive signal. Gradations in labeling density were common and the intensity of the signal varied according to the depth of the cell body in the slice. In the control female T1, most c-Fos protein was expressed in neurons outside of the VMH, in regions such as the arcuate hypothalamic nucleus (Arc) and the lateral hypothalamus (LH) (Figure 3.1).

This signal probably represents basal levels of neuronal activity. As expected, almost none c-Fos positive cells were found in the VMHvl (Figure 3.1A,B) and PMV (Figure 3.1C) a nucleus also known to exhibit neuronal activation in the first stages of social interaction. In female T2, the presentation and interaction with a conspecific female stimulus during five minutes induced c-Fos expression in approximately the same region as T1 (Figure 3.2). Although some c-Fos positive neurons can be seen in VMHvl (Figure 3.1A,B), the bulk of expression is located in the Arc, LH and other surrounding regions, similarly to the T1 control female. Moreover, the PMV also contains little to no c-Fos signal (Figure 3.2D).

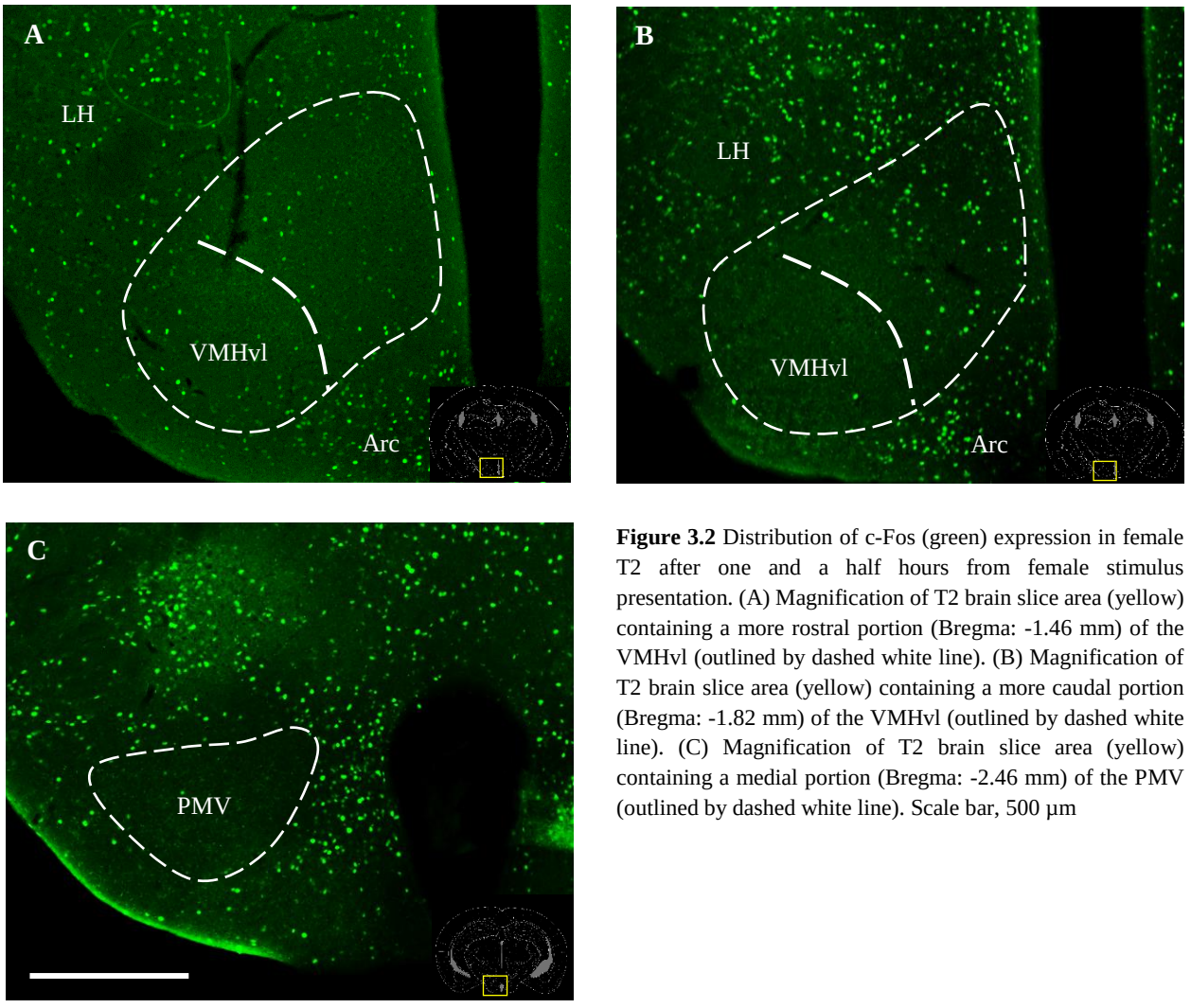


Figure 3.2 Distribution of c-Fos (green) expression in female T2 after one and a half hours from female stimulus presentation. (A) Magnification of T2 brain slice area (yellow) containing a more rostral portion (Bregma: -1.46 mm) of the VMHvl (outlined by dashed white line). (B) Magnification of T2 brain slice area (yellow) containing a more caudal portion (Bregma: -1.82 mm) of the VMHvl (outlined by dashed white line). (C) Magnification of T2 brain slice area (yellow) containing a medial portion (Bregma: -2.46 mm) of the PMV (outlined by dashed white line). Scale bar, 500 µm

For female T3 (Figure 3.3) the interaction with a conspecific male stimulus during five minutes once again induced c-Fos expression in the surrounding region LH and Arc in an apparently similar density. However, this time slightly more cells appear to express c-Fos in the rostral portion of the VMHvl (Figure 3.3A). And, interestingly, significantly more cells have c-Fos protein expression in the PMV (Figure 3.3C). This could indicate that the PMV had more neurons being activated than VMHvl at the moment of the social encounter with a male. In our lab, recordings conducted by António Dias have shown that PMV neurons are indeed activated upon female-male first stages of social interaction.

