Characterization of neuronal circuits processing specific sensory modalities in the spinal cord

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Summary

Somatosensory information is first detected by specialized sensory neurons in the periphery and then transmitted to the central nervous system (CNS) where sensory perception is formed in order to generate appropriate behavioral responses. The projections of afferents in defined layers of the spinal cord provide access to distinct neuronal pathway in the CNS, where spinal interneurons represent the first relay station controlling the coding of sensory stimuli. Despite its importance, little is known about the organization of spinal circuits involved in the integration of distinct sensory information. Here, we took advantage of the ability of the rabies virus to move from sensory neurons in the anterograde direction to directly link sensory modalities with its output connectivity (Zampieri et al., 2014). We used mouse genetic to conditionally express histone-tagged GFP, TVA receptor and rabies glycoprotein G (Rosa-HTB: Yan Li et al., 2013) under the control of Cre-recombinase activity. As proof of principle we focused on proprioception using the *PV::cre* mouse line to target proprioceptive sensory neurons (Hippenmeyer et al., 2005) and we performed stereotactic rabies injections (RVDG-mCherry/EnvA) in the spinal cord of *PV::cre +/- ; Rosa-HTB f/f* mice. We successfully obtained highly reproducible distribution patterns of connected neurons consistent with the known trajectory and termination of proprioceptive afferents in the spinal cord. In addition, we used this method to trace from a different sensory modality, focusing on *Trpv1::cre* mouse line known to label nociceptive/thermosensitive sensory neurons (Mishra et al., 2011). Comparing those sensory modalities, we observed a distinct laminar distribution pattern of second order neurons involved in proprioception and nociception reflecting the specific termination pattern of the sensory afferents in the spinal cord.

Zusammenfassung

Somatosensorische Informationen werden in der Peripherie durch spezielle sensorische Nervenzellen wahrgenommen und an das zentrale Nervensystem (ZNS) weitergeleitet. Im ZNS werden diese Informationen zu einer bewussten sensorischen Wahrnehmung verarbeitet, die schließlich zur Generierung einer Verhaltensantwort führt. Die afferenten Nervenfasern projizieren zu spinalen Interneuronen in definierten Schichten des Rückenmarks, die den Zugang zu verschiedenen neuronalen Leitungsbahnen zum ZNS gewähren. Spinale Interneurone stellen damit die erste Relaisstation dar, die die Kodierung von sensorischen Reizen kontrollieren. Dennoch ist nur wenig über die Organisation von spinalen Netzwerken, die bei der Integration von verschiedenen sensorischen Informationen involviert sind, bekannt. Basierend auf der wissenschaftlichen Publikation von Zampieri et al., 2014 wurde in der vorliegenden Dissertation ein genetisch modifizierter Rabies Virus verwendet, das ermöglicht anterograd Nervenzellen zu visualisieren, die direkt mit spezifischen sensorischen Nervenzellen verbunden sind. Um die initiale sowie die transsynaptische Infektion des Rabies Virus zu realisieren, wurde eine Mauslinie verwendet, die konditional unter der Kontrolle der Cre-Rekombinase nukleäres-GFP, den TVA-Rezeptor sowie das Rabies Glykoprotein G (Rosa-HTB: Yan Li et al., 2013) exprimiert. Zur Etablierung und Validierung der Methode wurde zunächst eine *PV::cre* Mauslinie (Hippenmeyer et al., 2005) verwendet um spezifisch propriozeptive sensorische Nervenzellen zu transduzieren und deren direkt verbundenen Nervenzellen im Rückenmark zu detektieren. Hierfür wurden stereotaktische Rabies Virus (RVDG-mCherry/EnvA) Injektionen in das Rückenmark von *PV::cre +/- ; Rosa-HTB f/f* Mäusen durchgeführt. Die analysierten Verteilungsmuster von infizierten Neuronen im Rückenmark war hoch reproduzierbar und übereinstimmend mit den Projektionsmustern von propriozeptiven Afferenzen. Anschließend wurden die gleichen tracing Experimente mit der *Trpv1::cre* Mauslinie durchgeführt, die spezifisch thermosensitive/nozizeptive sensorische Neurone (Mishra et al., 2011) markiert. Beim Vergleich der Positionen der infizierten Neurone im Rückenmark, nach initialer Infektion der sensorischen Neurone beider Modalitäten, konnte eine differenzierte laminare Organisation, die ebenfalls den Projektionsmustern der Afferenzen beider Modalitäten im Rückenmark entspricht, nachgewiesen werden.

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List of abbreviations

2D	Two-dimensional
3D	Three-dimensional
C°	Degree Celsius
AF	Alexa fluor
CB	Calbindin D-28K
CSN	Central nervous system
CHAT	Choline acetyltransferase
Chx10	Ce-10 homeodomain
Cy	Cyanin
DNA	Deoxynucleotide acid
DRG	Dorsal root ganglion
DsRed	Discosoma red
D-V	Dorso-ventral
e	embrionic day
EnvA	Avian sarcoma leucosis virus envelope
Fl	Flox
FoxP2	Forkhead box p2
CPG	Central pattern generator
GFP	Green fluorescent protein
HTB	H,histone Tag GFP,T, TVA, B, rabies glycoprotein B19G
IN	Interneuron
L1	Lumbar level 1
Lbx1	Ladybird Homeobox 1

Lhx1	Lim Homeobox 1
mCherry	Monomeric red fluorescence protein
M	Molar
M-L	Medio-lateral
mm	Millimeter
MN	Motorneuron
mRNA	Messenger RNA
n	Number of experiments or samples analysed
NaCl	Sodium chloride
NaOH	Sodium hydroxide
P	Postnatal day
PBS	Phosphate- buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PV	Parvalbumin
r	Coefficient of correlation
RNA	Ribonucleic acid
RV	Rabies virus
SN	Sensory neuron
tdTomato	Orange fluorescent protein
STT	Spinothalamic tract
TRIS	Tris (hydroyumethl) amino methane
TVA	Subgroup A avian leucosis viruses
Trpv1	Transient receptor potential cation channel subfamily V member 1
μ	Micro

1. Introduction

1.1. The somatosensory system

The somatosensory system is a complex system composed of sensory neurons and neuronal circuits that respond to environmental changes at the surface or inside the body, in order to generate appropriate behavioral responses. Somatosensory neurons comprise the largest sensory system in mammals and have nerve endings throughout the skin, muscle, bone and viscera. One of the first investigators of the body senses, Charles Sherrington, noted that the somatosensory system has three major functions: proprioception, exteroception and interoception (Fig.1). Proprioception, which will be discussed in more detail in later section of this thesis, is the sense of self. In contrast, exteroception is the sense of direct interaction with the external world as it affects the body. The principal modality of the exteroception is the sense of touch that includes sensations of contact, pressure and vibration. It also includes thermal senses of heat and cold and the sense of pain. The third component, interoception, provide the sense of the major organ systems of the body and therefore its internal state. Visceral receptors regulate multiple body functions, including blood pressure, heart rate and gastrointestinal movements and their activation play a critical role for regulating autonomic functions and do not become conscious sensations (Koch SC et al., 2018). The peripheral targets of the somatosensory neurons spread from the skin to the inner organs of the body where they detect the presence of conspicuous diversity of stimuli. Such diversity of sensation has fascinated scientists and philosophers alike for several centuries and shows the multiple functions of the somatosensory system. The richness of sensation can be well appreciated from the feeling of a breeze on a summer´s day that might involve the activation of sensory neurons (SNs) detecting cooling and the silencing of the ones detecting warm. Meanwhile, the gentle mechanical forces generated by the wind might stimulate mechanosensitive SNs that sense the bending of single hairs or the pressure changes across the skin. Such simultaneous detection of different stimuli imply the question: how are diverse stimuli from the periphery detected and

encoded by the somatosensory system and integrated in the central nervous system (CNS)?
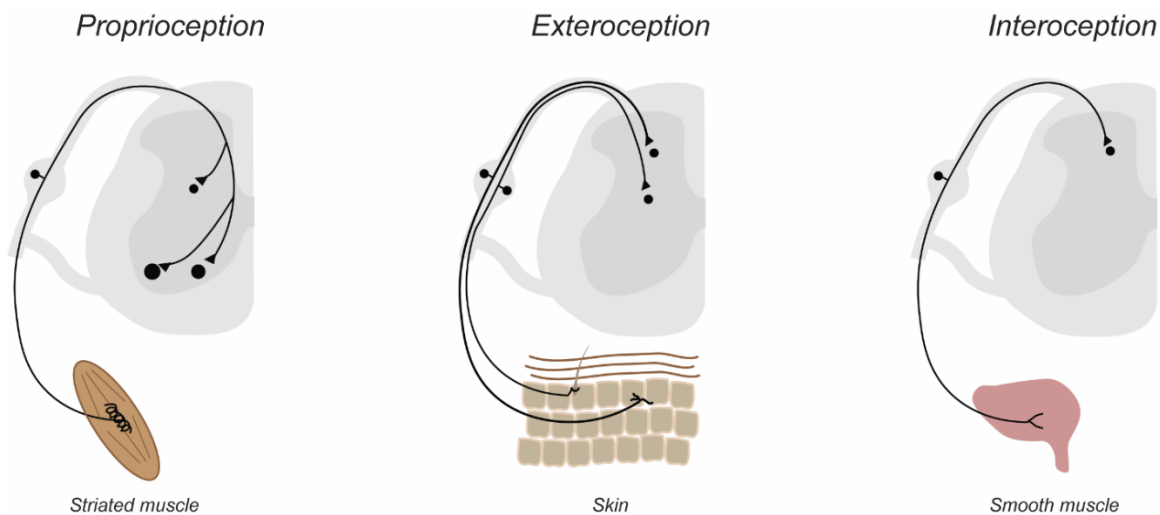
Figure 1.The somatosensory system: proprioception, exteroception and interoception. Left: proprioceptive afferents provide information about the position of the body in space. Center: cutaneous afferents innervate the hairy and glabrous skin provide information about the external environment. Right: autonomic afferents provide information about the activity of internal organs.

1.1.1. Peripheral organization of sensory neurons

Sensory neurons are specialized to detected peripheral stimuli, which are transmitted via afferents towards the CNS. The cell bodies of sensory neurons reside within the dorsal root ganglia (DRG) (Fig.2A) and the cranial sensory ganglia (Fig.2B). DRG sensory neurons are pseudo-unipolar, with a single axon divided in two branches: one innervating the periphery and responding to noxious and non-noxious stimuli, while the other goes to the spinal cord and forms synapses to second order neurons. All cutaneous sensory neurons are divided in four general fiber types: $A\beta, A\delta$ and C and proprioceptors. This classification is based on their cell body sizes, axon diameter, axonal conduction velocities and degree of myelination and generally there is a correlation between the type of sensory modality detected and the type of the neuronal afferents involved (Fig 1C).

C fibers represent half of all somatosensory neurons. They have small cell bodies and are unmyelinated fibers capable of responding of variety of stimuli including temperature, mechanical stimuli and tissue damage. Based on the expression of neuropeptides such as substance P or calcitonin gene related- peptide (CGRP) or the histological marker called the isolectin IB4 they are further divided into peptidergic and nonpeptidergig C-fibers. In the periphery, the C- fibers have free nerve endings in the skin, organs and bone. Aδ fibers have medium diameter cell bodies and are thinly myelinated. They are responsive to combination of temperature, irritants and force with a subset of them sensitive to innocuous temperature and play an important role in inflammatory pain (Basbaum et al., 2009). In term of size, the largest somatosensory neurons are the Aβ fibers and proprioceptors respectively involved in the detection of low-threshold mechanical stimuli and of muscle tension and contraction. They are heavily myelinated and have fast conduction velocities. Aβ fibers are low-threshold mechanoreceptors (LTMRs) and different subclasses are involved in specific types of mechanical stimuli such as touch, vibration, and hair deflection. The nerve endings associated to Aβ fibers are Meissner's corpuscles, Pacinian corpuscles, Merkel cell endings and the lanceolate nerve endings that surround the hair follicles (Kandel et al., 2012). On the other hand, proprioceptors are specialized endings that innervate muscle spindles or Golgi tendon organs. The difference in conduction velocity, due to the degree of myelination, allows signals of touch and proprioception to reach the spinal cord and higher brain centers earlier than noxious or thermal signals. The segregation of sensory neurons in different classes, such as proprioceptors/ mechanoreceptors versus thermoreceptors/ prurireceptors/ nociceptors starts at the very beginning of sensory neurogenesis. Neural crest cells (NCCs), under the control of specific signals from the neural tube, delaminate and some migrate ventrally between the dermamyotome and the neural tube (E9) to form the dorsal root ganglia (E11). During the migration and shortly after DRGs formation, NCCs cells diversify into different sensory neurons subtypes and central termination in the spinal cord are established (E12-birth). During development, the sensory neurogenesis is controlled by the expression of two genes, Neurog1 and Neurog2

along with a combination of transcription factors. Different studies showed the role of those genes in the generation of subsets of sensory neurons: with mice lacking both genes resulting in a complete loss of DRG neurons. However, the absence of Neurog2 gene implicate the initial loss of mechanoreceptors, compensated later by later neurons formed from Nuerog1- expressing precursors In contrast, Neurog2 is unable to compensate for the loss of Nueorg1 during development that results in lack of small diameter sensory neurons (TrkA+ SNs). Therefore, those studies suggested that the formation of proprioceptors and large mechanoreceptors mainly depends on Neurog2. Controversially the formation of nociceptors/ thermoreceptors and C-mechanoreceptor (TrkA+) depend of both genes (Ma et al., 1999).
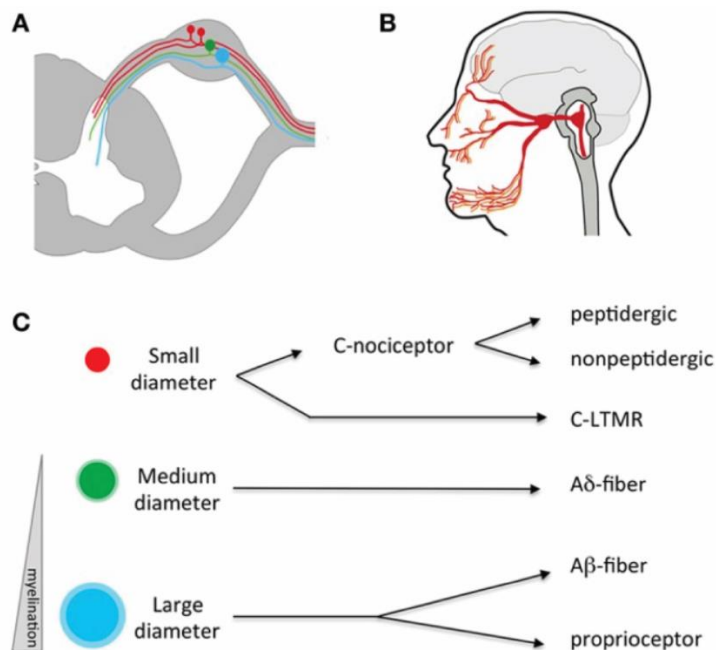
As taken from Le Pichon CE & Chesler AT, Front Neuroanat. 2014

Figure 2. Sensory neurons in the periphery. A) Sensory neuron cell bodies reside outside the spinal cord in the dorsal root ganglia (DRG). They have a single axon with two different targets: the periphery and the spinal cord. B) Sensory neurons residing in the trigeminal. C) Classification of the somatosensory neurons based on the size of their cell bodies and degree of myelination.

1.1.2. Proprioception

How do we distinguish our body from the rest of the world? How do we keep track of its posture and movements? Proprioception, named also the " sixth sense " by Sir Charles Bell, not only enables us to control the movements we make, but provide us the ability to perceive ourselves moving in space in relation to our surroundings. Proprioceptors endings located in the muscles, tendons and ligaments convey information about the sensation of muscular force and the position of the limbs. They can be classified in three different groups: muscle spindle, Golgi tendon organs (GTOs) and joint receptors. The muscle spindle consists of a bundle of thin muscle fibers, or intrafusal fibers, that are aligned parallel to the extrafusal muscle fibers innervated by the alpha motor neurons. Those afferents can be further divided, according to the size of their axons, into type I and type II fibers. The projections of primary sensory neurons, known as group Ia afferents, spiral around the central portion of intrafusal fibers, and respond to muscle stretch and the rate of stretch, whereas secondary afferents are found predominantly on chain intrafusal fibers with either spiral-like or flower-spray morphology and are involve to encoding static muscle length. The GTOs, on the other hand, are considered to be less complex structurally and lie at the interface between muscles and tendons. Each Golgi tendon organ contains sensory endings of proprioceptive sensory neurons surrounding strands of collagen, which are attached to individual muscle fibers. These groups Ib afferents innervate tendons organs and encode muscle force and increase their firing frequency as tension in the muscle rises, such as during resisted movements playing an important role in reflex circuits controlling muscle force. In contrast, joint receptor afferents as their name indicates, innervate receptors located in the joint capsules but also nearby ligaments. Joint afferents are not sensitive to muscle stimulation, but respond to moderate pressure applied over the joint, to joint movement and to contraction of the muscle inserting into the capsule. In mammals, three different receptors are found in the joints and are typically associated with tactile system: low-threshold mechanoreceptors such as Ruffini endings and Pacinian corpuscles. Those mechanoreceptors are generally considered to belong to three major types: type

I, slowly adapting receptors in the out layers of the joint capsule; type II, rapidly adapting receptors in the deeper layers of the joint capsule; type III, slowly adapting receptors embedded in the ligaments. All these receptors are distinguished from nociceptive free nerve endings distributed throughout the joint capsule, which have a higher mechanical threshold and respond to pain sensation.