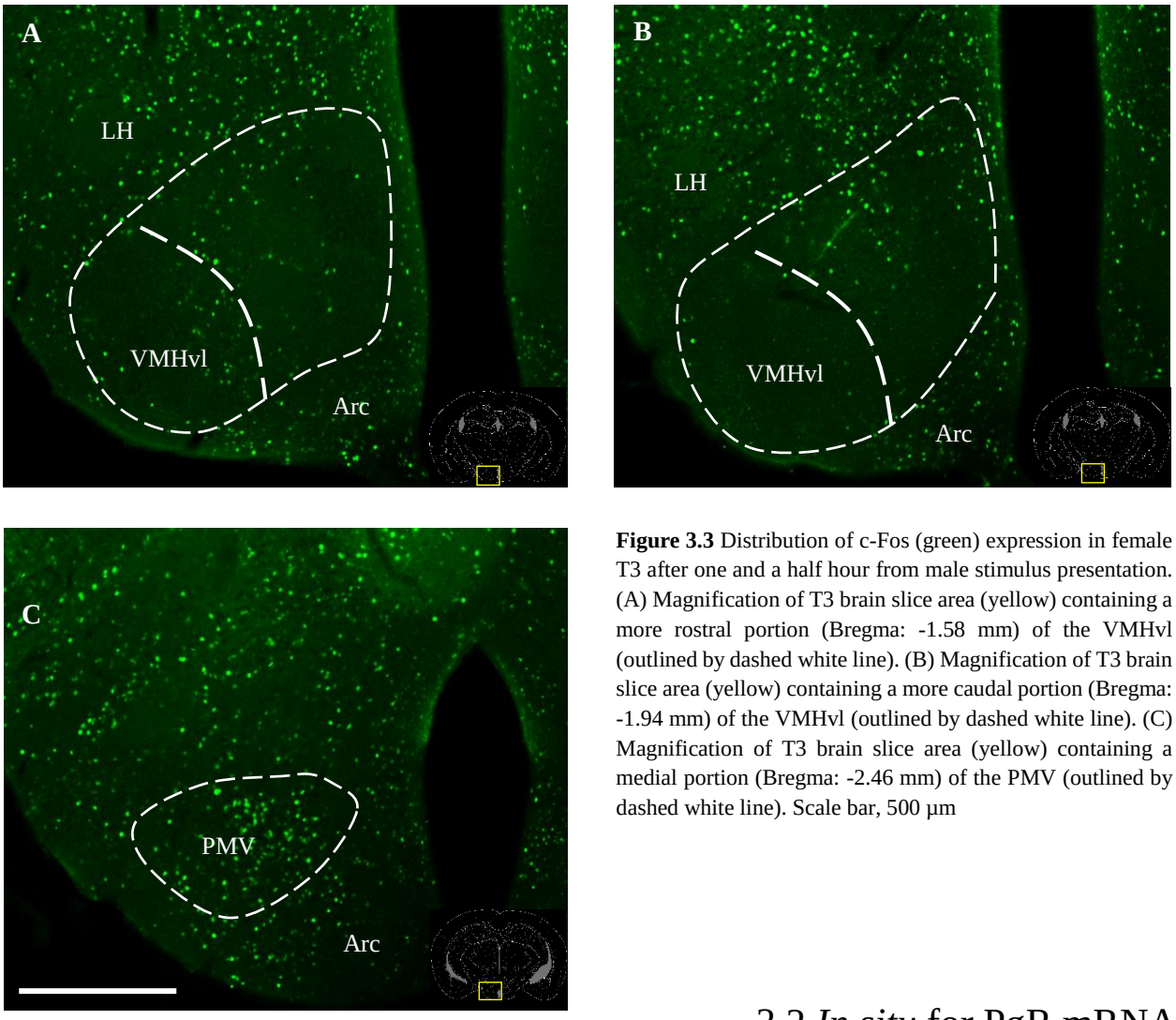


Figure 3.3 Distribution of c-Fos (green) expression in female T3 after one and a half hour from male stimulus presentation. (A) Magnification of T3 brain slice area (yellow) containing a more rostral portion (Bregma: -1.58 mm) of the VMHvl (outlined by dashed white line). (B) Magnification of T3 brain slice area (yellow) containing a more caudal portion (Bregma: -1.94 mm) of the VMHvl (outlined by dashed white line). (C) Magnification of T3 brain slice area (yellow) containing a medial portion (Bregma: -2.46 mm) of the PMV (outlined by dashed white line). Scale bar, 500 µm

3.2 *In situ* for PgR mRNA

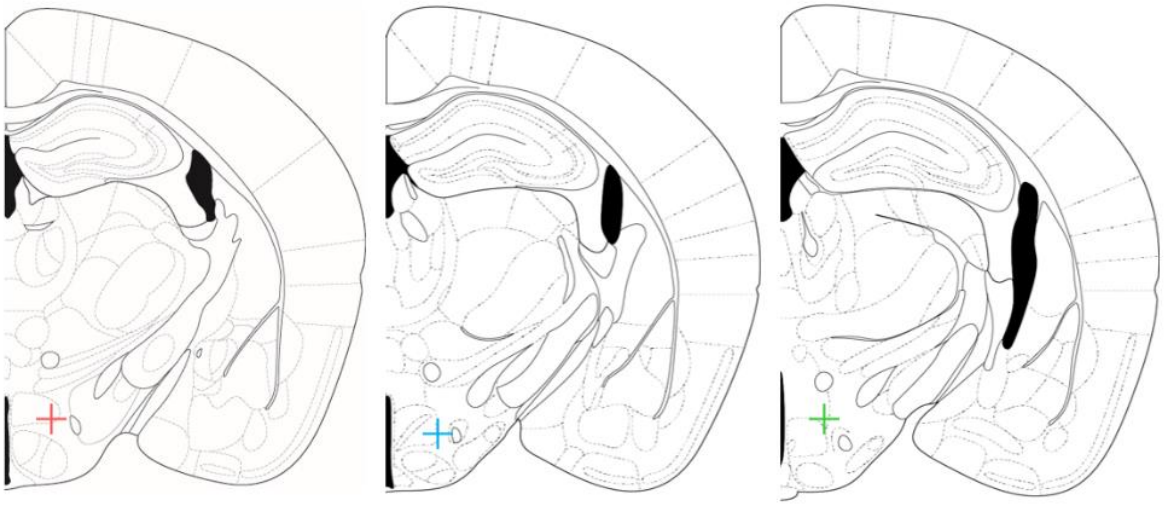
Having a viable PgR mRNA probe and an optimized *in situ* hybridization protocol would allow to study the percentage of female/male responding neurons which express progesterone receptors. This would help to enlighten the role of progesterone in modulating neural mechanisms underlying the first stages of social behavior. Unfortunately, this proposal of the present M.Sc.project cannot yet be tested due to an unsuccessful protocol. Even though the PgR probe appears to have been correctly produced, the adapted TAI-FISH protocol of Jianbo Xiu, 2014 is still being optimized.

3.3 Tracing of Monosynaptic Inputs to VMHvl

A combination of Cre/loxP FLEX gene expression and rabies-virus-based transsynaptic retrograde tracing was implemented to visualize inputs to PgR+ neurons in the VMHvl. Using this strategy, explained in detail in Chapter 2.3, it was possible to target a genetically defined population of cells in the VMHvl and infect them with a modified rabies virus that spread retrogradely to only their direct inputs.

3.3.1 Stereotaxic Viral Injections

The success of an intracranial viral injection depend on several variables, such as stereotaxic user experience, uncalibrated instruments and unique skull morphologies, to name a few. These variables usually lower the rate of a successful injected animal to 1:3 individuals. In this project, a success rate of 1:2 was achieved thanks to carefully optimization of coordinates and thoroughly implemented experimental protocols. From the six females used in this study, p02, p03 and p04 were injected in approximately the correct coordinate (Figure 3.4) and had most of the starter cell population confined to the VMHvl. Even if the injection points are not all completely similar, the virus spread was enough to encompass the target region. Conversely, females p01, p05 and p06 either had no viral expression or were injected to far from the VMHvl to be usable as reliable input data (data not shown).



p02: Bregma -1.46 mm p03: Bregma -1.82 mm p03: Bregma -2.06 mm

Figure 3.4 Schematic representation of injection locations for brains p01, p02 and p03.

Anatomical areas for starter cells and inputs were identified based on scalable vector graphics from a standard mouse atlas (Franklin and Paxinos, 2008) in superposition with DAPI channel (Figure 3.5B). To first examine the specificity of the tracing technique, the aforementioned procedure was first attempted in 3 female mice C57BL/6 (WT) that had no Cre expression (Figure 3.5A). The control brain had no cells expressing TVA or EGFP, indicating that the virus used are indeed Cre-dependent, not infecting ruptured cells from physical stress, a scenario that usually occurs during injection (Figure 3.5A). Nevertheless, different controls need to be performed to better understand the specificity of this procedure.

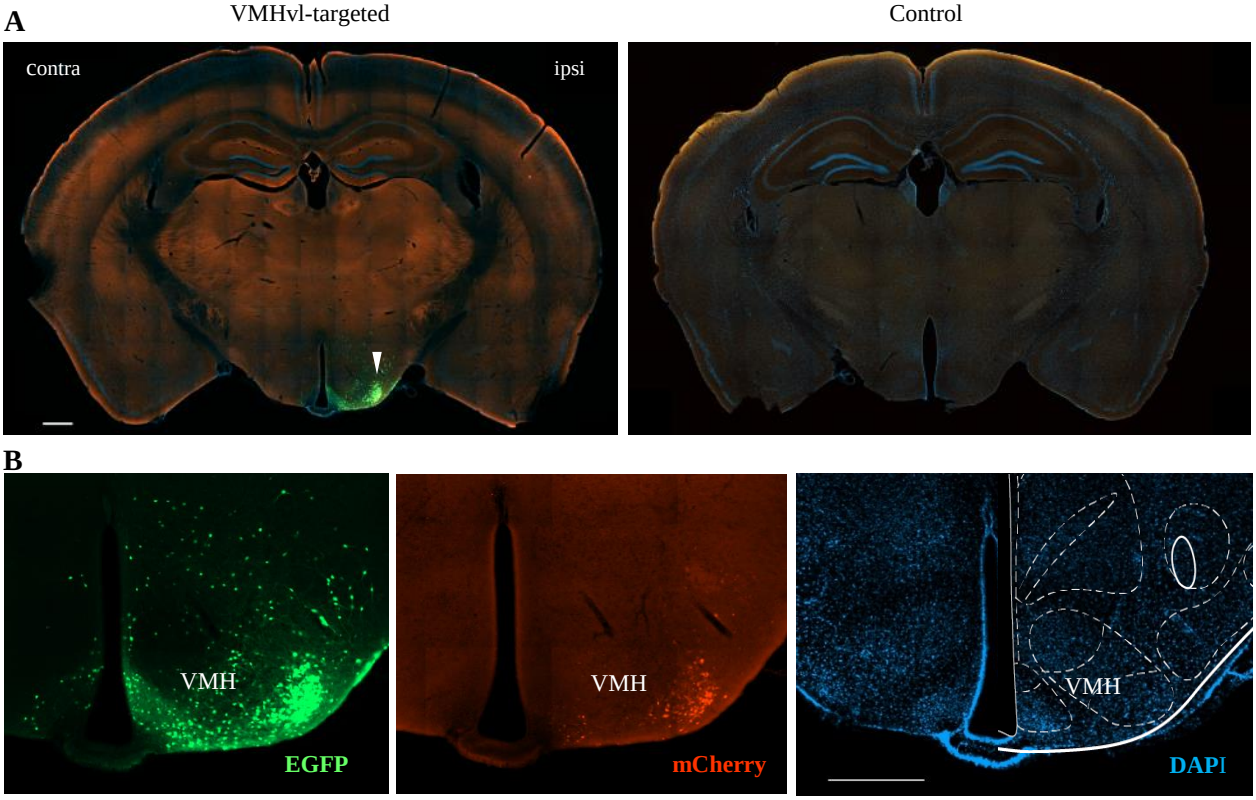


Figure 3.5 Identification of starter cells and input location to VMHvl using rabies virus and Cre-trangenic mice in brain p03. (A) Left: Transsynaptically labeled neurons in midbrain areas. Inputs (green) and starter cells in the VMHvl (yellow) can be seen in the right side of the slice (white arrow head). Righ: Control with wild-type mouse. Scale bar, 500 µm. (B) Characterization of cells in the injection site at the VMH for brain p03, for different wavelength channels Scale bar, 500 µm. In all images, the right side corresponds to the side ipsilateral to the injected side.

All brains presented a high density starter cell population on the VMHvl, and a surrounding, more dispersed, population in regions such as the Arc, LH and MTu (Figure 3.5B). Consistent results were obtained regarding the input patter distribution in the three analyzed brains, thus data from p03 will be shown as a representative. Across the whole brain, most of the inputs came from hypothalamic regions such as: the anteroventral periventricular nucleus (AVPV); medial preoptic area (MPO); anterior hypothalamic nucleus (AHN); supraoptic nucleus (SO); paraventricular hypothalamic nucleus (PVH); lateral hypothalamic area (LHA); arcuate hypothalamic nucleus (ARC); all three sub-regions of the ventromedial hypothalamic nucleus (VMH); tuberomammillary nucleus (TU); posterior hypothalamic nucleus (PH); dorsal and ventral premammillary nucleus (PMd, PMV); ventral part of the tuberomammillary nucleus (TMv); and medial mammillary nucleus (MM). The remaining inputs came from isolated regions, increasing the difficulty of its identification. Nevertheless, small populations were found in the cerebral bed nuclei of the stria terminalis (BST); in the medial and posterior amygdalar nuclei (MEA, PA) midbrain regions; in the midbrain reticular nucleus (MRN); and in periaqueductal gray (PAG) (Figure 3.6).

Labeled neurons were predominantly found ipsilateral to the injection site (Figure 3.6). Some nuclei, e.g. AVPV, PVH and PMV, seem to contain significantly larger quantity of neurons; while other regions have sparser distributions. Labeled neurons seem to form somewhat continuous bands spread across relatively long distances across the hypothalamus nuclei. Although these bands do not seem to reflect

anatomically identified regions (Franklin and Paxinos, 2008) the previously mentioned densely labeled regions do overlay with specific anatomical areas.