1.1.3. Thermosensation and pain

Thermosensation, the ability to estimate temperature, is one of the most important sensory modality. All organism, from bacteria to plants and animals, are able to react to changes in environmental temperature with adequate responses, crucial for survival. In the past two decades, important advances were made in understanding of mammalian thermosensation by the identification of various types of ion channels, transient receptor potential channels (TRP channels) highly sensitive molecular thermometers. The role of TRP receptors in thermal sensation was originally discovered analyzing the natural substance, such as capsaicin and menthol that produce respectively burning or cooling sensations when applied to the skin. TRP ion channels are nonselective cation receptor-channels with six transmembrane domains and they are distinguished between each other by their sensitivity to heat or cold. According with a role initiating temperature sensation, most of the thermoTRPs are normally found in subset of sensory neurons in the DRGs. In fact, a single DRG neuron nerve ending marks only a small spot on the skin that often senses a narrow range of temperature stimuli. Therefore, recording directly from DRG nerve fibers allowed to classify some of these neurons as hot, warm or cool- responsive (Patapoutian et al ., 2003). The first mammalian TRP identified, was the TRPV1 activated by the inflammatory vanilloid compound capsaicin, the active ingredient of chili peppers involved in nociception. In addition, TRPV1 receptor is activated by chemicals and elevated temperatures near 43°C perceived as painful or noxious heat stimulus by many organisms. Therefore, this receptor is a multiple signal integrator capable of transducing signals evoked by several noxious stimuli. Moreover, activation of heat is also a feature of TRPV2, TRPV3 and TRPV4 receptors. TRPV2 is activated by noxious heat (>52°C) but not by capsaicin or changes in pH and is expressed in distinct sensory neurons to

TRPV1, maintaining the accuracy to respond to different temperature range (Lewinter et al., 2004). TRPV3 and TRPV4 are activated at warm temperatures in the ranges of 33-39°C and 27-34°C, respectively. Similar to warm-sensitive fibers, a class of sensory neurons involved in moderately cool temperatures detection was identified. These sensory neurons were described as innocuous cold- specific and did not respond to warming or to non- thermal stimuli such as mechanical deformation. In particular, the cooling sensation of mint-derived menthol is well established and several studies indicated a strong correlation between menthol and cold sensitivity in the periphery (Patapoutian A et al., 2003; Tomiga et al., 2004). The most studied receptor activated by menthol and cooling is TRPM8, with an activation temperature of 25-28°C (Mckemy et al., 2002). This receptor is specifically expressed in DRG sensory neurons with the smallest diameter and in a subset of pain sensing neurons. However, all the TRP receptors that are activated within different range temperature, shared the characteristic to have free nerve endings in the periphery.

1.2. The central nervous system: from the body to the brain

The central nervous system controls body function by gathering sensory input, integrating that information internally and generating a proper motor output. The central nervous system consists of two main parts: brain and spinal cord. The brain, located rostral to the spinal cord, is composed of six regions: the medulla, pons, midbrain (collectively termed the brain stem), cerebellum, diencephalon and cerebral hemispheres. Each of these regions is found in both hemispheres of the brain. In contrast, the spinal cord, the main character on this thesis, connects the brain and the brain stem to all of the major nerves in the body. The general flow of information is that the peripheral nervous system (PNS) sends information through sensory neurons to the central nervous system (CNS) to be processed. Therefore, impulses from the receptors in the periphery reach the brain through the spinal cord, where they are processed and synthetized into instructions. Afterwards, the spinal cord transmits those instructions to the rest of the body via spinal nerves. However, even if the brain is considered to play the major role in elaborating

behavior responses, the spinal cord is the main region that connect the periphery and the brain essential to transmit the in and output information.

1.2.1. Role of the spinal cord

The sensory information transduced from the skin are processed in multiple laminae of the dorsal horn of the spinal cord, which is the main station for the integration of the sensory stimuli. The gray matter of the spinal cord can be divided in IX different regions, numbered from the dorsal to the ventral surface that differ from each other in the shape, size and classes of interneurons. The X region is the gray matter around the central canal (Rexed B. 1954; Fig 3A). Lamina I, also known as the marginal zone, is characterized by the presence of numerous small cell bodies of ascending projection neurons that relay information to supraspinal structures about nociception, including strong mechanical stimuli, and thermosensation. Lamina II can be subdivided into inner (IIi) involved in touch sensation and outer (IIo) layer for pain sensation. In contrast, laminae III to IV are mainly innervated by myelinated low-threshold mechanoreceptive afferents (Andrew J.Todd Review. 2010). While the dorsal horn is considered the main region for encoding sensation, the ventral horn of the spinal cord is involved in movement and arch reflexes. Laminae VIII and IX form the final motor pathway to initiate and modulate motor activity via motor neurons, which innervate striated muscle. Nonetheless, motor neurons are not the only one that plays the lead role. Recent developmental studies have shown classes of interneurons, in lamina VII and VIII of the ventral horn, are putative constituents of the locomotor CPG (Central pattern generator) (Jessell, TM, 2000, Goulding M. et al., 2002, Goulding M. and Pfaff S., 2005). Surrounding the gray matter is white matter containing two main tracts. A tract is a group of nerve fibers myelinated and unmyelinated, which usually has the same origin, destination and similar function. These fibers conduct information up or down the cord, respectively called ascending and descending tracts and their origin and termination give the name to the tract. For example, the corticospinal tract originates in the cortex and terminates in the spinal cord or the lateral spinothalamic tract originates in the lateral spinal cord and ends in the thalamus (Fig 3B). In conclusion, the spinal cord can be divided into three regions:

dorsal, intermediate and ventral. In the dorsal horn, cutaneous sensory neurons terminate in modality-specific patterns. However, intermediate spinal cord received convergent input from proprioceptive, exteroceptive, and supraspinal neurons (Fig 3A). Ventral spinal cord contains motor neurons and networks of interneurons that regulate motor neuron firing (Osseward, P.J., II, Pfaff, S.L., 2019). Clearly, the spinal cord is the most important structure between the body and the brain that allows the communication between them. It is evident how the spinal cord plays a central role in the integration of different sensory stimuli to generate specific motor responses or to send information to the brain, where our perception of sensation is formed.
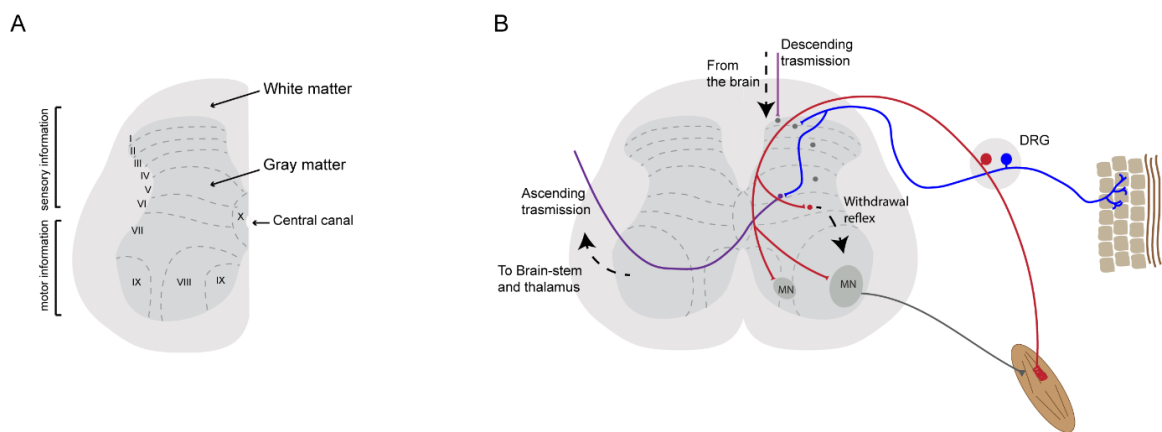
Figure 3. Spinal cord organization. (A) Cytoarchitecture of the spinal cord. The spinal cord consists of two different regions: white matter and gray matter. The gray matter is conventionally subdivided in X regions. The dorsal part of the spinal cord is involved in processing sensory information, while the ventral horn is involved in processing motor information. (B) Integration of different sensory stimuli in the spinal cord. The spinal cord receives and send sensory information from the periphery and the brain.

1.2.2. Central projections of sensory afferents

The projection pattern of the axons to specific targets in the spinal cord is crucial for the proper function of the nervous system. Depending on the sensory modalities, sensory neurons send afferent axons to different layers of the spinal cord. In particular, myelinated-low threshold mechanoreceptive afferents project between lamina IIIi-V, while nociceptive afferents in lamina I-II-V. It is known that thermoreceptive Aδ and C-fibers terminate in the superficial laminae of the dorsal

horn of the spinal cord and synapse with excitatory interneurons which in turn send ascending connections to the brain. In contrast, proprioceptive sensory neurons project centrally to the intermediate and ventral spinal cord, where they form both direct and indirect connections with motor neurons. For example, muscle spindle group Ia afferents directly excite alpha motor neurons (MNs) that innervate the same (homonymous) and synergistic (heteronymous) muscle but nonetheless they also recruit inhibitory interneurons that synapse to the antagonist motor pools. In contrast, GTOs group Ib afferents synapse onto excitatory and inhibitory interneurons that ultimately inhibit alpha MNs of homonymous muscle and the antagonist muscle. In contrast, group Ib afferents do not make monosynaptic connection with MNS. Together, these characteristic terminations are at the basis of the best-described circuit connectivity known to be the "spinal reflex pathway". Nonetheless, the characterization of the terminations of different sensory afferents in the spinal cord gave a better understanding of how specific sensory stimuli could be processed in the spinal cord moving the attention on the interneurons with whom they are linked.

1.2.3. Interneurons

A critical question in neuroscience is how neural circuits produce behavior. Several studies, including Thomas Graham Brown´s work, who was the first scientist demonstrating that the spinal cord has the intrinsic capacity to promote locomotor activity, remarked the importance of the role of the interneurons in the spinal cord. However, still little is known about the organization of spinal circuits in particular regarding the coding and integration of different sensory modalities. Incoming sensory information is processed by complex circuits involving excitatory and inhibitory interneurons in the spinal cord. The right balance between those two classes of neurons is crucial to maintain the normal sensory functions. Over the past decade, 11 distinct embryonic classes of neurons have been described, based on their generation, transcription factors, position and neurotransmitters. They can be broadly subdivided in two groups: the "V" (V0, V1, V2 and V3) interneurons with progenitors located in the ventral cord and typically associated with motor function, and a dorsal interneurons "dI" class, associated with sensory

processing. The V0, expressing the Dbx transcription factor, interneurons are characterized of contralateral, only few ipsilateral, projecting neurons with inhibitory and excitatory identities that send axons rostral to other spinal segments. This class of interneurons have been further divided in 4 subclasses (V0v, V0d, V0c and V0G) based on the expression of another transcription factors Evx1 and Pitx2 and at the functional level they are involved in left-right alternation at the hind limb level but not at the forelimb one. As a confirmation, recordings of the nerve motor activity from isolated mouse cord, the outcome was exactly what one would expect from hopping behavior: the hindlimb motor nerve burst synchronously instead of in alternating fashion in V0-ablated mice. The V1 interneurons are characterized by the expression of Engrailed-1 (En1) and located ventrally to the V0 interneurons. They define two type of inhibitory neurons in the spinal cord: Ia interneurons and Renshaw cells and some not yet identified interneurons, known to be involved in the regulation of the locomotor speed. The Ia interneurons receive inputs from muscle spindle Ia proprioceptive afferents carrying information about the muscle length and send inhibitory input onto motor neurons that innervate the antagonist muscle. On the other hand, Renshaw cells, expressing calbindin, have both motor neurons and Ia interneurons as target to modulate proprioceptive sensory input and motor neuron output. The V2 population emerge from Lhx3-progenitors cells and can be divided in two subsets, V2a and V2b interneurons. Unlike the V1 inhibitory INs, the V2 consists of a mix of GABAergic/glycinergic population (inhibitory V2b) as well as excitatory V2a cells expressing Chx10 while sharing with the previous interneurons population described (V1) the ipsilateral projection. The V2a cells were the first subset to be characterized and shown to be involved in left and right alternation by providing excitatory drive to commissural V0 neurons. Interestingly, recent work has demonstrated that this population can be further classify in different subset of neurons may be involved in locomotor rhythm generation. Nonetheless, the selective ablation of V2a interneurons also affects the variability (increase) of step cycle period and amplitude of locomotor rhythm (Crone SA et al., 2008). The role of V2b interneurons in flexor- extensor motor activity has come to light recently where in mice lacking V1 and V2b inhibitory interneurons are unable to articulate their joints and display deficits in

reflex movements even although the adult progeny of V2b neurons have not been identified (Zhang et al., 2014). The V3 cells are a heterogeneous population of excitatory commissural interneurons in mouse spinal cord that emerge from the most ventral NKx2.2-expressing progenitor domain of the neural tube. These cells, located ventral and intermediate regions of the spinal cord, make monosynaptic connections onto contralateral motor neurons, as well as ventrally located interneurons on either side of the spinal cord, playing and important role in establishing a stable and balanced locomotor rhythm (Zhang Y et al., 2008). In conclusion all these four subclasses of V interneurons described above, contribute to the spinal locomotor CGP and each one exhibits a unique phenotype signature. Moving our attention on the dorsal part of the spinal cord, were found eight canonical classes of dorsal progenitors, dI1-6 and dILa and dILb mainly grouped in two classes: A (dI1-3) and B (dI4-6, dILa and dILb). The dI1 interneurons, born from the dorsal-progenitors domain pd1, migrate to the deep dorsal horn and intermediate gray part of the spinal cord where they received proprioceptive input from the periphery and form commissural projections of dorsal and ventral spinal cord tracts. In the same intermediate region of the spinal cord, were found the dI2 ascending interneurons that project contralateral. These neurons, based on their location, have been suggested to convey information via the spinothalamic tract (STT) to the thalamus. The dI3 neurons are excitatory interneurons in the deep dorsal horn and intermediate spinal cord expressing Tlx3 and Isl1 transcription factors and recent rabies tracing experiments (Stepien et al., 2010) revealed their monosynaptic connection to motor neurons. As a matter of fact, in mice model, the dI3 appears to convey input from low threshold mechano afferents to the motor neurons, critical in hind/forelimb grip (Bui et al., 2013). In contrast, dI4 dI5 and dI6 interneurons, they all have in common the expression of Lbx1 but they can be distinguished from each other from their projection pattern: dI4 (in the lateral deep dorsal horn) are ipsilateral projecting neurons, dI5 (deep dorsal horn and ventral horn) contralateral glutamatergic neurons and dI6 commissural inhibitory interneurons. The late born dorsal interneurons (dIL) represent a second wave of neurogenesis from the dIL progenitor domain that constitutes most of the interneurons in the superficial lamina of the dorsal horn: dILA are GABAergic

ipsilateral projecting interneurons in laminae I-III expressing calbindin, while the dILB interneurons are glutamatergic ipsilateral projecting neurons expressing Lbx1 located in laminae I-III. Although, even if this general classification of dorsal interneurons in laminae I-III, we still do not have a general accepted scheme that covers all of these cells. Several studies tried to identify interneurons in lamina II by their morphology and they identified four main classes: islet, central, vertical and radial cells, which differed in dendritic morphology. These findings arise the question whether the morphological identity could represent genuine functional population in lamina II. However, this can be only confirmed after further analysis on their synaptic inputs and outputs even if certain markers show a restricted distribution that may represent functional populations of interneurons in the superficial laminae. Recently, different studies have begun to shed light on the neuronal circuits in the spinal cord horn by the identification of interneurons involved in specific sensory modalities and different cell type in the spinal cord applying snRNA-seq following behavior experiments (Sathyamurthy et al.,2018). Nonetheless, we still know little about the neuronal circuits and different interneurons involved in processing sensory information in the dorsal horn, mainly because of their heterogeneity.

1.2.4. Specificity and pattern theories

Two main theories have been popular for explaining how sensory information is encoded and processed by the central nervous system. One, the specificity theory, based on the observation that primary sensory neurons in the periphery are specialized to detect specific sensory stimuli, implies that different sensory modalities are processed in parallel on fixed neural circuits, termed labeled lines (Ma Q J Clin.Invest.2010). The existence of "specific labeled lines" was suggested first by Blix, Goldscheider and Donaldson and later confirmed by Von Frey, finding that discrete spots in human skin were activated with a specific sensation: cool, warm and pain. Soon after this discovery, electrophysiological recordings demonstrated the existence of specific primary afferents that exclusively respond to certain stimuli (Green 2004). Molecular, genetic and behavioral studies supported this idea by the identification of families of channels and receptors,

expressed in subset of primary sensory neurons. The most studied of those molecules is the non-selective cation channel TRPV1. Initially identified as the capsaicin receptor, TRPV1 is the principle detector of noxious heat (> 42°C). As matter of fact, TRPV1-knockout mice exhibit deficits in heat detection and have shown to develop less thermal hyperalgesia during inflammation (Caterina et al., 2000). However, in a similar manner, other TRP channels present in the skin were characterized and several of them were gated by a range temperature between 52°C down to 18°C. For their capability to respond to different temperature threshold, it was proposed that each channels or receptors are selectively involved in the propagation of different stimuli, including innocuous or noxious temperature across the extensive temperature range (-10°C to 52°C) (Pier et al., 2002, Banham et al.,2003, Dhaka A. Annu Rev Neurosci 2006). In contrast, the criticism of Weddell and colleagues of Von Frey's theory pointed out that is unlikely that a naturally occurring stimulus applied to just 1mm of the skin can activate only one specific single afferent fiber, underling that the different sensitivity of endings is taken in account. Nevertheless, Burgess et al. (1974) estimated that application of a weak stimulus activates rapidly adapting mechanoreceptive afferent fibers while strong stimulus would also activate slowly adapting mechanoreceptors. Thus, pressure spots are likely to be more complex and to involve more than one sensory fiber types. Because of these difficulties with the specificity theory, another theory of cutaneous sensation seemed to be required and an alternative one was the so called "pattern theory". This theory was based on the observation that sensory stimuli are encoded by train of nerve impulses reaching a particular threshold of activation delivered and recognized by the central nervous system. However, this opposite view indicates that the generation of somatosensory sensation involves a cross-talk between sensory fiber input at the level of the central nervous system due to polymodal nature of most sensory neurons. Nonetheless, we still know little about the anatomical and functional organization of the neural circuits that link incoming primary afferents to second-order neurons (interneurons) in the spinal cord. To better understand which theory holds true for sensory perception and how networks of cells mediate behaviour in response to incoming sensory stimuli, it is

necessary to classify the various cell types in the spinal cord and know which population of cells are involved in specific processing stimuli.

1.3. Rabies virus: powerful tool to trace neuronal circuits

The CNS is comprised of infinitely complex network of neurons that coordinate the flow of information. Over many decades, neuroscientists have sought to understand how diverse neurons in the central nervous system generate perception and behavior. Unraveling specific neuronal connections is essential to understand the complex wiring of the central nervous system. Genetic tools delivered by viral vectors or in transgenic animals have become a powerful resource. In particular, rabies virus is a valuable and powerful tool for study neuronal circuits due to its ability to selectively infect neurons and to propagate exclusively between synaptically connected neurons. Rabies virus is a single-strand negative RNA virus with a small genome (less than 12kb) encoding only five proteins, i.e., a nucleoprotein (N), a matrix protein (M), RNA polymerase (L), a polymerase cofactor phosphorylated protein (the phosphoprotein P) and a single external glycoprotein (G). This virus unlike other tracing viruses, such as herpes viruses, can amplify from even a single viral particle and it remains detectable in the cells for weeks. It is the only viral trans neuronal tracer that is entirely specific to propagate exclusively between connected neurons (Ugolini et al., 1995). However, intact RV infects nonspecifically and spread across multiple synapses creating ambiguity in determining neural pathways. For this reason, two main changes have been adopted to create a pseudotyped recombinant RV (Etessami et al., 2000; Wickersham et al., 2007b). The first was to modify the viral genome by deletion of the G glycoprotein (Rv∆G), replaced with a fluorescent protein, that would allow the viral spread to be monosynaptically restricted using the trans complementation approach. The second was to alter the tropism of the virus so that it could infect specific neuronal population. To achieve that, the rabies virus is pseudotyped with an envelope protein from an avian virus, the avian sarcoma and leucosis virus envelope protein: EnvA. By doing this, rabies virus expressing the viral protein EnvA on its surface only infects the cells with the TVA, an avian receptor for the envelope protein EnvA. As mammalian cells lack an endogenous

receptor for EnvA, only the cells of interest that express TVA will be susceptible to RV infection (Fig.4). Therefore, the resulting modified virus (RvΔG/EnvA) infects specific starter cells types and spreads only to monosynaptically connected inputs. This rabies virus allow controlling the tracing of the neuronal circuits. There are different ways to trans-complement and restrict the infection of the RVDG/EnvA. One option is injecting helper viruses to restrict the expression of G and TVA proteins into cells in a specific location. On the other hand, is possible to use mouse line that express the same proteins in specific cells under the control of the Cre- recombinase. Ideally, in this way the gene expression should be restricted to Cre-expressing neurons.
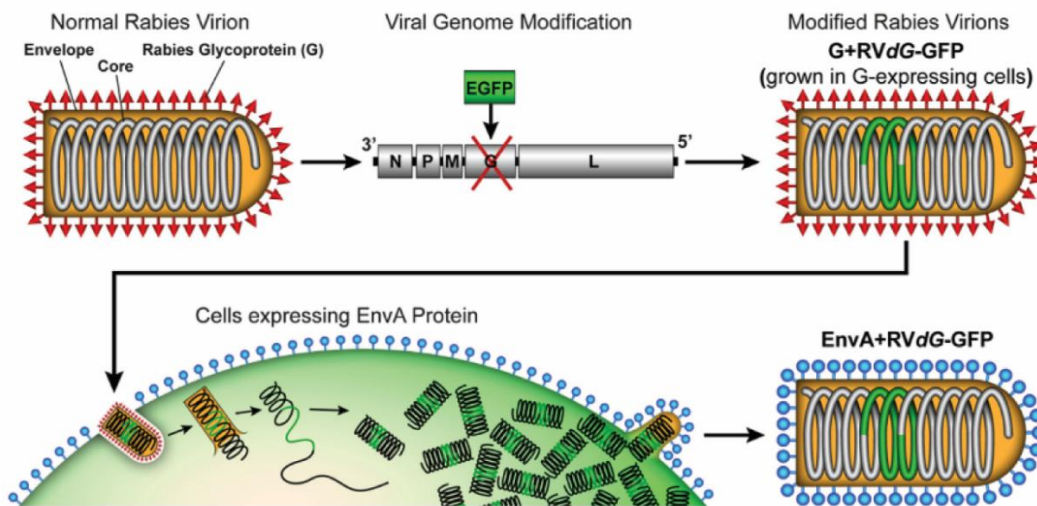
As taken from Callaway & Luo. J. Neurosc.20015

Figure 4. Rabies virus: Glycoprotein deletion and EnvA pseudotyping. Top left: Normal rabies viral expressing the G glycoprotein. Top middle: the viral genome codes for five different proteins, including G. The viral genome can be modified deleting the G glycoprotein and it can be replaced with the coding sequence for a transgene, such as GFP. Bottom left: to generate the pseudotyped RVDG, cell expressing TVA is infected with G Glycoprotrein and RVdG. Bottom right: rabies virus particles that emerge will have EnvA on their envelope.

One main question when working with the rabies virus is in which direction it spreads. Since the beginning, rabies virus was considered a retrograde trans-neuronal marker within CNS (Fig.5A) (Ugolini G. Review. Adv Virus R. 2011;

Ugolini G. 2010). However, this is not the case for at least SNs (Fig.5B). Several studies have shown how the RvΔG virus is able to infect SNs in the periphery and olfactory sensory neurons (Tsiang et al., 1989; Astic Let al., 1993; Bauer et al., 2014) and spread anterogradelly to neurons into the spinal cord (Zampieri et al., 2014). However, this tool has advantages and disadvantages to take in consideration when performing experiments. First, using the RV, not only as tool to trace neuronal circuits, give the possibility to link connectivity with function. For example by expressing optogenetic or chemogenetic tools from the rabies virus genome (Osakada et al., 2011) allows activation or inhibition of neurons connected with particular starter cells.
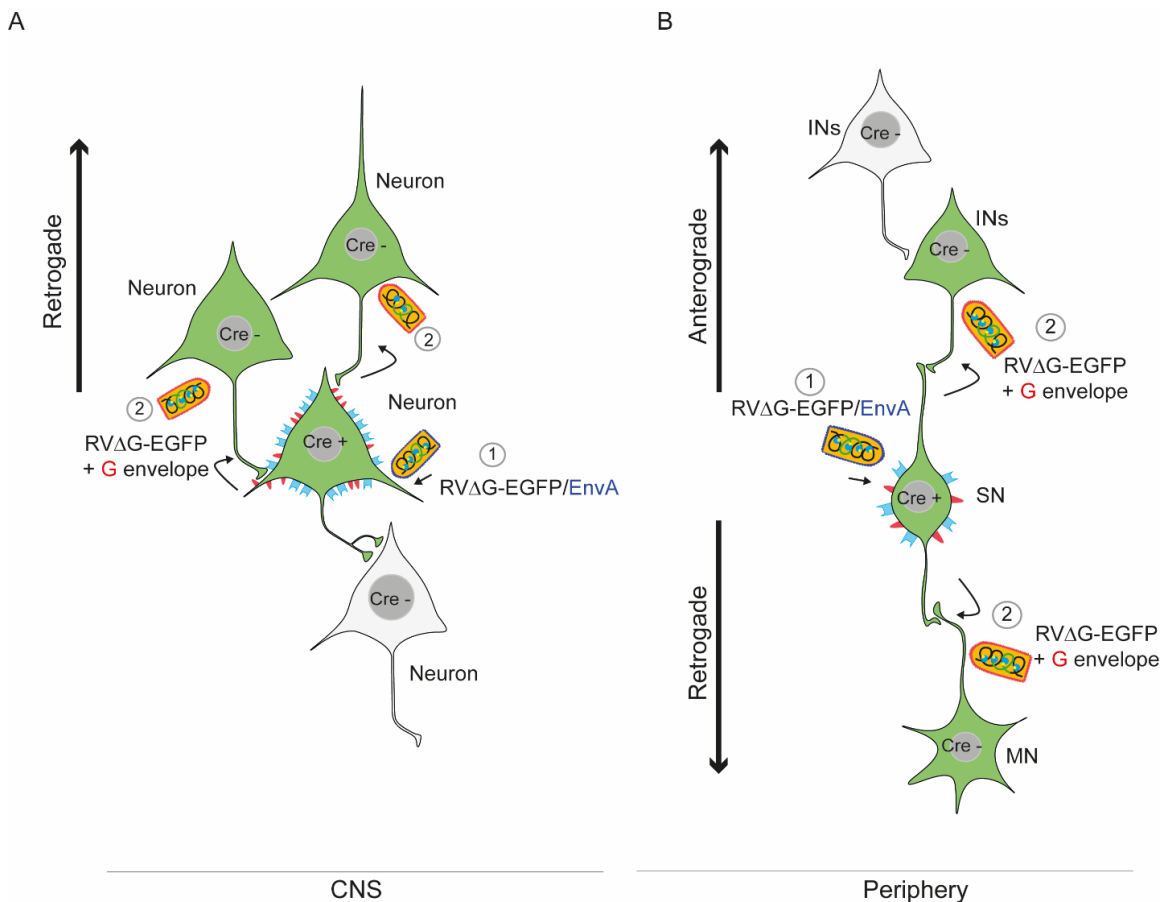
Figure 5. Spread of the RV in the CNS and in the periphery. A) In the CNS, RV has the ability to spread only in retrograde direction. Neuron of interest (Cre+) has TVA receptors and expresses G-Glycoprotein. The pseudotyped (RV RVΔG-EGFP/EnvA) infects Cre+ neuron (1) and move retrograde (2) to neurons monosynaptically connected

to the starter cell. B) In the SNs, the Rv has the ability to spread in both direction. SN of interest (Cre+) has the TVA receptors and expresses G-glycoprotein. The pseudotyped (RV RVΔG-EGFP/EnvA) infects Cre+ SN (1) and move in anterograde and retrograde directions (2) to neurons monosynaptically connected.

However, important limitations occur using rabies virus monosynaptic tracing. In certain conditions, it only labels a fraction of cells connected with the starter cells and in the case of SNs not all of them, NP neurons (NP DRG neurons with unmyelinated axons) and TH-expressing neurons (unmyelinated c-LTMRs) for example, cannot be efficiently infected (Albisetti et al., 2017). Furthermore, due to its cytotoxicity leading to the death of infected cells, its use has been confined only to short-term experiments. In particular, it has been shown (Wickersham et al., 2007) morphological changes related to cytotoxicity in RvΔG transduced cells after 16 day of infection. Recently Chatterjee et al. have described a monosynaptic tracing system based on double-deletion-mutant rabies viral vectors. In addition to deletion of the G glycoprotein gene, viral polymerase, which is required both for transcription of viral genes and for replication of the viral genome has been deleted. They demonstrated that deletion of the viral polymerase gene abolished the cytotoxicity and infected neurons survived for almost 4 months without morphological changes. This version provides sufficient time for the study and manipulation of select neural circuit. However, the rabies virus technique is likely to evolve more in the future as a genetic tool used to target networks without perturbing neighboring neural circuits providing a complete insight into connection of neurons and their functions.

2. Aim of the study

The spinal cord plays an important role in sensory processing, yet we still know little about the neuronal circuits that process sensory information, mainly due to the heterogeneity of the neural components that partecipate in these circuits. Recently, 43 neural population that contribute to spinal sensory-modality circuits have been identified (Sathyamurthy et al., 2018) using a parallel single nucleus RNA-seq. However, how those cells are linked to the incoming sensory information in the spinal cord remains unclear. The main question that scientist are trying to answer is : which neuronal cells decode incoming sensory inputs in order to produce the right behaviour outputs? In order to address this question using the Rabies virus tracing strategy,we focused our attention in the spinal cord and in particular in the interneurons that receive different sensory information from the periphery. We seek to understand the functional organization of somatosensory circuits in the spinal cord according to the following specific aims:

1) Characterization of circuit level organization of second order neurons (INs) receiving input from distinct modalities

2) Identification and molecular classification of second order   neurons

3. Material

3.1. Antibodies

Table1.Primary antibodies used for immunohistochemistry.

Target	Host	Source	Dilution
Calbindin-28K	Rabbit	SWANT	1:2000
CHAT	Goat	Millipore	1:250
Chx10	Sheep	Abcam	1:500
DsRed	Rabbit	TaKara	1:1000
FoxP2	Goat	Abcam	1:250
GFP	Sheep	BioRad	1:2000
Lbx1	Guinea Pig	Birchmeier Lab	1:10000
Lhx1	rabbit	Jessell Lab (*)	1:10000
PKCgamma	rabbit	Cell Signaling Technology	1:500
PV	Chicken	CU1664	1:10000
VGLUT1	Guinea Pig	Millipore	1:5000

*Self-made"antibodies were generated and used as previously described (Zampieri et al., 2014)

Table 2. Secondary antibodies used for immunohistochemistry

Secondary antibodies were obtained from Jackson ImmunoResearch.

Target	Host	Conjugate (*)	Dilution
Chicken	Donkey	AF-488	1:1000
		Cy3	1:1000
		Cy5	1:500
Guinea-Pig	Donkey	AF-488	1:1000
		Cy3	1:1000
		Cy5	1:500
Rabbit	Donkey	AF-488	1:1000
		Cy3	1:1000
		Cy5	1:500
Sheep	Donkey	AF-488	1:1000
		Cy3	1:1000
		Cy5	1:500

*Alexa=AF; Cyanin =Cy

3.2. Oligonucleotides and PCR programs

Table 3. List of DNA primers used for genotyping PCR

Name of the Gene	Name of the mouse line	Primer sequence	Fragment length
Cre 83 Cre 85	PV::cre TRPV1::cre	GTC CAA TTT ACT GAC CGT ACA CC GTT ATT CGG ATC ATC AGC TAC ACC	600bp
Rosa4 *Rosa 10* *Rosa 11*	Rosa-lsl-HTB	TCA ATG GGC GGG GGT CGT T CTC TGC TGC CTC CTG GCT TCT CGA GGC GGA TCA CAA GCA ATA	Wt allele: 330 bp Mt allele:250 bp
oIMR9020 oIMR9021 oIMR9103 oIMR9105	Rosa-lsl-tdTomato	AAG GGA GCT GCA GTG GAG TA CCG AAA ATC TGT GGG AAG TC GGC ATT AAA GCA GCG TAT CC CTG TTC CTG TAC GGC ATG G	Wt allele:297 bp Mt allele:196 bp Heteroz allele: 297bp and 196 bp

3.3. Mouse strains

Table 4. Mouse lines used for the in vivo experiments

Mouse line	Official nomenclature	Published by
PV::cre	Pvalb[tm1(cre)Arbr]	*Hippenmeyer et al.,2007*
Trpv1::cre	*TRPV1- cre*	Mishra and *Hoon*, 2010
Rosa-lsl-HTB	Lox-stop-lox-histone GFP-2A-TVA-2A-G	Bourane et al., 2015

Rosa-lsl-TdTomato	CAG-lox-stop-lox-tdTomato	Ai14, Jackson laboratory

3.4. Devices and chemicals

Table 5. Devices used to perform the experiments

Name of the device	Model	Brand
PCR Cycle	Mastercycler nexus GX2	Eppendorf
Pipettes	Research plus	Eppendorf
Thermomixer	Thermomixer comfort	Eppendorf
Confocal	LSM 800	Zeiss
Fluorescence Microscope	DFC3000 G	Leica
Cryostat	CM3050 S	Leica
Gel Electrophoresis System	Owl Easycast B1 and B2	Thermo Scientific
Gel imager	C150	Azure biosystems
Hotplate stirrer	VMS-C7 advanced	VWR
Thermomixer	Thermomixer comfort	Eppendorf
Microcentrifuge	PerfectSpin Mini	Peqlab
Nanoject III Auto-Nanoliter Injector	Nanoject III	Drummond Scientific Company
Hamilton	7634-01	Hamilton Company
Water bath	Alpha A6	Lauda
Light sheet microscope	Zeiss Z1	Zeiss
Scale	PF	Schinko Denish
Vibratome	VT1000S	Leica

Table 6. Chemicals used for the experiments

Name	Company
Agarose Standard	Roth
D(+)-Saccharose	Roth
Ethanol	Roth
EthidiumBromide	Roth
Formamide	Sigma
Gene Ruler 1kb Plus	Thermo Scientific
KAPA2GFast ReadyMix + dye (2x)	KAPA Biosystems
Methanol	Roth
Mineral oil	Roth
Nissl	Thermo Scientific
Paraformaldehyde	Roth
10x PBS Liquid Concentrate	MerkMillipore
TRIS	Roth
Tissue-Tek OCT	Labtech
Vectashield	Vector

3.5 Rabies Virus

Table 7. Rabies viruses used for the experiments

Name	titer
RV ΔG mCherry/EnvA	$1.0*10^8$
Rv ΔG H2B mCherry/ EnvA	$5.0*10^8$

3.6. Software

Table 8. Software used for analyses

Name	Description
Adobe Illustrator	CS6
Adobe Photoshop	CS6
Fiji	ImageJ 2.0.0-rc-68/1.52e
GraphPad	Prism 7
IMARIS	Bitplane IMARIS 9.4
Microsoft Office	Excel, PowerPoint, Word,2011
R	R.app GUI 1.68 R Foundation for Statistical Computing, 2016
R studio	1.0.136 2009 -2016 RStudio, Inc.
ZEN	ZEN 2.3(blue edition)

4. Method

4.1. In vivo experiments

All animal experiments were approved by local ethics committees (LAGESO, Berlin Germany). Animals were housed in the facility with controlled environmental parameters under a 12h light / 12 h dark cycle. Mice were fed with standard chow. Only males were used to generate the experimental animals.