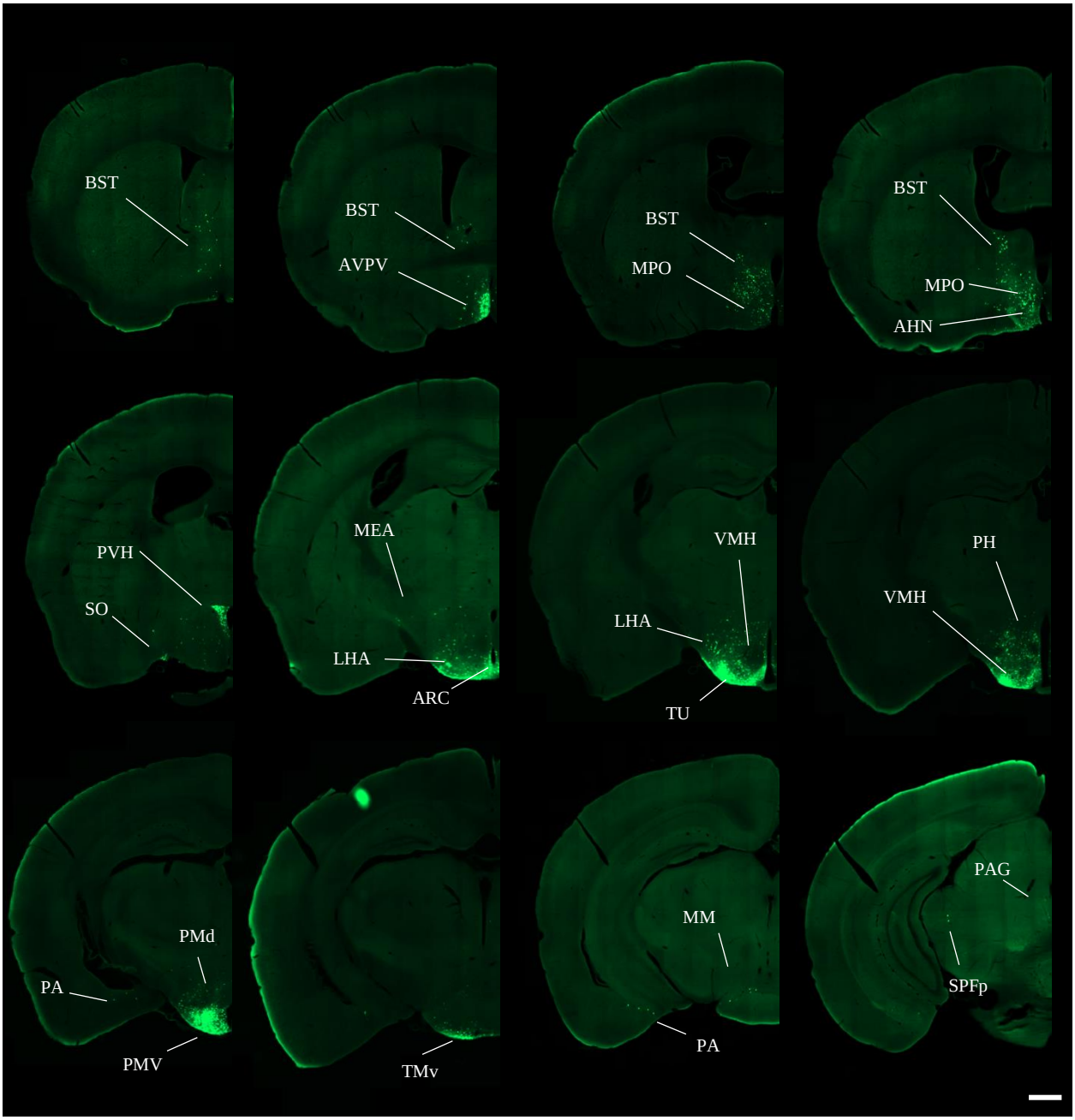


Figure 3.6 Series of coronal sections for VMHvl-targeted cases from p03. The slices are organized from a more rostral to a more caudal location in a left to right and top to bottom fashion. Only the side ipsilateral to the injection site is shown. AVPV, anteroventral periventricular nucleus; MPO, medial preoptic area; AHN, anterior hypothalamic nucleus; SO, supraoptic nucleus; BST, bed nuclei of the stria terminalis PVH, paraventricular hypothalamic nucleus; LHA, lateral hypothalamic area; ARC, arcuate hypothalamic nucleus; VMH, all 3 sub-regions of the ventromedial hypothalamic nucleus; TU, tuberomammillary nucleus; PH, posterior hypothalamic nucleus; PMd, PMV, dorsal and ventral premammillary nucleus; TMv, ventral part of the tuberomammillary nucleus; MM, medial mammillary nucleus; MEA, PA, medial and posterior amygdalar; SPFp, midbrain reticular nucleus; PAG, periaqueductal gray. Scale bar, 1 mm.

What are the neuronal mechanisms responsible for integrating gender specific neuronal activity? Is this activity internal state dependent? And if yes to what extent do neurons that express sex hormone receptors play a role in modulating this activity?

This M.Sc. project aimed to approach these questions by performing double-labeling experiments using markers for PgR mRNA and c-Fos protein in females exposed to a social stimulus. Such method would help to simultaneously visualize the neuronal response of a stimulus across the whole-brain and characterize the activated neurons as being or not progesterone positive (Farivar et al.; 2004; Xiu et al., 2014). The project focused on the VMHvl nucleus due to its role in several social behaviors such as male aggression and mating (Lin et al., 2011); its integration in neural circuitry that receives information from conspecifics (Pfaus et al., 1993); and because several of its neuronal populations express sex hormones rceptors, such as progesterone and estrogen. Also, an anatomical tracing study was performed to understand which regions input to the PgR+ cells of the VMHvl and who those inputs relate with the rest of the nucleus. Comparing these results with already known outputs of the PgR+ cells of the VMHvl (Yang et al., 2013) might help clarify how the sensory information is being integrated with the internal state of a female.

From the social exposure tests there was no apparent difference in c-Fos protein expression in the VMHvl of females exposed to male/female stimulus or no stimulus (Figures 3.1, 3.2, 3.3). This is intriguing because other studies have found significant neuronal activity in this region during the first stages of social investigatory behavior towards male or female (Nomoto & Lima, 2015). Despite this, there appears to be clear c-Fos expression in the PMV of female T3 (Figure 3.3C) a result that is in conformity with the neuronal activity seen by António Dias, a PhD student of our lab, when females are exposed to male in similar behavioral conditions. Then why is c-Fos being expressed in PMV and not VMHvl of male exposed females?

One explanation could be that investigatory behavior driven activity is not significant enough to be represented by c-Fos immunofluorescence. This is a strong possibility when we take into account that several studies have shown c-Fos expression in the VMHvl when investigating mating or aggression behaviors in females, these could be considered as more intense or consistent behaviors over time that increase synaptic activity and, consequently, calcium intake (Labiner et al., 1993). Another possibility is that c-Fos is simply not a good reporter for male induced neuronal activity in the VMHvl. Despite its widespread use as a tool to indirectly identify neuronal activation (Cruz et al., 2013) c-Fos protein detection is known to have false negative results (Bepari et al., 2012) which can lead to erroneous conclusions about the role of a specific circuit or brain region in a behavior.

In order to test these possibilities further experiments would need to be performed. Knowing that there is socially evoked activity in the VMHvl of female mice when their presented to male stimulus one approach would be testing other IEG reporters of neuronal activity such as EGR1 or Arc (Steward et al., 1998; Thiriet et al., 2001). This way, we could pair a reliable marker for neuronal activity with in situ for PgR receptors and start understanding what percentage of this genetically defined neuronal population is active during which socials behaviors.

Unfortunately, the protocol used for visualizing PgR+ neurons is not yet optimized and could not produce any usable results. So, for the time being, we have no means of characterizing cells that respond to specific stimuli. Another method that could be used to characterize these neurons is having PgR-Cre mouse lines crossed with a reporter line that expresses a fluorescent protein, such as EYFP, upon recombination (Abe & Fujimori, 2013) in order to have mice that continuously express yellow fluorescent proteins in neurons with progesterone receptor. Using this system would, in principle, only require a simple immunohistochemical staining for an IEG in order to have double-labeled neurons minus the complexity of

a TAI-FISH protocol. Still, most literature relies on in situ to reliably mark PgR in neurons (Yang et al., 2013) so if our PgR-EYFP-Cre method were to be used we would first have to make sure that it correctly portrays expression of PgR mRNA.

We aim to soon be able to find a consistent IEG for representation of neuronal activity and use it in parallel with a reliable marker of PgR. This way we can implement a more complete and thorough experimental design for the exposure tests and start to functionally characterize the VMHvl neurons that respond to social stimuli. Not only that, thanks to a recent study (Sørensen et al., 2016) we might be able to use Cre-dependent marker for robust induced neuronal activity of ensembles of neurons. This technique addresses some of the problems of regular IEG by achieving a greater temporal control and being able to be used in parallel with AAV and possibly anatomical tracing studies. This would eventually allow us to study neuronal activity from a specific population and its direct connections.

The rabies-virus-based tracing method has been used before (Watabe-Uchida et al., 2012; Wall et al., 2016) and shows great promise in helping to understand brain function in terms of how neuronal circuits integrate perception and behavior (Wickersham et al., 2007b). But there are still several controls that we should implement in our study before more complex analysis and experiments can be performed. To test for the specificity of the initial injection and to make sure that the transsynaptic spread is under strict control of RG expression more PgR-Cre females should be injected the using the same protocol only this time without AAV-RG. Moreover, given that nearly all VMH neurons, including those in the sexually dimorphic VMHvl sub region, release the excitatory neurotransmitter glutamate (Cheung et al., 2015) and use the vesicular glutamate transporter 2 (Vglut2) we aim for a future anatomical study of direct inputs done in the VMHvl of Vglut2-Ires-Cre. Also. This would allow to better understand how inputs to PgR+ neurons in the VMHvl relate to the rest of the nucleus.

The anatomy studies resulted in three brains with a population of starter cells mostly confined to the VMHvl and identical patterns of direct inputs. Thus, we had a fairly solid source in which to base our interpretation and description of regions that input to the PgR+ cells of the VMHvl. Subjective observations seem to indicate that the relative amount of inputs seemed is correlated with the number of starter cells, although this claim need quantification to be taken into account. Most of these direct inputs came from regions that comprise the social brain network (Goodson, 2005; Pfaff et al., 2008; Maney et al., 2011), such as the, MPO, POA, MEA, VMH, PMV and PAG. This result adds to findings from Yang and colleagues (2013) who found outputs from PgR+ neurons in the VMHvl of female mice to the AVPV, POA and PAG, we found that these regions also present strong inputs to the PgR+ neurons of the VMHvl, allowing some crosstalk between them that may play a major role in modulating socio-sexual behavior. Interestingly, almost all of the input regions described also express a high density population of PgR cells (Yang et al. 2013) indicating that other nuclei might be modulated by internal states and help to filter a behavior or decision making process.