4.1.1. Genotyping

The genomic DNA for PCR was obtained from mouse tail or toe. The biopsies were incubated in 200µl of NaOH (0.05M) for 1h at 95°C on a shaking heat block. Samples were then vortexed briefly and 20µl of 1M Tris-HCl (pH 7.5) was added. Subsequently, samples were vortexed and used for PCR.

Biopsies lysis buffer

- *200µl NaOH 0.05M*
- 20µl of 1M Tris/HCl pH7.5

Oligonucleotides were used according to the mouse lineas listed in Table… The following protocol was used:

KAPA2G Fast ReadyMix	12.5µl
50xPrimer mix (20µM of each primer)	0.5µl (0.5mM each final)
DNA	1.6µl
Milli-Q H2O	to 25µl

The following PCR program was used:

1 step	95°C	3min
2 step	95°C	15 sec

3 step	60°C	30 sec
4 step	72°C	15 sec
5 step	Go to step 2	34X (repeat)
6 step	72°C	1min/1kb
7 step	4°C	hold

PCR products were then loaded next to a DNA ladder onto a 1.5% agarose gel containing ethidium bromide for 40min at 110V and imaged at basic gel documentation system.

4.1.2. Spinal cord injection

For rabies tracing experiments, wild-type and PV::cre; Rosa-lsl-HTB; TRPV1::cre; Rosa-lsl-HTB mice were used. Mice at postnatal day 9 were anesthetized with isoflurane and placed on a stereotaxic frame. Under anesthesia, a skin incision was made to expose the back of the mouse and target the last ribs (thoracic) to identify the lumbar spinal cord level L1. RvDGmCherry/EnvA (300nl deltaV/deltaD = 50nl/50 μm) was injected into the L1 using Hamilton syringe (0.5μl) on the left side (400μm from the midline) of the spinal cord of the extent of lamina I –II of the dorsal horn (300μm). Skin was then sutured. Animals were kept for 7 days to label the SNs (DRGs) and INs (CNS), then sacrificed and perfused with 4% paraformaldehyde (PFA).

4.1.3. Spinal cord dissection

Animals were perfused transcardially with 4% PFA. After the head was cut off and the rib cage was opened, all the internal organs (Heart, lungs and guts) were removed in order to expose the spinal cord. The spinal cord was pinned down on the dorsal side in a dish coated with sylgard gel filled with cold PBS 1X to allow the ventral laminectomy.

4% PFA (100 ml)

- to 50 ml of ddH2O add 20 μl 10 N NaOH

- microwave for 30 sec

- add 4 g PFA and stir under hood until dissolved

- add 50 ml of 0.2 M PB

- mix and filter

- store at 4°C and use within 48 hrs

4.1.4. Immunohistochemistry

Spinal cords were dissected and post-fixed in PFA over-night at 4C°. The tissue (lumbar level) was then cryopreserved in 30% sucrose in phosphate buffer overnight and embedded in Tissue-Tek OCT compound. 30μm-thick consecutive sections were made with a cryostat and mounted on Superfrost Plus slides (VWR). For immunohistochemical staining, sections were rehydrated with PBS (1x) for 20min and permeabilized with 0.1% Triton X-100 / PBS for 10 min at RT. Then primary antibodies (see Table 1) diluted in Triton X-100 / PBS were added overnight at 4C. After washing (3 times) with Triton X-100 / PBS, sections were incubated with their corresponding secondary antibodies (see Table 2) for 1h at RT. Sections were then washed twice with PBX for 5min each and once with 1x PBS for 10min. Slides were cover-slipped using Vectashield as a mounting medium. Images were acquired by using confocal microscope (Zeiss LSM 800).

PBX

- 0.1 % Triton X 100 inPBS (1x)
- stir and store at room temperature

0.2M PB (1 L)

- 6.2 g NaH2PO4* 2H2O
- 42.88 g Na2HPO4* 7H2O
- filter and store at room temperature

30 % sucrose

- 30 % saccharose in 0.1 M PB
- filter and store at 4°C

4.1.5. Tissue clearing

After transcardial perfusion with 4% PFA as described above, spinal cords were post fixed in 4% PFA and then washed in PBS (both overnight at 4 °C). The dura was carefully and completely removed to prevent bubbles formation. 2-mm-long segments of the lumbar spinal cord was cut. Samples were incubated (on a rotating shaker 200 rpm) at 37°C in ½ Reagent 1 with water for 3-6h and then were incubated with Pure Reagent 1 overnight at 37°C on a rotating shaker 300. On the 2nd day, the reagent 1 was changed with a fresh one and leaved for other 2 days. Then, samples were washed with PBS (1X) overnight and ½ Reagent 2 with PBS 3-6h 37°C- 300rpm was added. The next day, samples were transferred in Pure Reagent 2 overnight at 37°C on a rotating shaker 300 rpm. After clearing, samples incubated in mineral oil were immediately imaged with Light sheet miscroscope (Zeiss Z1).

ScaleCUBIC 1 (Reagent 1)

- 7.5 g of 25 wt% Urea
- 7.5 g of 25wt% N,N,N´,N´- tetrakis(2-hydroxypropyl) ethyleediamine
- 2ml of dH20

Waterbath: 80°C till it dissolves

- 4.5 g of 15 wt% polyethylene glycol mono-p-isooctylphenyl
- Up to 30ml of dH20

Sonification "degas" function

ScaleCUBIC 2 (Reagent 2)

- 50 wt% sucrose
- 25 wt% urea
- 10 wt% 2,20,20´- nitrilotriethanol
- 2ml of dH2O

Waterbath: 80°C till it dissolves

- Up to 30ml of dH20

Sonification "degas" function

4.1.6. Interneuron Positional Analysis

Acquisition of 30μm consecutive spinal cord section using confocal microscope LSM 800 (Zeiss). The position of each Interneuron was analysed using the "spot" function in IMARIS software and 3D coordinates calculated relative to the central canal and normalized to a standard spinal cord dimensions.

4.2 Statistical analysis

Positional analyses were performed using custom script in "R project" (R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Contour and Density plot were generated using "ggplot2" package. The heat maps were used to compare the 2D spatial distribution of interneurons within each experiment and generated with the "corrplot" function. The similarity between experiments was measured by the Pearson correlation coefficient "r".

5. Results

5.1. Assessment of Transgenic Animal Model

In this study, we focused our attention on two main sensory modalities, proprioception and thermosensation, based on their different projection pattern in the spinal cord as well due to their differences in physiological properties and peripheral innervation. We first decided to test two different Cre-mouse lines that would enable us to identify sensory neurons cell body in the DRGs and the projection patterns in the spinal cord. In order to describe proprio- and thermo-sensation in somatosensory circuits, we took advantage of the availability of *PV*- and *Trpv1-cre* mouse lines (Hippenmeyer et al., 2005, Mishra et al., 2011). We therefore employed the Cre-lox system of recombination in existing transgenic mouse models to produce *PV::cre; Rosa--lsl-tdTomato* and *Trpv1::cre; Rosa--lsl-tdTomato* mice in which all the sensory neurons are labeled with a red fluorescent protein. Focusing first on *PV::cre ; Rosa-lsl-tdTomato* mouse line, we observed, as previously described, that the distribution of the proprioceptive afferents were consistent with their known trajectory and termination. These afferents convey information from sensory neurons in the periphery directly to motor neurons area in the ventral horn and to INs in the intermediate spinal cord. In the DRGs (Fig.6A), the number of PV+ cells cover 10% of the total number of the sensory neurons. Interesting, in the spinal cord we did not detect PV+ interneurons until postnatal day 15 (P15; data not shown). Thus as expected, based on our results, genetic tracing from *PV::cre; Rosa-lsl-tdTomato* shows that it recapitulates the endogenous PV expression in the DRG and spinal cord.
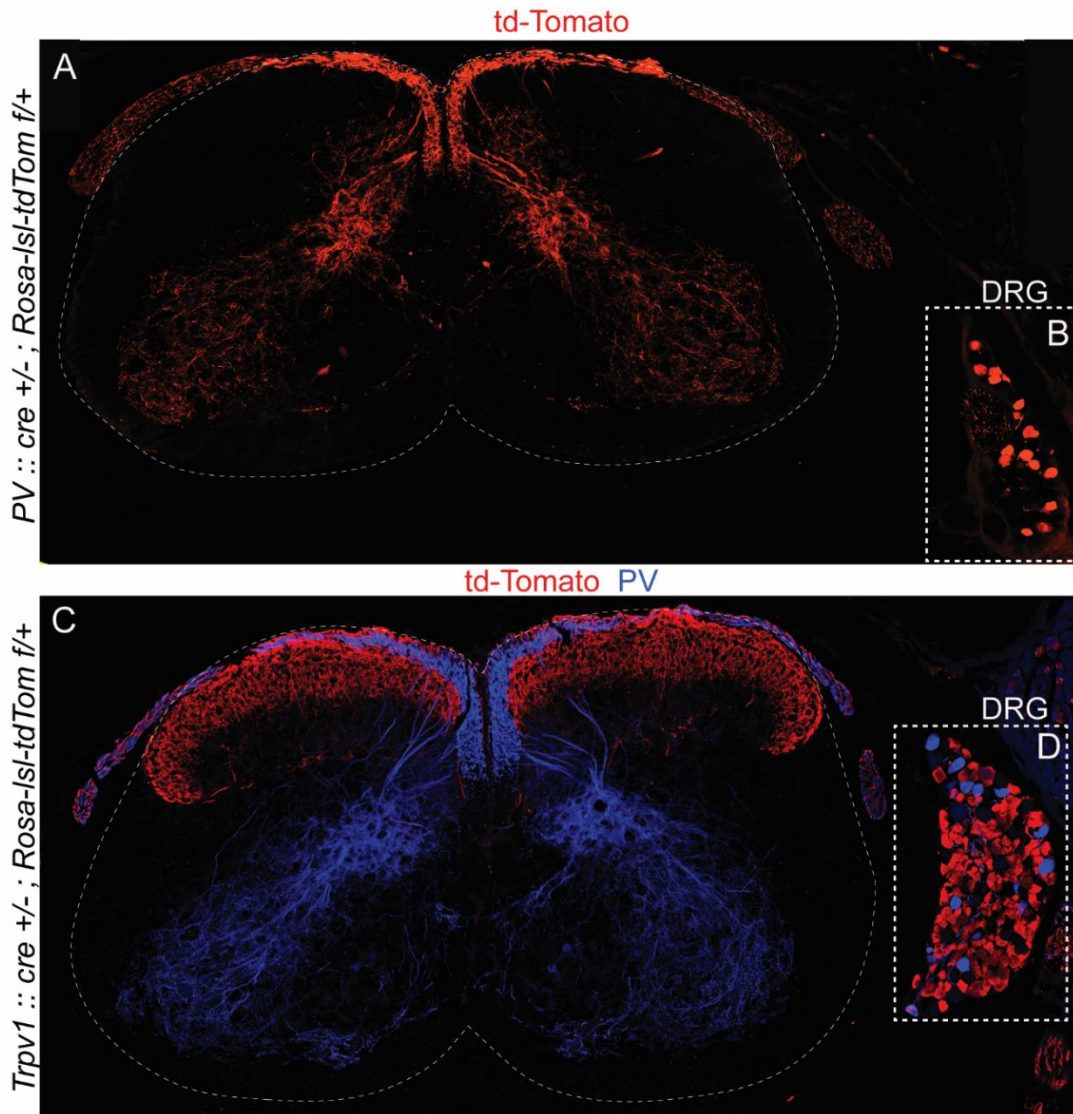
Figure 6. Characterization of *PV::cre* and *Trpv1::cre* mouse lines. (A) Projection pattern of PV+ SNs in *Pv::cre; Rosa-lsl-tdTomato* spinal cord at P15. (B) Proprioceptive SNs (red, TdTomato+) in the DRG of *PV::cre; Rosa-lsl-tdTomato* mouse at P15. (C) Projection pattern of Trpv1+ SNs in *Trpv1::cre; Rosa-lsl-tdTomato* spinal cord at P15. (D) Noxious/thermal SNs (red, TdTomato+) in the DRG of *Trpv1::cre; Rosa-lsl-tdTomato* mouse at P15.

Next, we analyzed the spinal cord and the DRGs of *Trpv1::cre* mouse lines involved in noxious-heat thermosensation, crossed with a reporter line Rosa-lsl-tdTomato mice to generate Trpv1::cre; td-Tomato mice (Fig.6C). Because of the intense fluorescence of tdTomato in these mice, peripheral and central projections of the labelled sensory neurons were revealed. In the DRGs, the number of Trpv1

+ cells cover more than 80% of the total number of sensory neurons, underling how those sensory mouse line represents the majority of sensory neurons involved in thermosensation and a subset of nociceptive SNs(Fig.6D). In the spinal cord, as expected, the sensory afferents terminate in the superficial laminae of the dorsal horn and no positive tdTomato INs were detected. We further characterized the specificity of Cre expression performing a double staining against td-Tomato and PV to remark selectively the difference in the spinal cord and in the DRGs. In the Fig. 6C, clearly we observed different projection patterns of the sensory neurons in the spinal cord and different expression in terms of number of SNs between those two Cre lines in the DRGs.