One unexpected result we had was a small population of inputs that seem to be located in the parvicellular part of the subparfascicular nucleus (SPFp) in the thalamus. This region appears to receive afferents from several regions, including the VMH, and be involved in gonadal activity sensitivity during sexual behavior (Normandin & Murphy, 2008).

The data from these experiments is immense and complex so we are also aiming to implement a more objective and semi automated way of analyzing it. Giving more emphasis on the quantitative nature of input distribution by means of cell densities and ROI based studies. Soon methods like clarity and light sheet microscopy might help gather and quantitatively analyze data from anatomical tracing studies in a high throughput fashion.

With this project, we have strong indications that c-Fos is not a good reporter for VMHvl neuronal activity induced in the first stages of social interaction. More work will be needed in order use a better IEG in pair with procedures that characterize neuronal molecular markers. This will be relevant to determine the identity of neurons whose activity is being modulated by internal states and mainly, to understand how they are integrated with other VMHvl cell populations. We applied a recent tracing technique to study the direct inputs to PgR+ cells of the VMHvl and discovered that most inputs come from nuclei belonging to the SBN that also express PgR, emphasizing the importance of these cells and the VMHvl in this fundamental network for socio-sexual behavior. Comprehending how modulation of activity in the VVMHvl can underline changes in female receptivity and how the functional organization of this nucleus and its circuit of inputs might play a role in this modulation was a major goal. Our aims are still far from being completed but this challenging project is moving on a direction that might help to clarify these important questions in a near future.

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6. Appendix

6.1 Stereotactic Intracranial Injection of Viral Vectors

Adapted from Kensaku Nomoto 2015/09

Virus Handling

Always use a lab coat and gloves in accordance with procedures for handling BSL-1 agents. Take aliquot of virus in ice box, store it in the fridge to allow thaw for later usage.

Materials

- Scissors
- Two forceps (curved and straight)
- Scalpel
- Eye opener (to extend the skin)
- Suture kit
- Ear-bars
- Nanoject II (Programmable Nanoliter Injector)
- Oscilloscope for pulse generation
- 2.5-uL pipette
- Paraffin sheet
- Glass pipette (200 µm diameter)
- Eppendorf with virus in Styrofoambox with ice (stored in fridge)
- Shaver
- Eye ointment
- Betadine
- Hydrogen peroxide
- Kimwipes
- 1-mL syringe of saline
- A pile of paper (to support an animal)
- Isoflurane
- Buprenorphin

Preparations before Surgeries

1. Note weight, age, strain and gender;

2. Weight to be used in calculations for administration doses of analgesic and anesthetic. Consider postponing surgery if animal shows: signs of dehydration; bad fur condition; porphyrin (excess of blood) around the eyes; apathy; weight loss;

3. Make sure O2 and air tubing connections are normal, the air must flow from the valves to the induction box, gas mask, and stereotaxic instrument. Then from there to the isoflurane collection box;

4. Open O2 and air valves;

5. Check if there is enough isoflurane, best if above 50% of total volume;

6. Cover the surgical area with absorbent lab bench paper. Surgical tools should be sterilized before usage;

7. Set one ear-bar at 3.8 (stereotaxic scale) of the left arm of the equipment, and another ear-bar on the right arm (it can be from 3.5 to 3.9);

8. Choose an area adjacent to the surgical area that will be dedicated to loading the micropipettes with virus, and cover it with absorbent lab bench paper;

9. Setup a waste container of 10% bleach in the dedicated virus handling area for disposing of pipette tips, etc. that come in contact with the virus;

10. Attach a heating blanket to the base of the stereotaxic equipment to maintain a body temperature of 35-37°C throughout the surgery (use of rectal thermometer is optional);

Animal preparation

1. Anesthetize the animal in the isoflurane box;

2. (3-4% isoflurane in 2 l/min oxygen, and 2 l/min suction);

3. Move the animal to the gas mask and shave hair of the head from a line between the eyes to the middle of the ears, clean excess air from the animal;

4. Move the animal to the stereotaxic, and check anesthesia by toe and tail pinch;

5. Fix the animal with ear-bars and a tooth holder, the ear-bars should be at the same distance, usually they stand at the 3,8 mark (stereotaxic scale);

6. (Make sure the tongue is out of the mouth so that he won't suffocate);

7. Reduce isoflurane and air flow (2% isoflurane in 1 l/min oxygen, and 1 l/min suction) – maintenance levels;

8. Cover eyes with eye ointment using Q-tips;

9. Verify tail and toe pinch;

10. Bathe the head with three alternating scrubs of ethanol and betadine to sterilize the area. From the center to the periphery in a spiral clockwise motion;

11. Cut the skin down the middle of the head with a surgical scalpel;

12. Extend the skin with the eye opener;

13. Clean the bone with hydrogen peroxide, using Q-tips (Skull sutures take white shades and become more visible);

14. Decrease isoflurane to 1% in 1 L/min oxygen (remember to keep hydrating the skull with sterile saline);

15. Inject 10 µl/g of anti-inflammatory drug Rimadyl;

Alignment of skull for injection

1. Back fill a glass pipette (200um in diameter) with mineral oil (make sure there are no air bubbles inside of the pipette);

2. Set the pipette to the Nanoject, and mount the Nanoject to the stereotaxic left arm;

3. Determine the midline by aligning the pipette tip with the midline anterior to the bregma;

4. Align the depths of two selected points 2 mm lateral to the bregma. Depth difference should be less than 100 µm on the dial, if not, readjust the position of the animal.

5. Align the depths of the bregma and the lambda. Depth difference should be less than 100 µm on the dial, if not, adjust the height of the stereotaxic mouth piece;

6. Write down the coordinates of the bregma and the lambda from anteroposterior (AP), medial lateral (ML), and dorsal ventral (DV).

Drilling

1. Calculate the coordinate of the injection point using subtractions of the stereotaxic coordinates based on information from Franklin and Paxinos mouse brain atlas:
   AP: -1.45 to -1.5 from bregma
   ML: -0.7 to -0.75 from the midline.
   DV: -5.8 from brain surface.

2. Using a standard dentist drill open a 1,5mm x 1,5mm hole, gently. Stop any bleeding with a sterile piece of paper;

3. Check if the pipette is within the hole;

4. Take notes of the relative position of the pipette to the hole;

5. Keep the surface of the brain moist with a drop of saline;

Virus preparation and Injection

1. The injection solution contains 2 parts of AAV-TVA to 1 of TVA-RG – In general 2ul of TVA and 1ul of RG were mix in a 3ul AAV solution that should be placed on a paraffin sheet;

2. Connect the control unit to the Nanoliter injector, then to the provided power cord;

3. Set up the pulse generator (square wave, 0.1 Hz);

4. Empty mineral oil from the pipette. Then, fill the pipette with virus (make sure there are no air bubbles) – If there is a space between the metal plunger and the oil replace the micropipette, otherwise the injection will not work;

5. Move the pipette to the hole (remember the relative position of the pipette to the hole) – using the same coordinates;

6. Take notes on the depth when the pipette touches the surface of the brain. Apply a drop of mineral oil to the tip of the micropipette to prevent clogging and tissue damage related injection as the micropipette is lowered into the brain;

7. Lower the pipette to the target coordinate at a steady rate of 1mm per minute. After the tip reaches the injection point wait for 10 minutes;

8. Inject virus by connecting the control unit with the pulse generator, the amount of time the injection last depends on the quantity injected. To inject between 0.1 to 0.2 µl of AAV8 TVA + RG and 0.1 to 0.2 ul of SADΔG-GFP(EnvA) wait approx. 3:30 to 7:30 min (0.1 Hz = 1 pulse per 10 seconds; 1 pulse is equivalent to 4.6 nl); throughout the injection keep verifying if the virus is being injected.

9. Wait for 10 min. after injectionis finished then remove the micropipette at a rate of 1mm per minute;

Post-Injection and Cleanup

1. Suture the skin whit a suture kit, use double knots in opposite directions for increased stability. (Remove stitches after 10 days);

2. Stop isoflurane and air suction the dismount the animal;

3. Inject subcutaneously a 50/50 combination of an analgesic and saline supplement (0.1ml per 10mg)

4. Inject the animal subcutaneously with buprenorphine at a dose of 0.1 mg/kg every 8-12 hours over the next 72 hours, or as long as the animal is exhibiting signs of pain;

5. Place the animal in the box with a heating pad beneath and with moist pellets or gel for later feeding;

6. Close oxygen and air valves;

7. Clean the surgery station and tools with ethanol 70%, detergent or Clidox. Autocalve material at least every week;

8. Dispose of plastic waste in the correct biohazard container;

9. Rinse the micropipette with 10% bleach and discard it in a sharp waste container.

10. Clean any equipment or surfaces that came in contact with the virus with 10% bleach. Discard gloves.

11. Store aliquots back in -80°C freezer.

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6.2 Whole-brain Slices from Anatomical Study of p03 Female

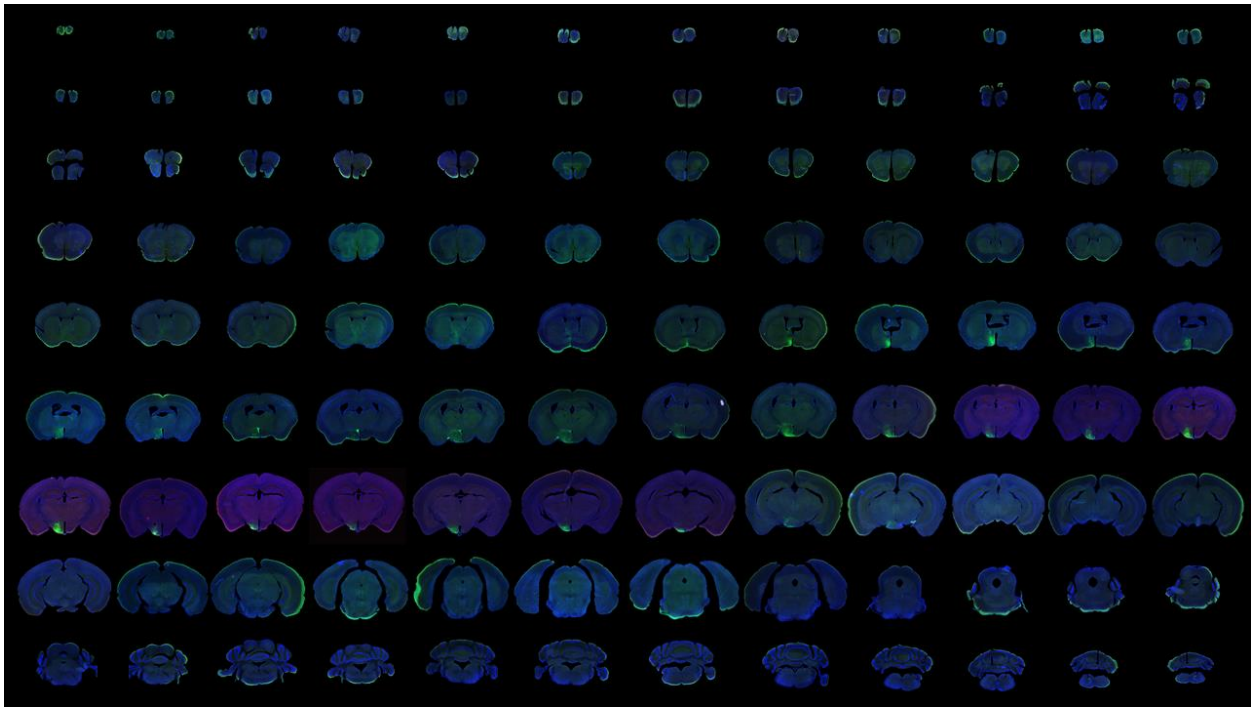

Figure 6.1 Representative partial serie of whole-brain coronal slices**:** A sample 108-serial section data set of a PgR-Cre female mouse (pr01) used for a monosynaptic input tracing study targeting the VMHvl. Afferent regions (EGFP-labeled) are clearly visible in slices from the 5th row to the 7th (ignore peripheral autofluorescence green signal in other slices). These and all other samples were imaged by AxioScan Z1: Fluorescence Slide Scanner.

6.3 Anti-c-Fos Immunofluorescence protocol

Owner: Silvana Costa Araujo

Antibodies:

Anti-c-Fos – Synaptic Systems, Germany, Cat. No. 226 003 polyclonal rabbit antidoby

Goat polyclonal secondary Anti-Rabbit IgG H&L (Alexa Fluor® 488) – Abcam, ab150077

Procedure:

(This protocol is done using always the Heidolph Unimax 1010 shaker at approximately 100 rpm)

Day 1:

1. Place desired slices series in 12-Well Plates from NEST® Cell Culture with phosphate buffer saline **PBS 1x** pH 7.4 and wash **1x** for **10 min**;
2. Wash **1x** for **15 min** in **PBS 1x + Sodium Borohydride** (Aldrich Chemistry 452882-25G) 1% (0.1g in 10 ml PBS 1x) to remove background fluorescence, use gloves and work under hood!
3. Wash **3x** in **PBS 1x** for another **2 min**, **20 min** and **30 min**;
4. Incubate the sections in a solution containing **10% weight/volume (w/v) bovine serum albumin (BSA)** (Millipore 82-045-1) **and 0.3 % Triton X-100** (Sigma-Aldrich T9284-100ML) **in PBS 1x** for **60 min** to block unspecific binding of primary antibody
   e.g. 1g of BSA in 10 ml of PBS-T (1000 ml PBS 1x + 3 ml Triton X-100)
5. Incubate the sections with diluted primary **anti-c-Fos** (1:7500, SySy) in a solution of **10% w/s BSA, 0.1% v/v sodium azide and 0.3 % Triton X-100 in PBS 1x** (e. g. 1g BSA in 10 ml PBS-T + 100 µl sodium azide 10%), leave **overnight** (≈ 20h) at room temperature (25°C)

Day 2:

1. Wash **3x** in **PBS 1x** for another **10 min, 30 min, 40 min**;
2. Incubate the slices in a solution containing **10 % w/v BSA and 0.3 % Triton X-100 in PBS 1x for 120 min**;
3. Incubate the sections with diluted secondary **goat anti-rabbit Alexa 488** (1:1000, Abcam) in a solution of **10 % w/v BSA 0.1% v/v sodium azide and 0.3 % Triton X-100 in PBS 1x for 120 min**;
4. Wash **3x** in **PBS 1x** for another **10 min, 30 min, 40 min**;
5. Incubate section with diluted **DAPI counterstaining** (1:1000) in a solution of **PBS 1x** for **15 min**;
6. Wash 3x in phosphate buffer **PB 1x** for another **10 min**, **30 min**, **40 min**;
7. Wash sections **1x** for **10 min** with **Milli-Q water**
8. Mount section in Superfrost® plus microscope slides (Thermo Scientific) and let them dry at RT
9. Apply Mowiol mounting medium to the slides, cover them with a cover slip (VWR International cover glass) and seal them with nail polish
10. Store prepared slides in cold room at 4°C

PgR RNA Probe adapted from Yang and colleagues (2013)

Sales Nbr: 2492533
Ref ID: 70055106
Gene Name: pIDTBlue: PgR probe
Gene Size: 985 bp
Plasmid weight: 2,432,999.6 g/mole