5.2. Rabies transsynaptic tracing technique restricted to specific primary sensory neurons

In order to identify the interneurons monosynaptically connected to the sensory afferents in the spinal cord, we used a rabies virus (RV) transsynaptic tracing strategy that allows to link directly sensory modality of primary sensory neurons with its output connectivity. It was previously shown (Zampieri et al., 2014) that RV infects primary sensory neurons from peripheral axon and moves to the central nervous system via anterograde axonal transport. The ability of the RV to spread across the synapse is conferred by the rabies glycoprotein G. We planned to infect the cell population of interest with a virus lacking G (RvΔG), which will instead be provided in trans in neurons of interest. To target the initial RvΔG infection selectively to specific primary sensory neurons, we pseudotyped RvΔG with the avian sarcoma leucosis virus envelope protein EnvA. The presence of this protein (EnvA) can direct virus infection specifically to cells expressing the TVA receptor, a protein found only in birds but not in mammals. First, to make the sensory neurons competent for rabies transsynaptic spread and to restrict the infection only to a subset of sensory neurons of choice, we used *PV- TRPV1::cre* mouse lines in a combination with the *Rosa-HTB* mouse line to conditionally express histone-tagged GFP in the nucleus, TVA receptor, and rabies glycoprotein G. Thus, we generated *PV::cre (+/-), Rosa-HTB (f/f), TRPV1::cre (+/-); Rosa-HTB (f/f)* to be

used in our rabies tracing experiments to label interneurons in the spinal cord involved in receiving information from proprioceptive and thermoreceptive afferents respectively. Previous study (Zampieri et al., 2015; Zhang et al., 2015) have shown how the rabies virus, injected in the periphery in neonatal mice, infects primary SNs and in turn spreads monosynaptically into interneurons in the spinal cord. In particular, Zampieri et al showed how the RV tracing could permit to map in-output organization of sensory recipient neurons. However, depending on where the rabies is injected (periphery or CNS) the outcome changes. In particular, Zhang et al in their study showed a dramatic decrease of transsynaptic spread of the virus upon plantar skin injection. Both studies suggested that RV is a powerful tool to trace neurons but they also underlined the reduce efficiency of the rabies to spread in the CNS when injected in the periphery. Based on these observations, we decide to inject the rabies virus directly in the spinal cord, avoiding the time limitation step, to infect the terminations of the sensory afferents and trace more efficiently receiving interneurons in the spinal cord. Previous studies have shown the presence of PV+ interneurons in the dorsal horn, mostly distributed between lamina II and III, starting from the third postnatal week (D.I. Hunghes et al.;2012; M.Petitjean et al.;2015). Therefore, based on our previous results in *PV::cre;td.Tomato* mice and to avoid the tracing in the spinal cord from those positive interneurons (starter cell in the spinal cord), we decide to perform the rabies injections at P9. To avoid variability between experiments, we injected 300nl (deltaV/deltaD = 50nl/50 µm) of the RvΔGmCherry/EnvA unilaterally (400µm from the central canal) on the left side of the extent of lamina I–II of the dorsal horn (300µm) where sensory afferents terminate. We sacrificed and analyzed the spinal cord after 7 day of the incubation of the RV (P16) (Fig.7). Our results confirmed that the rabies virus is able to infect sensory terminals, carrying the TVA receptors, when injected directly in the spinal cord.
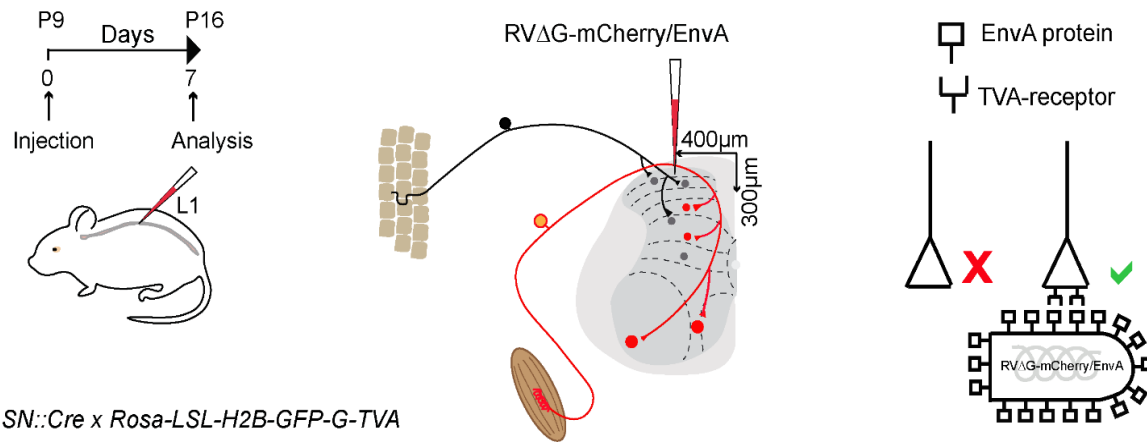
SN::Cre x Rosa-LSL-H2B-GFP-G-TVA

Figure 7. **Rabies virus injection directly into the spinal cord**. (A) Time course of spinal cord injection in the mouse. The rabies virus is injected at P9 the lumbar level (L1), after 1 week of incubation (P16), the mouse is sacrificed and the spinal cord is analyzed. (B) Spinal cord injection procedures. The capillary attached to the Hamilton syringe is filled with the RvDGmCherry/EnvA. The point of injection is 400μm from the midline (central canal) and deep 300μm into the dorsal horn. (C) Strategy to target the infection of specific SNs. Only sensory afferents in the spinal cord expressing TVA receptor can be infected from the RvDGmCherry/EnvA (X). Sensory afferents do not express TVA receptor on the surface are not infected by the rabies (√).

5.3. Specificity of the mouse line used for the tracing experiments

First, we asked whether performing the RV injections allowed us to trace from our sensory neurons of interest. We focused on the DRGs in *PV::cre (+/-), Rosa-HTB (f/f)* mice at the time of injection (P9) to determine whether these neurons were PV+ sensory neurons. To address this issue we analyzed the co-expression of the nuclear GFP, carried by the conditional mouse line, with a molecular marker specific for proprioceptive sensory neurons. First, we observed that the number of HTB+ cells in the DRGs reflected the one previously observed in *PV::cre; Rosa-tdTomato* mice. Then, we stained DRG sections (30μm) with antibodies against PV and GFP. We found that more than 95% of the HTB+ cells were PV+. This observation showed that the expression of nuclear GFP in the PV::cre; Rosa-HTB mouse line recapitulates the endogenous expression of PV in proprioceptive neurons (Fig.8)
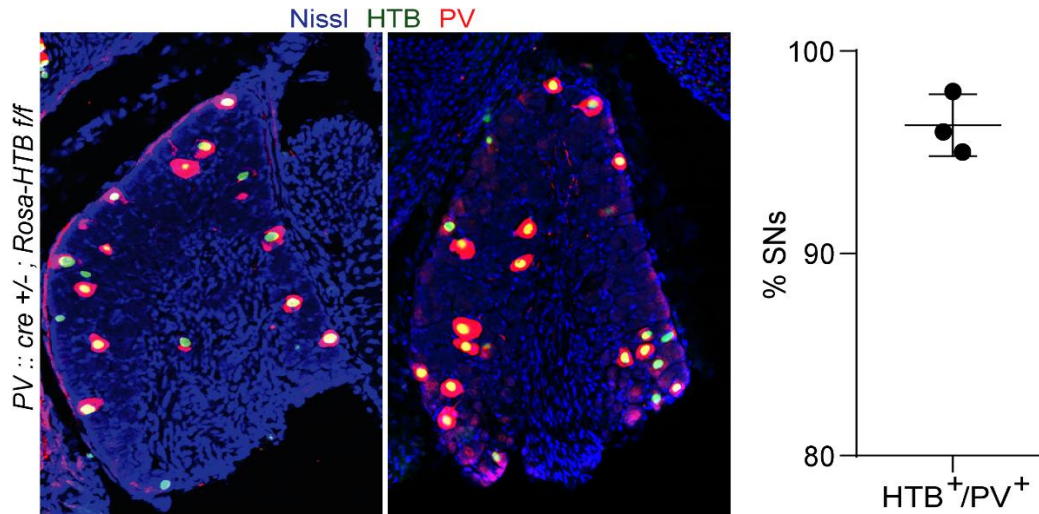
Figure 8. Quantification of PV+ HTB+ sensory neurons in the DRG. DRGs sections stained with antibodies against PV (red) and Nuclear GFP (HTB, green). 95 % of the HTB+ (green) cells are PV+ (red).

The observation that almost all the Pv+ sensory neurons in the DRG were HTB+ opens the way to specifically trace the interneurons involved in proprioception. Thus, we decided to use the reporter line *Rosa-lsl-HTB* crossed with *PV::cre* mouse line to complement the RVΔGmcherry/EnvA specifically in proprioceptors and then perform the rabies spinal cord injections.

5.4. Specificity and efficiency of Rabies primary infection of proprioceptive (PV+) sensory neuron

To determine whether RvΔGmCherry/ EnvA can infect sensory neurons retrogradely, we performed viral injection in the spinal cord and identified sensory neurons in the DRGs. We focused our initially analysis in *PV::cre; Rosa-HTB* DRGs 7 days after injection. We observed retrogradely labeled SNs in 3 DRGs at the lumbar/sacral levels in agreement with their innervation of the spinal cord at the injections point (L1). The expression of mCherry remained almost confined to HTB+ SNs. Thus, we observed that the 92% of the RV+ cells were HTB+. The ability to distinguish starter cells (both GFP+ and RV+) from the other proprioceptive cells (GFP+ only) allowed us to estimate the efficiency with which RV infects proprioceptive SNs. The ratio between the number of the starter cells

(GFP+ and RV+) over the number of the GFP+ cells, showed us that the efficiency of the primary infection of proprioceptive SNs was approximately 12% (Fig.9). These findings indicate that the RvΔGmCherry/ EnvA can be complemented successfully only in HTB+ SN carrying the TVA receptor providing the evidence that selectively infects sensory neurons (PV+) of interest.
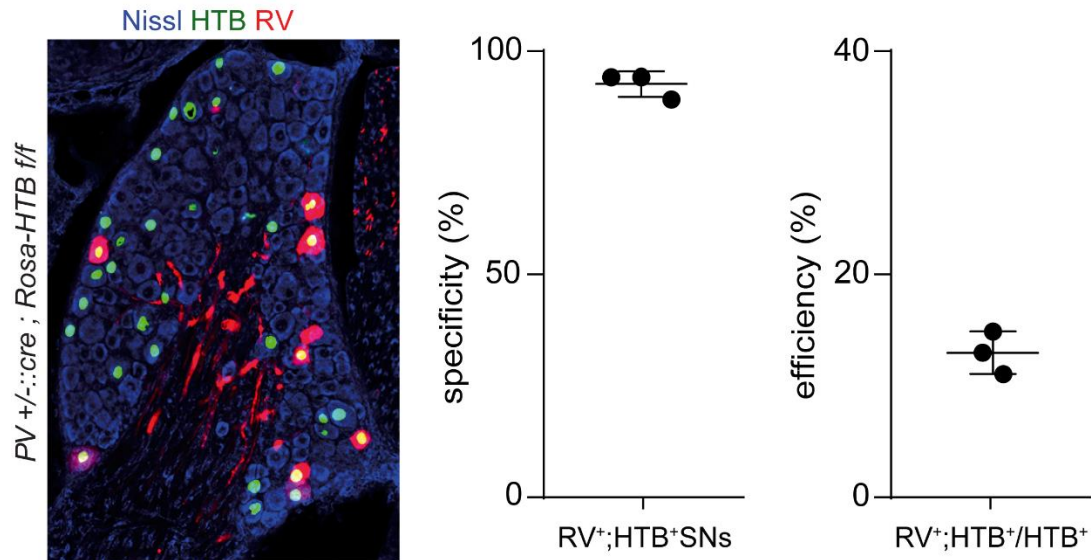
Figure 9. Specificity and efficiency of rabies primary infection in *Pv::cre;Rosa-HTB* DRGs. Lumbar and sacral section of DRGs (30um) showed that the 92% of RV+ cells (red) were HTB+ (green) with an efficiency of infection of proprioceptive neurons around 12%.

5.5. Complementation of Rabies virus in sensory neurons permits trans-synaptic viral transfer into spinal interneurons linked with a specific sensory modality

Next, we relied on the known precision of sensory-motor circuitry in *PV::cre* mouse line to examine the specificity of anterograde trans-synaptic transfer of RV into INs and MNs in the spinal cord. We compared *PV::cre; td-Tomato* spinal cord with the interneuron patterns obtained after rabies injection in *PV::cre; Rosa-HTB* mouse line (Fig.10 A,B). As mentioned before, proprioceptive sensory afferents enter in the dorsal horn of the spinal cord and some of them terminate in the intermediate area while others synapse directly into motor neurons and INs in the ventral spinal

cord as it is shown in *PV::cre; Rosa- tdTomato* spinal section (Fig.10 A). First, we checked if the position of RV+ INs reflect the termination pattern of proprioceptive afferents in the spinal cord. Indeed, we observed that the distribution of proprioceptive sensory receiving interneurons in *Pv::cre; Rosa-HTB* cord (Fig.10 B) was consistent with the known trajectory and termination of proprioceptive afferents in the spinal cord. In particular, the confirmation of a successful tracing from proprioceptive afferents, was proved not only from the position of the interneurons (Fig.10 Ba-Bb) but also by the presence of labeled MMC and LMC MNs (Fig.10 Bc-Bd). These findings confirmed that the rabies virus, complemented successfully with the G glycoprotein using a conditional mouse line, acquire transsynaptic spreading activity and can be used to map sensory recipient interneurons population in the spinal cord linked with a specific sensory modality.
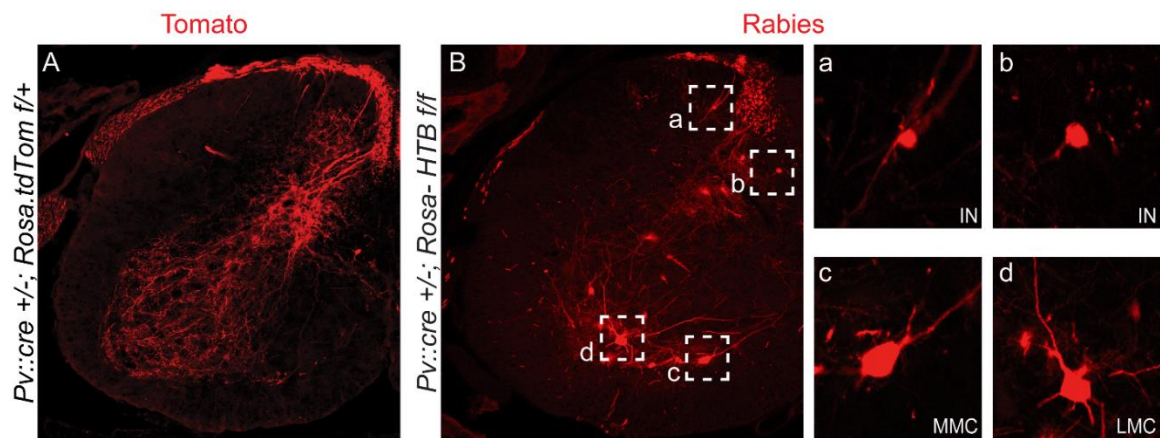
Figure 10. The distribution of the 2° INs reflect the projection pattern of proprioceptive afferents in the spinal cord. (A) *Pv::cre; Rosa-lsl-TdTomato* spinal cord showing the projection pattern of proprioceptive SNs. (B) *Pv::cre; Rosa-lsl-tdTomato* injected spinal cord showing INs monosynaptically connected with the proprioceptive sensory afferents. (a, b) RV+ INs in the spinal cord. (c, d) RV+ MMC and LMC MNs in the spinal cord.

To further investigate the connectivity of cellular network, we used a clarification protocol to make the intact spinal cord transparent, which allows us to image the rabies mCherry fluorescence of INs with a light sheet microscope. Serial image stack were obtained throughout the entire lumbar level of the spinal cord (2mm) on the medio-lateral, dorso ventral axis and were stitched together using a

software to generate the reconstruction of all the tissue. As illustrated in the reconstructed spinal cord, acquired from the lateral side (medio-lateral), the distribution pattern of labeled INs corroborated our previous findings. Thus, some INs were located dorsally and linked with the incoming proprioceptive afferents and the majority of them were distributed in the intermediate and ventral spinal cord. Nevertheless, we were able to visualize the presence of the MNs distinguished from the INs by size (Fig.11). This result allows us to assess the consistency of the distribution of the receiving proprioceptive INs in the spinal cord. These findings confirmed that the RV can also be used to map sensory recipient interneurons population in the spinal cord linked with a specific sensory modality.
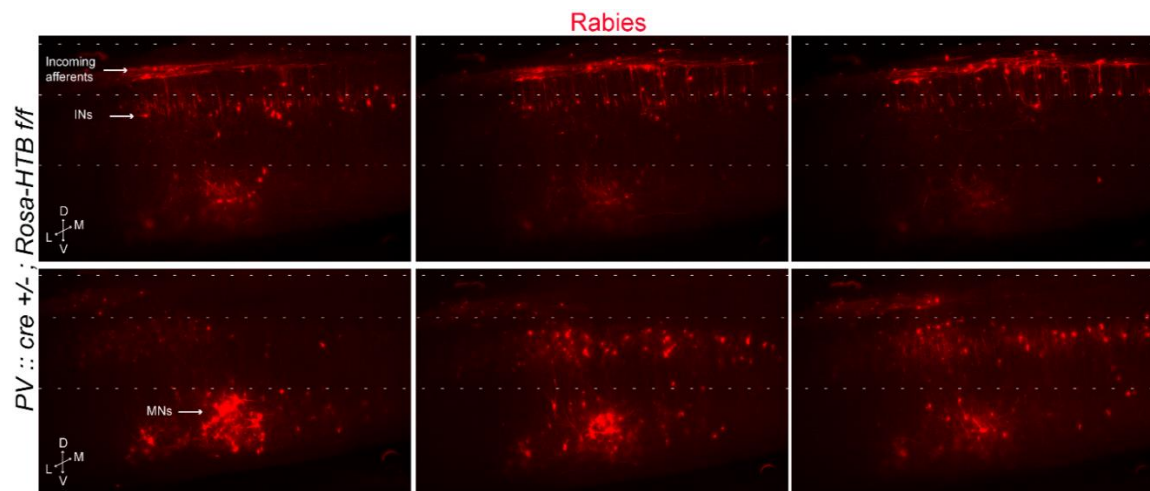
Figure 11. 3D visualization of RV+ INs and MNs in cleared mouse spinal cord. Lateral projections from the cleared spinal cord at different depths showing INs and MNs directly connected with the incoming proprioceptive afferents in the spinal cord.