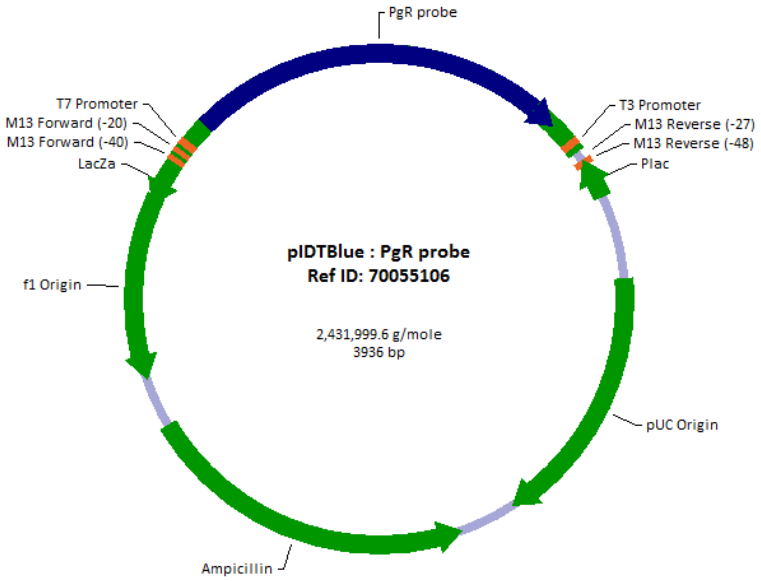

Figure 6.2 Scheme of pIDTBlue plasmid with the PgR probe insert

Name	Sequence	Recognition Sites
Acc65I	GGTACC	1677
ApaLI	GTGCAC	2442, 3688
Asp718I	GGTACC	1677
AvaI	CYCGRG	704
BglII	AGATCT	746
BspDI	ATCGAT	659
ClaI	ATCGAT	659
DraII	RGGNCCY	1052
DraIII	CACNNNGTG	222
EagI	CGGCCG	666
EcoRI	GAATTC	1501
HindIII	AAGCTT	1683
KpnI	**GGTACC**	**1677**
MluI	ACGCGT	710
PvuI	CGATCG	497, 657, 3388
PvuII	CAGCTG	527, 1950
SmaI	**CCCGGG**	**704**
TspMI	CCCGGG	704
XmaI	CCCGGG	704

Sequence Detail

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00001 CTAAATTGTA AGCGTTAATA TTTTGTTAAA ATTCGCGTTA AATTTTTGTT AAATCAGCTC ATTTTTTAAC CAATAGGCCG
00081 AAATCGGCAA AATCCCTTAT AAATCAAAAG AATAGACCGA GATAGGGTTG AGTGTTGTTC CAGTTTGGAA CAAGAGTCCA
00161 CTATTAAAGA ACGTGGACTC CAACGTCAAA GGGCGAAAAA CCGTCTATCA GGGCGATGGC CCACTACGTG AACCATCACC
00241 CTAATCAAGT TTTTTGGGGT CGAGGTGCCG TAAAGCACTA AATCGGAACC CTAAAGGGAG CCCCCGATTT AGAGCTTGAC
00321 GGGGAAAGCC GGCGAACGTG GCGAGAAAGG AAGGGAAGAA AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG
00401 GTCACGCTGC GCGTAACCAC CACACCCGCC GCGCTTAATG CGCCGCTACA GGGCGCGTCC CATTCGCCAT TCAGGCTGCG
00481 CAACTGTTGG GAAGGGCGAT CGGTGCGGGC CTCTTCGCTA TTACGCCAGC TGGCGAAAGG GGGATGTGCT GCAAGGCGAT
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00801 CTGTGGGAGC TGCAAGGTCT TCTTTAAGAG GGCAATGGAA GGGCAGCATA ACTATTTATG TGCTGGAAGA AATGACTGCA
00881 TTGTTGATAA AATTCGCAGA AAAAACTGCC CAGCATGTCG TCTGAGAAAG TGTTGTCAGG CTGGCATGGT CCTTGGAGGT
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01041 TAACGAGAGC CAGGCCCTGG GCCAGAGAAT CACCTTTTCA CCAAATCAAG AAATTCAACT GGTCCCGCCA CTCATCAACC
01121 TGCTCATGAG CATTGAGCCT GATGTGGTCT ATGCAGGGCA TGACAACACA AAGCCTGACA CTTCCAGCTC TTTGCTGACC
01201 AGTCTCAACC AACTAGGCGA GAGACAACTG CTTTCAGTAG TCAAATGGTC TAAATCTCTG CCAGGTTTCC GGAACTTACA
01281 CATTGATGAC CAGATAACCC TGATTCAGTA CTCCTGGATG AGCCTGATGG TGTTTGGCCT GGGGTGGAGG TCGTACAAGC
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01521 CTTACTACTT CTTAACACAA TTCCTTTGGA AGGACTGAGG AGTCAAAGCC AGTTTGAAGA GATGAGATCA AGCTATATCC
01601 GCGAATTGAT CAAGGCAATT GGTTTAAGAC AGAAAGGGGT TGTCCCCAGC TCACAGCGCT TCTACCAACT CACAAAGGTA
01681 CCAAGCTTAT CACTACTGGA CCGCGAGCTG TGCTGCGACC CGTGATATGC AGCTCCAGCT TTTGTTCCCT TTAGTGAGGG
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01841 ACGAGCCGGA AGCATAAAGT GTAAAGCCTG GGGTGCCTAA TGAGTGAGCT AACTCACATT AATTGCGTTG CGCTCACTGC
01921 CCGCTTTCCA GTCGGGAAAC CTGTCGTGCC AGCTGCATTA ATGAATCGGC CAACGCGCGG GGAGAGGCGG TTTGCGTATT
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02161 ACCGTAAAAA GGCCGCGTTG CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCG ACGCTCAAGT
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02641 GTATCTGCGC TCTGCTGAAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC TCTTGATCCG GCAAACAAAC CACCGCTGGT
02721 AGCGGTGGTT TTTTTGTTTG CAAGCAGCAG ATTACGCGCA GAAAAAAAGG ATCTCAAGAA GATCCTTTGA TCTTTTCTAC
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02961 ATCAGTGAGG CACCTATCTC AGCGATCTGT CTATTTCGTT CATCCATAGT TGCCTGACTC CCCGTCGTGT AGATAACTAC
03041 GATACGGGAG GGCTTACCAT CTGGCCCCAG TGCTGCAATG ATACCGCGAG ACCCACGCTC ACCGGCTCCA GATTTATCAG
03121 CAATAAACCA GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT TTATCCGCCT CCATCCAGTC TATTAATTGT
03201 TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA GTTAATAGTT TGCGCAACGT TGTTGCCATT GCTACAGGCA TCGTGGTGTC
03281 ACGCTCGTCG TTTGGTATGG CTTCATTCAG CTCCGGTTCC CAACGATCAA GGCGAGTTAC ATGATCCCCC ATGTTGTGCA
03361 AAAAAGCGGT TAGCTCCTTC GGTCCTCCGA TCGTTGTCAG AAGTAAGTTG GCCGCAGTGT TATCACTCAT GGTTATGGCA
03441 GCACTGCATA ATTCTCTTAC TGTCATGCCA TCCGTAAGAT GCTTTTCTGT GACTGGTGAG TACTCAACCA AGTCATTCTG
03521 AGAATAGTGT ATGCGGCGAC CGAGTTGCTC TTGCCCGGCG TCAATACGGG ATAATACCGC GCCACATAGC AGAACTTTAA
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03841 ATCAGGGTTA TTGTCTCATG AGCGGATACA TATTTGAATG TATTTAGAAA AATAAACAAA TAGGGGTTCC GCGCACATTT
03921 CCCCGAAAAG TGCCAC
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Antisense

Transcription using RNA polymerase corresponding to promotor 2 (T3) will produce antisense RNA (The cut is made at 5' prime).
Restriction enzyme: **SmaI** (New England Bio Labs®)

Sense

Transcription using RNA polymerase corresponding to promotor 1 (T7) will produce sense RNA with the same sequence as the target mRNA (The cut is made at 3' prime)
Restriction enzyme: **KpnI** (New England Bio Labs®)
All the following protocol steps were performed in a RNAse free environment inserted in fully prepared microbiology wing of the lab

DNA Purification using QIAGEN Plasmid Midi Kit (adapted protocol)

1. Transform competent bacteria (rapid transformation) for One Shot E.coli from Invitrogen. Note: it is essential that selective plates be pre-warmed prior to spreading.

    a. Thaw, on ice, one 50 µl vial of One Shot® Cells for each transformation.

    b. Pipet 1 µl of plasmid directly into the vial of competent cells and mix by tapping gently. Do not mix by pipetting up and down.

    c. Incubate the vial(s) on ice for 5 min.

    d. Spread 50 µL of cells on a pre-warmed, labeled LB agar plate containing 100 µg/ml ampicillin and incubate at 37°C overnight.

    e. Select 2 colonies and analyze by plasmid isolation.