5.6. Positional analysis of labeled INs in the spinal cord upon viral injection

Next, we assessed the overall 3D distribution pattern of labeled INs in the spinal cord using a quantitative method previously established in our laboratory (Dewitz et al., 2018). Lumbar levels L1 to L3 at P16, were analyzed by cutting 30um consecutive sections and performing immunohistochemistry with specific antibodies against the mCherry and the GFP proteins expressed respectively from the RvΔGmCherry/ EnvA and the conditional mouse line *Rosa-lsl-HTB* crossed

with Cre-mouse line. The high-resolution images of the spinal cord were acquired at confocal microscope and processed with the imaging software IMARIS using the "spots" function to assigns Cartesian coordinates to all labeled INs. We set the central canal as the 0-0 coordinate for the medio-lateral (x-axis) and dorso-ventral (y-axis) axes. These coordinates (x and y) were rotated and normalized to a standardized spinal cord, whose dimensions were obtained by calculating the average size of spinal cords at P16, to avoid the variability in size and orientation of the spinal cord between experiments. The rostro-caudal position (longitudinal; z-axis) of each INs was calculated by considering the histological order of the sections thick 30μm. Datasets were aligned on the z-axis by starting analysis from the section where the first labeled INs appeared (z=0) in the L1 segment and progressed caudally for 2 mm, covering approximately two lumbar segments of the spinal cord. Using this method, we assigned 3D coordinates to all interneurons labeled transsynaptically upon viral injections in the spinal cord. To visualized longitudinal and transverse distributions of labelled INs, we used the statistic software R to run those datasets derived from individual injections. Comparison of positional coordinates in 3D gave information about the rostro-caudal, medio-lateral and dorso-ventral distribution of RV-mCherry labeled spinal INs. Thus, using the method described above, we reconstruct the position of the INs involved in two different modalities: proprioception and thermosensation.

5.7. Transsynaptic labeling of INs and MNs from rabies infected proprioceptive sensory neurons

We next explored the organization of labeled INs involved in proprioception in the spinal cord by plotting the medio-later, dorso-ventral and rostro-caudal positions. We found the 96% of mCherry+ INs were mostly distributed ipsilateral to the point of injection in all rostro-caudal extent of the spinal cord. We detected the majority of mCherry+ INs (49%) in the intermediate laminae of the spinal cord, while 27% in the dorsal laminae and 23% in the ventral horn. Indeed, we also observed few (4%) mCherry+ INs in the contralateral side of the point of injection that could reflect midline crossing of sensory afferents or of interneuron dendrites. The

number of the virally labeled MNs is constant in all the three experimental replicates. To further investigate the position of virally labeled INs, we also surveyed their distribution on the dorso-ventral axis (Fig.13 A-B). As expected, we observed a subset of virally labeled interneurons in lamina II, which are involved in modulation and transmission of touch sensory information, interneurons in intermediated and ventral spinal cord and motor neurons directly connected with the proprioceptive afferents. Thus, to quantitatively assess variability in the overall interneuron distribution, we compared individual experiments using correlation analysis, a method suitable for extraction of common features from pairs of multivariate data. We found that the distribution analysis were highly correlated (r>0.8) (Fig. 13C). The high overall reproducibility of the method is further remarked by the visualization of the density plots on the medio-lateral and dorso-ventral axis showing a dense network of labeled interneurons in the vicinity of the central canal where the majority of proprioceptive afferents terminate (Fig.12 B-H; C-I) .
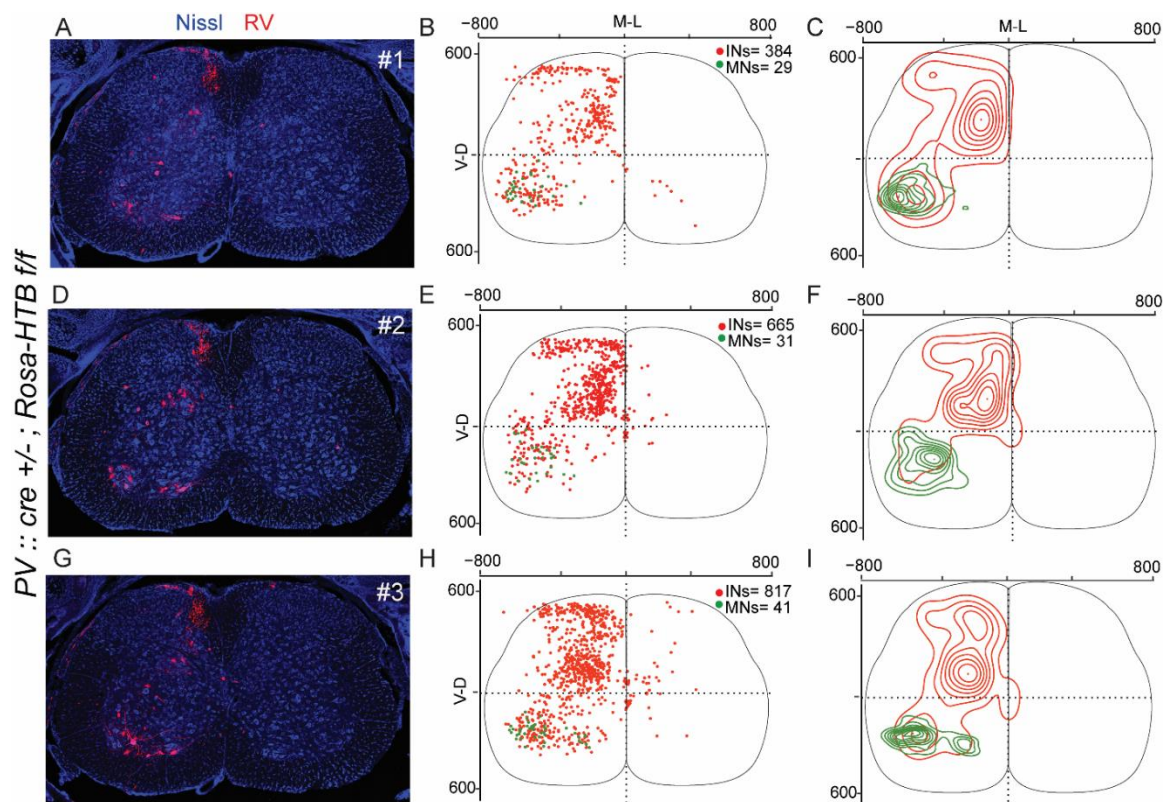

Figure 12. Three-dimensional analysis of RV+ cells in the spinal cord (P16). (A, D, G) Representative *Pv::cre; Rosa-HTB* injected spinal cord (n=3). (B, E, H) Digitally

reconstruction of RV+INs (red) and RV+ MNs (green) upon spinal rabies injection in *Pv::cre; Rosa-HTB* mice (n=3). (C, F, I) Transverse contour density plot of RV+ INs (red) and RV+ MNs (green).

Interestingly, to evaluate the "link" between SNs and INs/MNs in the spinal cord, we calculated the connectivity index (Fig.13 F). The ratio between the total number of labeled neurons in the spinal cord (INs; MNs RV+) over the number of the starter cells in the DRGs was consistent in each experiments (around 4-5). This data suggested that infecting one single SN in the DRG we can label 4/5 neurons in the spinal cord. Together, these results confirm that we were able to identify RV+ interneurons receiving proprioceptive information. Therefore, RV+ INs exhibit a highly reproducible distribution pattern that was consistent with the known trajectory and termination of proprioceptive afferents in the spinal cord. Moreover, they also confirmed that we used reliable and reproducible methods to label and map INs in the spinal cord linked with a specific modality.
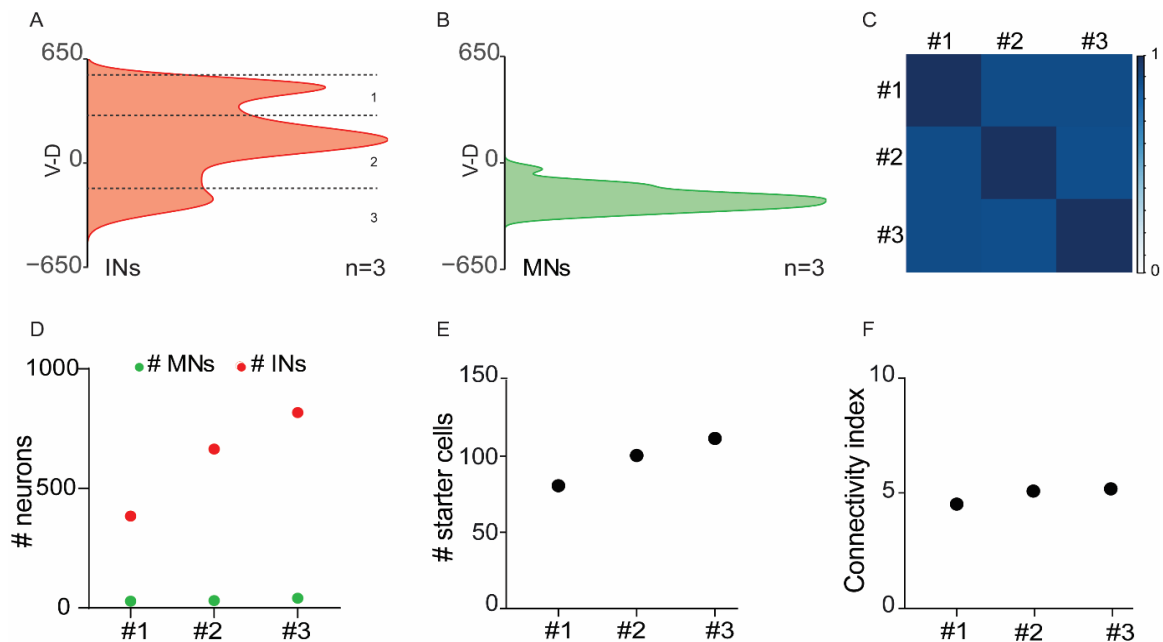
Figure 13. Distribution pattern of RV+ INs and MN in the spinal cord is reproducible. (A) Dorso-ventral distribution plot of RV+ INs (red) in *PV::cre; Rosa-HTB* spinal cord. (B) Dorso-ventral distribution plot of RV+ MNs (green) in *PV::cre; Rosa-HTB* spinal cord. (C) Correlation analysis of RV+ INs and MNs on the medio-lateral and dorso-ventral Cartesian coordinates in *PV::cre; Rosa-HTB* mice. D) Number of RV+ INs and RV+ MNs in *PV::cre; Rosa-HTB* injected mice. E) Number of starter cells in DRGs of *PV::cre; Rosa-HTB*

injected mice. F) Connectivity index: ratio between the total number of labeled neurons in the spinal cord (INs; MNs RV+) over the number of the starter cells in the DRGs.

5.8. Molecular identification of Interneurons involved in proprioception

We next examined whether it was feasible to delineate the molecular identity of sensory recipient INs infected with the rabies. Previous study (Zampieri et al., 2014) showed that after rabies injection into the GS muscle of *PV::cre+/-; RGT+/-* mice, different classes of INs were labeled: V2a, V1 and V0. Based on the literature (Zampieri et al., 2014), we analyzed the expression of Chx10, FoxP2 and Lhx1: transcription factors that mark a subpopulation of V2a, V1, V0 and dI4 interneuron subtypes at both embryonic and post-natal stages respectively. As expected, after RVΔG-mCherry/EnvA injection into the spinal cord of PV::cre+/-; Rosa-HTB f/f mice we detected mCherry expression in Chx10+ V2a interneurons ,FoxP2+ V1 interneurons and dorsal Lhx1+ interneurons (Fig.14). Thus, molecularly defined subpopulations of interneurons known to be involved in proprioception can be identified after Rabies spinal injection. The observation that we were able to identify and in the meantime confirmed our tracing, opens the way to characterize different classes of INs using antibodies against specific transcription factors.
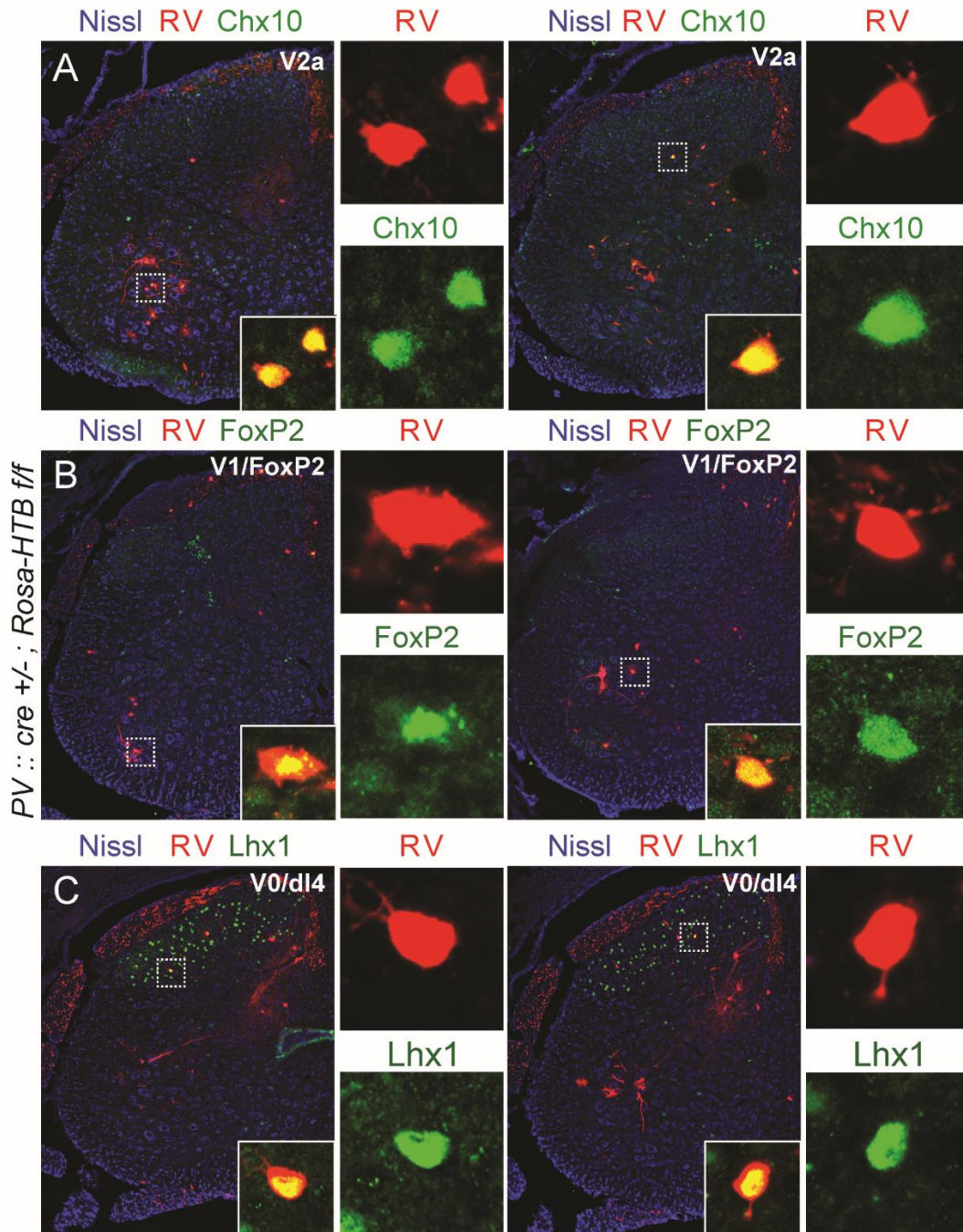
Figure 14. **Subclasses of V2a, V1, V0 and dI4 interneurons are monosynaptically connected to incoming proprioceptive afferents in the spinal cord.** A) Representative images of Chx10+/RV+ INs upon rabies spinal injection in *Pv::cre; Rosa- HTB* mice. B) Representative images of FoxP2+/RV+ INs upon rabies spinal injection in *Pv::cre; Rosa- HTB* mice. C) Representative images of Lhx1+/ RV+INs upon rabies spinal injection in *Pv::cre; Rosa- HTB mice*

As showed before, the infection of proprioceptive SNs upon rabies virus injection allowed us to trace MNs in the ventral spinal cord, directly connected with the sensory afferents. Thus, we performed a double-label immunohistochemistry using antibodies against mCherry and ChAT to detect specifically double positive MNs (Rv+ and ChAT+) (Fig.15).
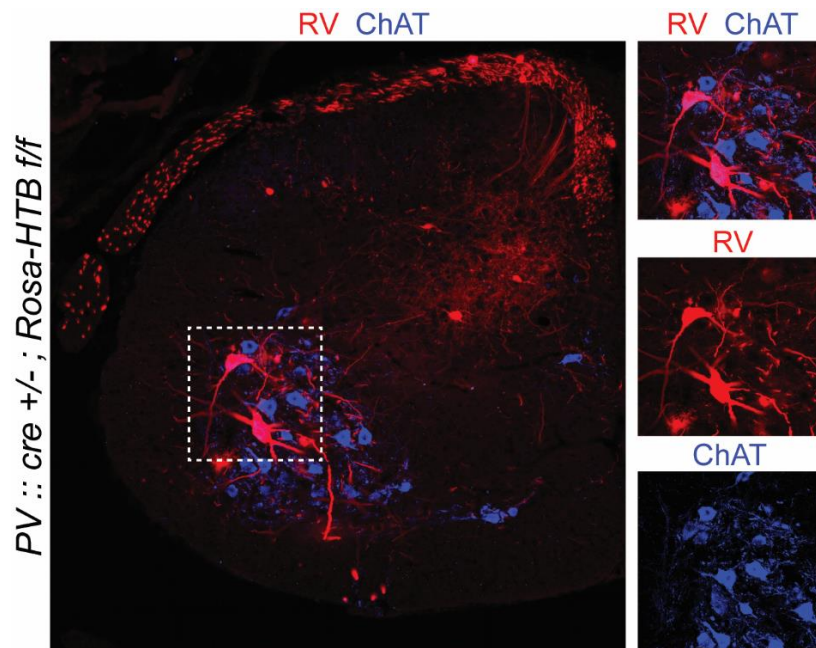
Figure 15. MNs in the spinal cord monosynaptically connected with proprioceptive sensory afferents in the spinal cord. Representative image of RV+ and ChAT+ MNs upon rabies spinal injection in *PV::cre; Rosa-HTB* mice.