2. Pick 2 colonies and incubate starter cultures of 5 ml LB medium containing Ampicillin. Incubate for approx. 8h at 37°C with vigorous shaking (approx. 300 rpm).

3. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. Inoculate 500 ml of medium with 500–1000 µl of starter culture. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm).

4. Isolate the DNA from the two minipreps using the QIAGEN miniprep kit:

    pIDTBlue is a high-copy plasmid so 25 ml was the maximum volume used for culture, QIAGEN-tip 100 were used

    P1 buffer already has RNase A added

    a. Harvest overnight bacterial culture by centrifuging at 6000 x g for 15 min at 4°C.

    b. Re-suspend the bacterial pellet in 4 ml of Buffer P1 (already contains RNase A).

    c. Add 4 ml of Buffer P2, mix thoroughly by vigorously inverting 4–6 times, and incubate at room temperature (15–25°C) for 5 min.

    d. Add 4 ml of pre-chilled Buffer P3, mix thoroughly by vigorously inverting 4–6 times. Incubate on ice for 15 min.

    e. Centrifuge at 10,000 x g for 10 min at 4°C. Re-centrifuge if the supernatant is not clear. Remove supernatant containing plasmid DNA promptly.

    f. Equilibrate a QIAGEN-tip 100 by applying 4 ml of Buffer QBT, and allow column to empty by gravity flow.

    g. Apply the supernatant from step e. to the QIAGEN-tip and allow it to enter the resin by gravity flow.

h. Wash the QIAGEN-tip with 2 x 10 ml of Buffer QC. Allow Buffer QC to move through the QIAGEN-tip by gravity flow.

i. Elute DNA with 5 ml of Buffer QF into a clean 15 ml vessel.
j. Precipitate DNA by adding 3.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA and mix. Centrifuge at ≥15,000 x g for 30 min at 4°C. Carefully decant the supernatant.

k. Wash the DNA pellet with 2 ml of room-temperature 70% ethanol and centrifuge at ≥15,000 x g for 10 min. Carefully decant supernatant.

l. Air-dry pellet for 5–10 min and redissolve DNA in 1 ml of appropriate buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5).

Determining of yield:

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. Spectrophotometric DNA quantification showed that the solution contained purified DNA with 420 ng/µl concentration.

Gel electrophoresis analysis of linearized DNA:

The plasmid was digested to check if construct was correct (1 µl of DNA from the miniprep; 980bp insert; 2950bp vector). For the digestion process the restriction enzymes KpnI and SmaI were used independently to produce the sense and antisense strands, correspondingly. For the sense digestion 1ul of DNA was added to 1.5 µl of NE Buffer 1.1 (New England Bio Labs®) followed by 1 µl of KpnI restriction enzyme, finally add 13 µl of Milli-Q water for a final volume of 15 µl. For the antisense follow the same steps but add NE Buffer 3.1 (New England Bio Labs®) and restriction enzyme SmaI instead. Place both eppendorfs containing the digestion solutions in an incubator for 1h at 37°C

For the electrophoresis analysis prepare an agarose gel 1% by diluting 1g of ultra-pure™ agarose from Invitrogen in 100 ml of TAE 1x and adding 2 µl of GelRed™ Nuclei Acid Gel Stain 10,000x (Biotium), heat the preparation in a microwave hoven until liquid and let it solidify on an acrylic bed. Load the first well of the gel with a GeneRuler 1 kb DNA Ladder (Thermo scientific), the second with a mixture of 1 µl of KpnI digested DNA + 2 µl of Gel loading dye and the third with a mixture of 1 µl of SmaI digested DNA + 2 µl of Gel loading dye. Run the gel for approx. 2h at 90V.

Following this protocol and after the electrophoresis procedure the gel was visualized with Molecular Imager® Gel Doc™ XR system (Figure x), two fairly distinct strands are visible for both digestions at approx. 4000 bp step (980bp insert + 2950bp vector), indicating successful linearization of the plasmid in both sense and antisense fashion. Nevertheless, the SmaI antisense digestion was not as successful as the Kpn sense one, as nicked and supercoiled strands can be seen on the gel.

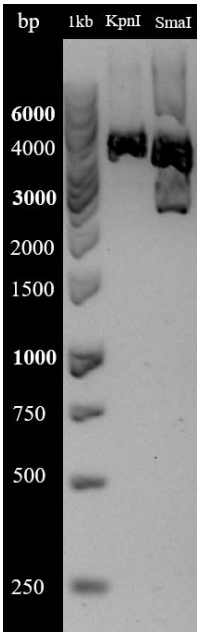

Figure 6.3 Electrophoresis gel run for probe viability assessment

DNA Purification for Probe Production

To previously tested digestion solutions purify DNA by phenol-chlorophorm extraction:

1. Add 151µL of RNAse free water
2. Extract once with 200 µL of 1:1 phenol:chloroform (Vortex and spin for 4 minutes at maximum speed at room temperature)
3. Extract once with 200 µL of chloroform (Vortex and spin for 4 minutes at maximum speed)
4. Measure volume and precipitate eluate with 10% µL sodium acetate (3M, pH5.2) and 250% µL ethanol.
5. Spin out DNA, 30 minutes at 4°C.
6. Wash pellet with 70% ethanol. Dry and resuspend in 11 µl of RNAse free water

The electrophoresis and determination of yield after this step returned the same results regarding the rate of success of the digestion process and the concentration of DNA.

In Vitro Transcription

Use 1 µg of template for the in vitro transcription. Mix the following: 6.5 µL H2O (nuclease-free); 2 µL BSA 10mg/ml; 2 µL Dig-labelled nucleotides 10x; 1 µL DTT 100mM; 1 µL RNase inhibitor (RNAsin ribonuclease inhibitor); 2 µL RNApol buffer 10x;1 µL RNA pol; 5 µL DNA. Add milli-Q water for a final volume of 20 µL and incubate the solution at 37ºC for 4h. After the synthesis, add 1ul of DNAse and incubate 15min at 37ºC to remove template.

To estimate the transcript amount, run 1µl on a 1 % agarose gel. A faint template band and an RNA band ten times more intense than the plasmid band should be observed, indicating that approximately 10µg of probe has been synthesized (this can be seen if you keep 1 µl of the reaction mixture to run at the same time, before you add the DNAse). Two or more RNA bands are commonly observed.

To clean RNA

1. Add 100 µL TE, 5.3 µL of 7.5 M LiCl, and 300 µL 100 % ethanol, mix, and precipitate RNA at −80 °C for 1 h.

2. Spin in a microcentrifuge at 4 °C for 30 min. 7. Wash the pellet twice with 70 % ethanol and air-dry.

3. Dissolve RNA in hybridization solution (100 µL)

The *in situ* hibridization steps and the detection protocol were adapted from Xiu and coleagues (2014) protocol, the adaptation was very experimental and done in trial and error fashion (data not shown).