To further confirmed the connection between MNs and proprioceptive afferents we checked on the MNs (ChAT+) surface the sensory terminal of the afferents (PV+) known to be also VGLUT1 +. To assess whether these boutons were in apposition to MNs, we used antibodies against mCherry, PV, VGLUT1 and ChAT (Fig.16 A-C). In all stained sections, mCherry / PV[+] terminals (yellow) were only seen scattered throughout the MNs infected with the rabies, while on the not infected MNs we did not detect double positive sensory terminals (only green) (Fig.16 Ba´-Bf´; Fig.16 Cg´-Ci´). Therefore, we observed that the majority of mCherry/ PV + terminals were also VGLUT1+ (Fig.16 Cg´´- Ci´´).
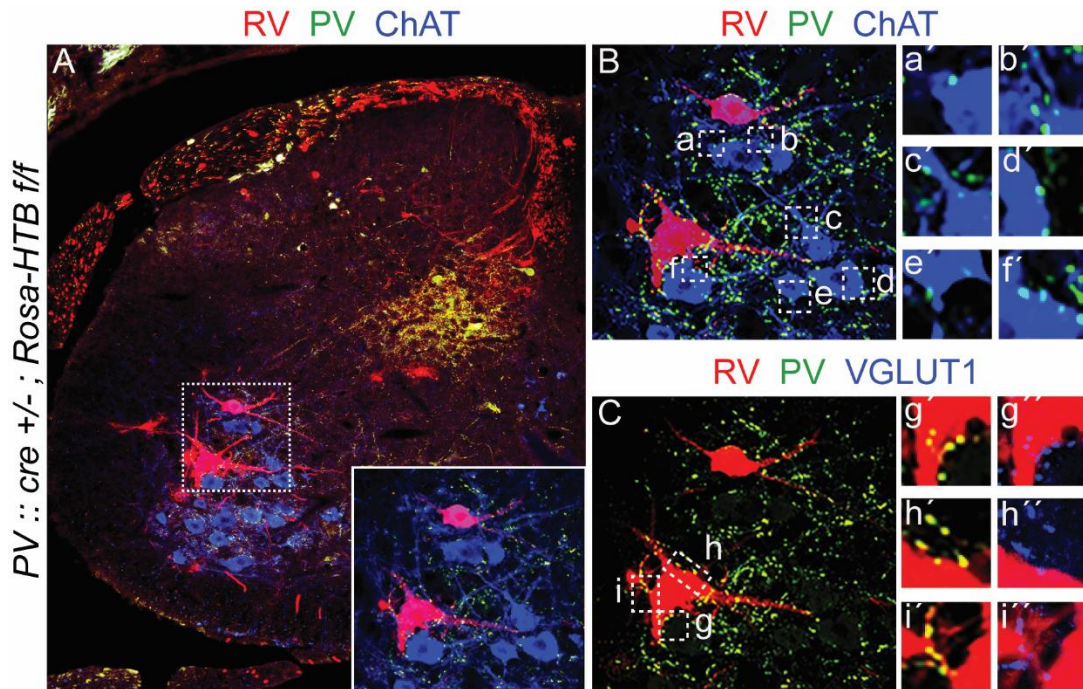
Figure 16. **Boutons on RV+MNs are PV+ and VGLUT1+.** Representative image of boutons on the RV+ and RV- MNs surface. A) Representative image RV+ MNs in the spinal cord upon viral injection. B) RV- MNs (ChAT+, blue) in the spinal cord contacted by proprioceptive sensory afferents (PV+, green only) not infected with the RV. C) RV+ MNs (ChAT+/RV+, purple) in the spinal cord contacted by proprioceptive sensory afferents (PV+/ RV+, yellow) infected with the RV. a, a´, b ,b´, c ,c´, d, d´, e, e´, f, f´,) Boutons on RV- MNs (blue) surface are PV+ and RV- . g, g´, i, i´,h, h´) Boutons on RV+ MNs (purple) surface are PV+ RV+ (yellow) and VGLUT1+.

These results showed that all mCherry+ MNs in the ventral horn of the spinal cord are monosynaptically connected with the PV+;mCherry+ sensory afferents. However, we detected also MNs in the same section not infected with the rabies that, as expected, showed only PV+ sensory afferents meaning that those afferents were not infected with the rabies that could not jump to those MNs. As previously shown, we observed mCherry+ INs distributed in lamina II of the dorsal spinal cord. Thus, we stained the sections with a specific lamina marker PKCγ which delineates the lamina II and as expected, we observed INs located in and below that lamina (Fig.17).
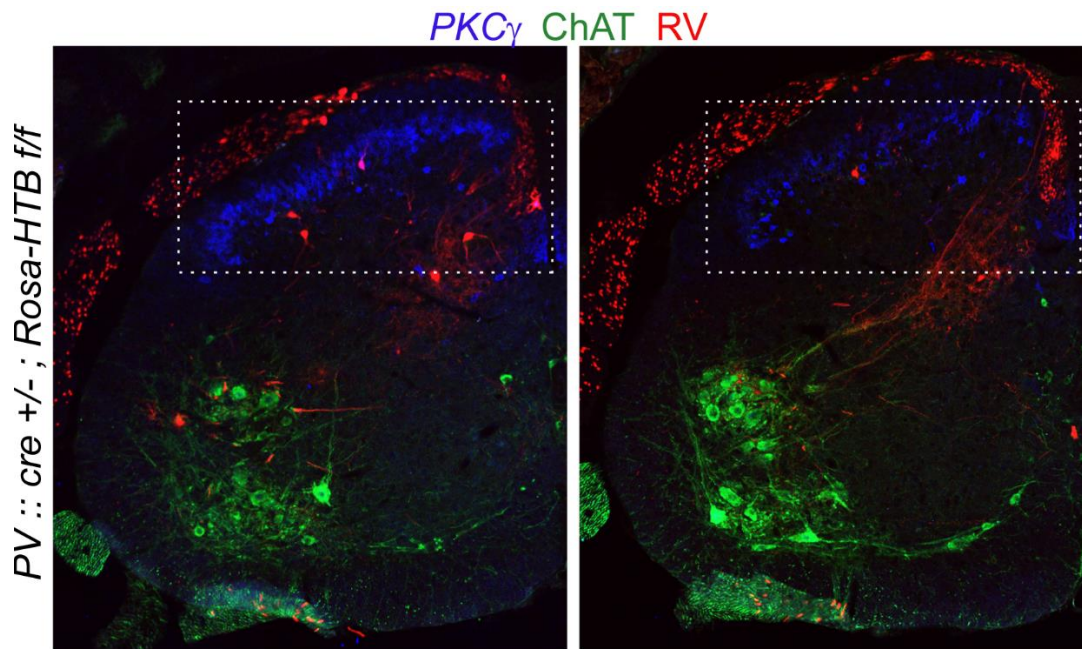
Figure 17. RV+ INs located in lamina II of the spinal cord. Representative images of spinal cord sections stained with lamina specific marker PKCγ (blue) and CHAT (green) showing RV+ INs (red) located in and below lamina II.

Next, to further investigate the molecular identity of labeled INs, we decided to stain for different transcription factors, Lbx1 and Calbindin D28k (CB), based on our positional analysis data. Lbx1 expressing INs in the spinal cord are dl4-dl6 interneurons, while CB + INs are located in the dorsal horn, near the central canal and predominately in Renshaw cells in the ventral spinal cord. Thus, we found some Lbx1+ ;mCherry+ INs (Fig.18), CB+;mCherry+ INs (Fig 19B) in the dorsal part of the spinal cord and mCherry+ Renshaw cells (Fig 19A).
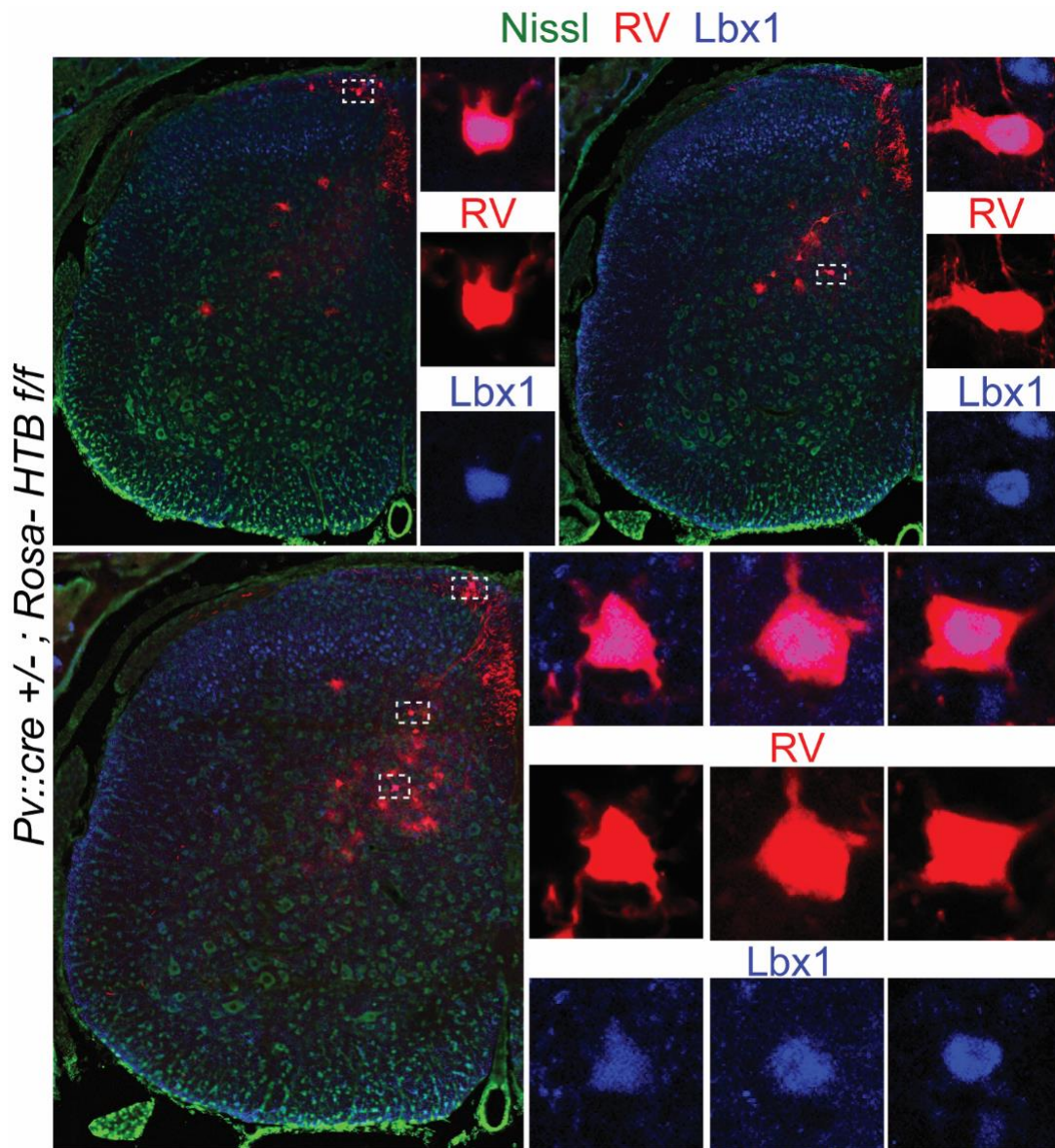
Figure 18. **dl4-dl6 interneurons monosynaptically connected with incoming proprioceptive afferents in the spinal cord.** Representative images of Lbx1+/ RV+ INs upon rabies spinal injection in *Pv::cre; Rosa- HTB* mice.
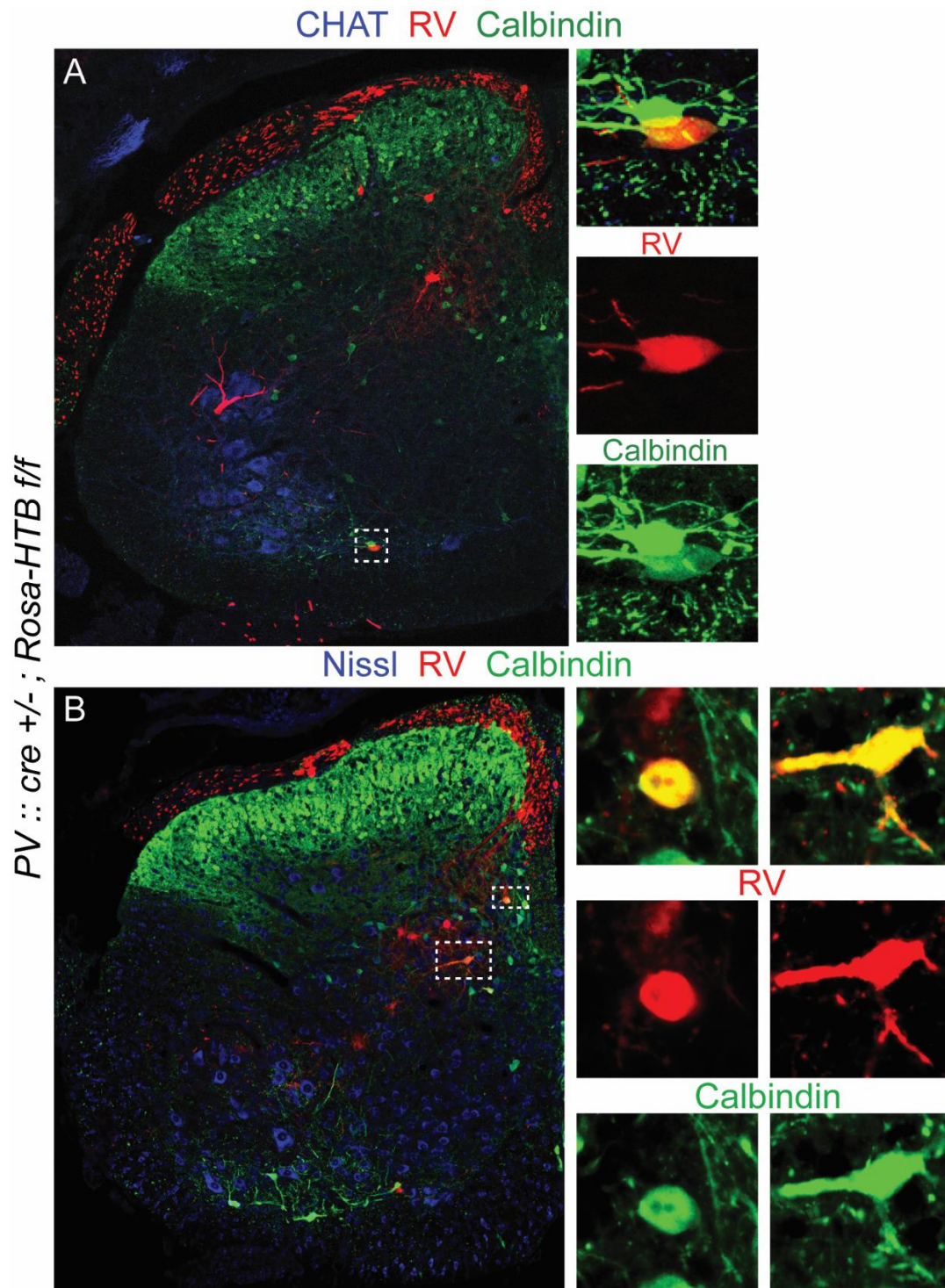
Figure 19. Calbidin+ interneurons and Renshaw cell monosynaptically connected with incoming proprioceptive afferents in the spinal cord. A) Representative images of Calbindin (green) +/RV+ INs upon rabies spinal injection in *Pv::cre; Rosa- HTB* mice. B) Representative images of Renshaw cell infected with the rabies spinal injection in *Pv::cre; Rosa- HTB* mice.

5.9. Specificity and efficiency of rabies primary infection of Trpv1+ sensory neurons

Next, following successful tracing from sensory neurons involved in proprioception, we asked whether it would be possible to trace from other sensory modalities focusing on noxious/thermal sensation. First, we looked on the specificity and efficiency of the primary infection in *Trpv1::cre ; Rosa-HTB* mouse line. To assess whether the primary infection of TRPV1+ sensory neurons was achieved after the injections, we analyzed lumbar/sacral DRGs of a *Trpv1::cre; Rosa-HTB* mice (n=3). As before, by following the expression of the nuclear GFP and the mCherry, we observed that 98% of RV+ (mCherry) cells were HTB+ (GFP) (Fig.20 B). Next, we examined the efficiency of primary infection observing that, in overall population of Trpv1+ SNs (GFP+), it was around the 20%. These findings, suggested that the rabies virus could infect specifically different types of sensory neurons of interest with a "variable" efficiency (Fig.20 C).
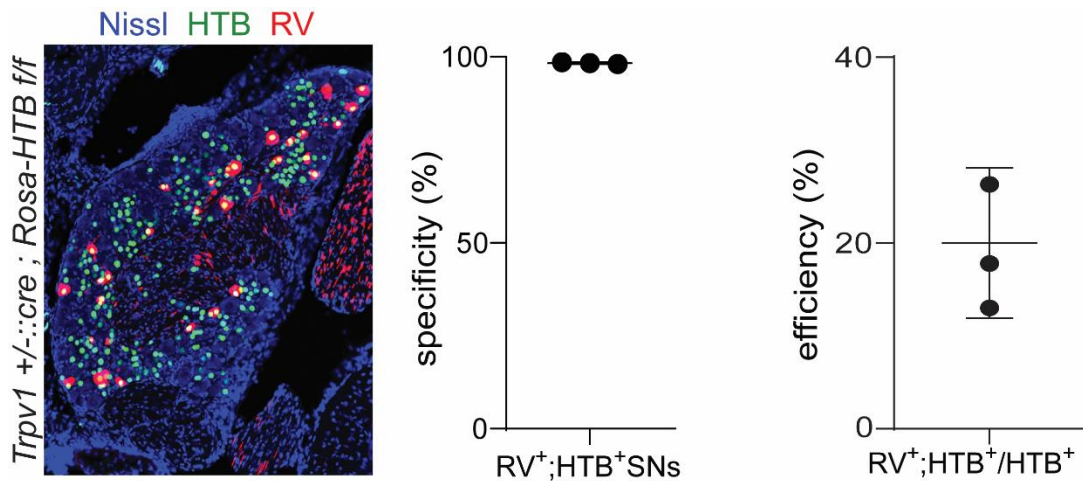
Figure 20. Specificity and efficiency of rabies primary infection in *Trpv1::cre;Rosa-HTB* DRGs. Lumbar and sacral sections of DRGs (30um) showed that the 98% of RV+ cells (red) were HTB+ (green) with an efficiency of infection of proprioceptive neurons around 20%.

5.10. Transsynaptic tracing of INs in the spinal cord involved in noxious/thermal sensation

As shown in our previous rabies tracing experiment, here we used the same strategy to identify the position of the INs in the spinal cord involved in noxious/thermal sensation. We injected the rabies virus unilaterally in the dorsal horn of the spinal cord (L1) where the Trpv1 + afferents terminate. By plotting the medio-later, dorso-ventral and rostro-caudal positions we investigated the organization of labeled spinal INs. We found that 92% of mCherry INs were mainly confined in the dorsal horn of the spinal cord and only the 7% were much more broadly distributed in the ventral part (Fig.21 B-H). This finding was suggested by the density plots (Fig. 21 C-I) of the interneurons in the spinal cord in each experiment, that takes in consideration where the majority of the INs are located. Furthermore, the distribution plot (Fig. 22 A) clearly underlines the presence of the dorsal INs confined in the superficial laminae of the spinal cord.
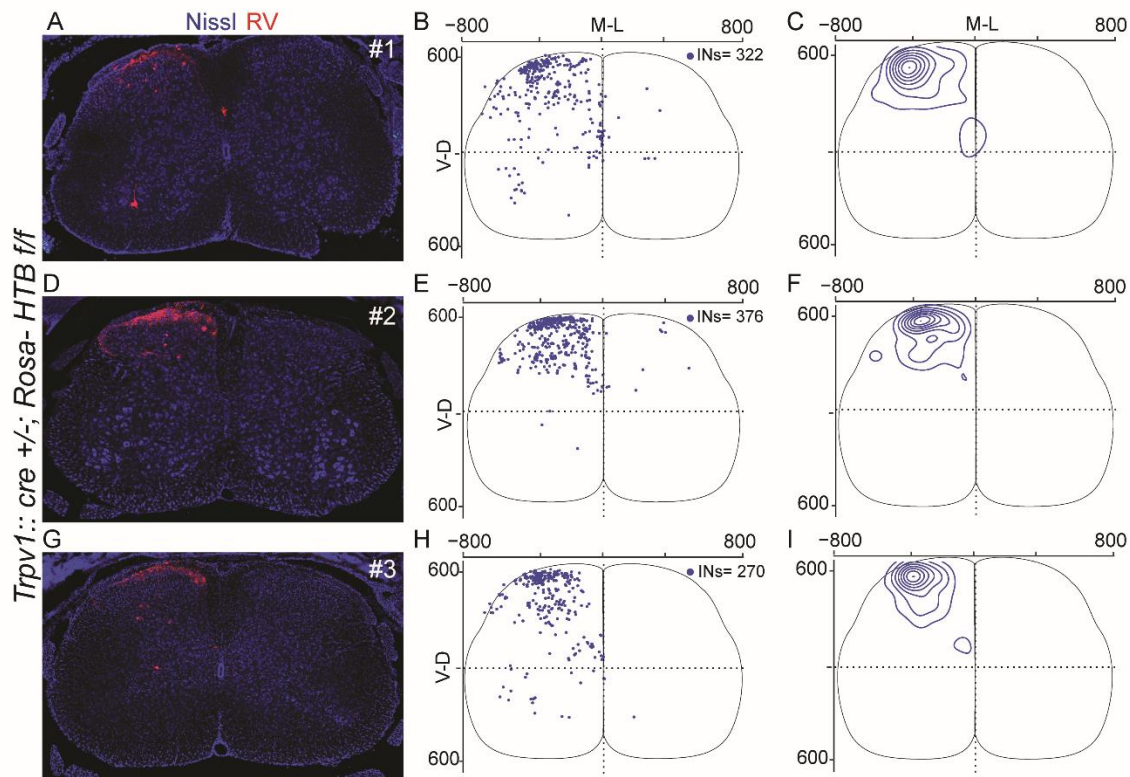
Figure 21.Three- dimensional analysis of RV+ cells in the spinal cord involved in noxious/heat sensation. (A, D, G) Representative *Trpv1::cre; Rosa-HTB* injected spinal

cord. (B, E, H) Digitally reconstruction of RV+ INs (blue) upon rabies spinal injection in *TRPV1::cre; Rosa-HTB* mice. (C, F, I) transverse contour density plot of RV+ INs (blue).

Consequently, when we compared individual experiments using correlation analysis we found that the distribution analysis were highly correlated (r>0.9) (Fig.22 B). Surprisingly, the values of the connectivity index dropped dramatically compare to the one observed in PV experiments. In *Trpv1::cre, Rosa-HTB* experiments the connectivity index was around 0.3 in average meaning that the ability of the rabies to spread from these sensory neurons is reduced (Fig. 22 D).
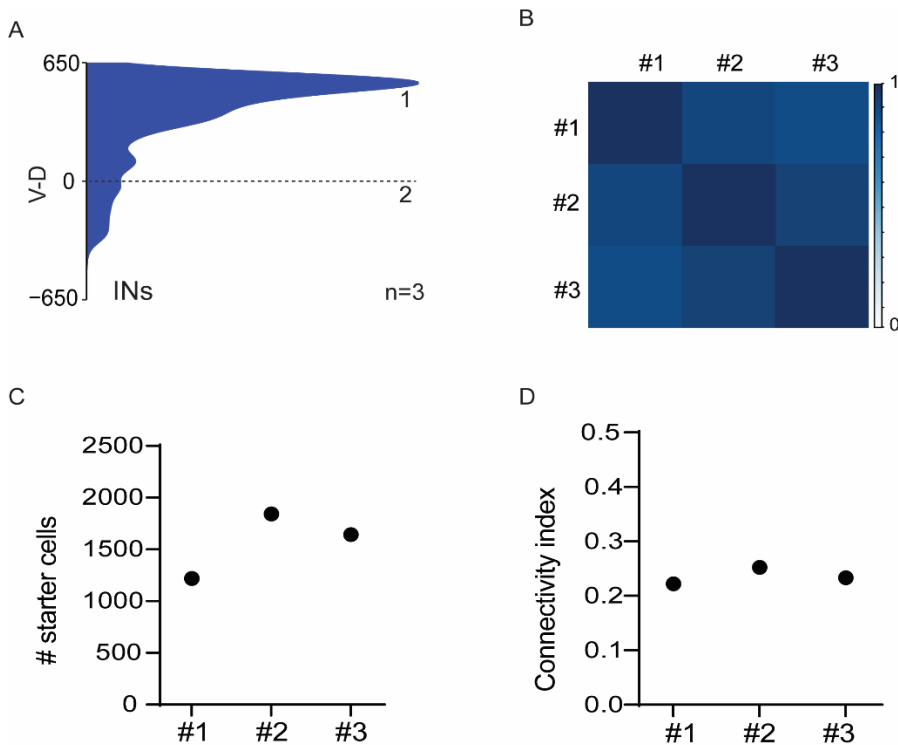
Figure 22. Dorsal distribution of RV+ INs involved in noxious/heat sensation. (A) Dorso-ventral distribution plot of RV+ INs (blue) in *Trpv1::cre; Rosa-HTB* spinal cords (n=3). (B) Correlation analysis of RV+ INs on the medio-lateral and dorso-ventral Cartesian coordinates in *Trpv1::cre; Rosa-HTB* injected mice. (C) Number of starter cells in DRGs of *Trpv1::cre; Rosa-HTB* injected mice. (D) Connectivity index: ratio between the total number of labeled INs in the spinal cord (RV+ INs) over the total number of the starter cells in the DRGs.

Together, these results showed that spinal injections of RVΔG-mCherry/EnvA in *Trpv1::cre; Rosa-HTB* mice reveal an overall different distribution of INs in the

spinal cord involved in noxious/thermal sensation, mainly located in the superficial laminae of the dorsal horn. However, the ability of the rabies virus to spread from those different sensory neurons is dramatically reduced indicating that the tracing might not cover the all the entire population of INs involved in this specific sensory modality.

5.11. Interneurons in the spinal cord encoding different sensory modalities

Next, to understand how the somatosensory circuits are organized in the spinal cord we decide to compare in more detail the distribution pattern of the INs involved in different modalities. From our previous results, we knew that the distribution of the INs involved in proprioception could be divide in three general populations: dorsal INs (Lamina II), intermediate INs and ventral INs. In contrast, in *Trpv1::cre; Rosa- HTB* mice we observed that the majority of the INs connected with the sensory afferents were distributed mainly in the dorsal horn of the spinal cord and in particular in the superficial laminae. These findings were confirmed by comparing the overall distribution of the INs on the medio-lateral and dorso-ventral axes (Fig. 23 C). Thus, we quantitatively assess the variability in overall INs distributions involved in proprioception and noxious/thermal sensation using the cross correlation. We found that the distribution of INs involved in proprioception do not correlate with the ones involved in noxious/thermal sensory modality (r<0.4) (Fig.23 D).
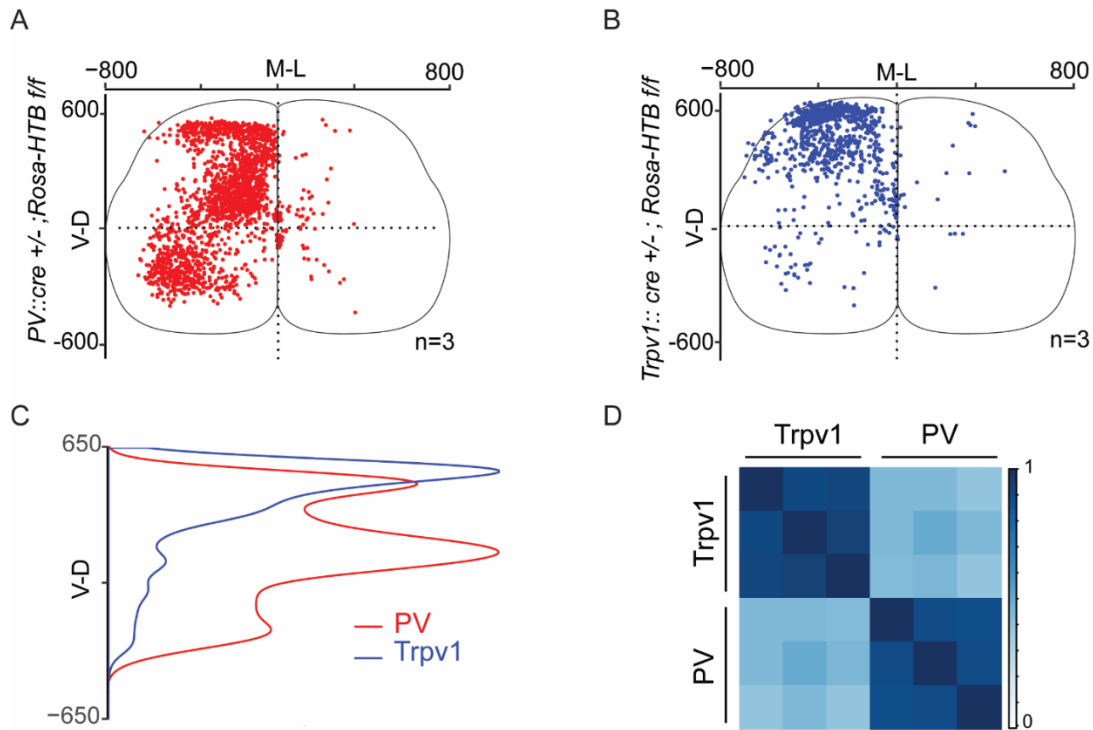
Figure 23. Different distribution pattern of labeled INs in the spinal cord involved in proprioception and noxious/heat sensation. (A) Digital reconstruction of the position of all INs labeled in *PV::cre; Rosa-HTB* injected spinal cord (n=3). (B) Digital reconstruction of the position of all INs labeled in *Trpv1::cre; Rosa-HTB* injected spinal cord (n=3). (C) Different distribution of the dorso-ventral axis of INs involved in proprioception (red) and noxious/heat sensation (blue). (D) Cross-correlation analysis comparing the distribution pattern of INs involved in proprioception and noxious/heat sensation.

5.12. V2a Interneurons are involved in noxious/heat sensation

Next, to further investigate whether these differences in the distribution of the INs involved in proprioception and heat/noxious sensation can be revealed also at the molecular level, we stained sections of *Trpv1: cre; Rosa-HTB* injected spinal cords with all the molecular markers used to identify INs involved in proprioception in the previous experiments (Fig. 14 A-C). We observed that all the INs involved in noxious/heat sensation were not Lhx1+/RV+ and FoxP2+/RV+ (data not shown). However, when we stained the *TRPV1::cre, Rosa-HTB* spinal cord with the antibody against V2a Chx10+ INs, we observed Chx10+/ RV+ cell (Fig.24). Together these results showed that only this population of INs (Chx10+) are

involved and monosynaptically connected with both sensory modalities: proprioception and noxious/heat sensation.
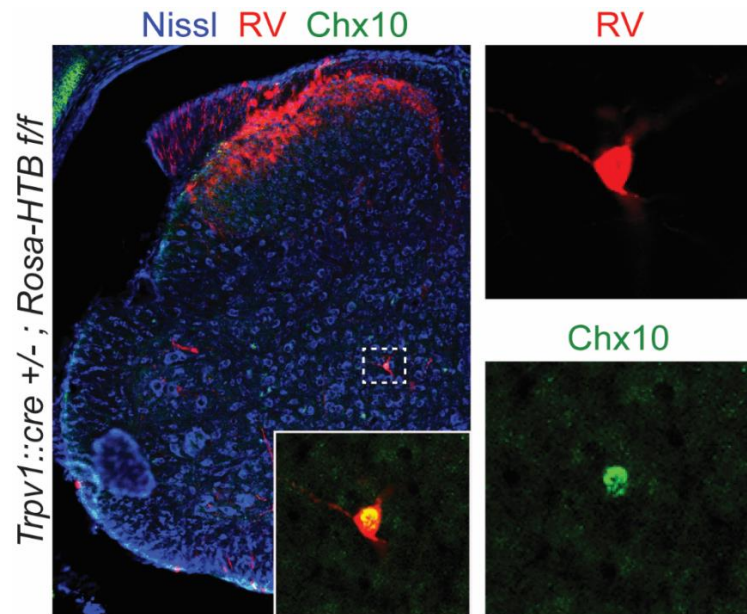
Figure 24. V2a Chx10+ INs monosynaptically connected to incoming trpv1+ afferents in the spinal cord. Representative image of Chx10+/RV+ INs upon rabies spinal injection in *Trpv1::cre; Rosa- HTB* mice.

5.13. Laminar distribution of INs involved in proprioception and noxious/heat sensation on the dorsal horn of the spinal cord

Next, we decided to have a closer look only on the superficial laminae of the dorsal horn. Therefore, we analyzed the INs distribution within the first two laminae of the spinal cord. Interestingly, we observed a specific laminar distribution of the INs involved in different sensory modality. We plotted the coordinates of labeled INs and PKCγ+ INs that delimitate specifically the lamina II of the spinal cord (Fig.25). We observed that noxious/thermal recipient labeled INs were located mainly in lamina I and lamina II while proprioceptive recipient labeled INs were in lamina II (Fig.25 B). As expected, the different laminae distribution was confirmed by the cross correlation analyses that results in r-value equal 0.45 (Fig. 25C)
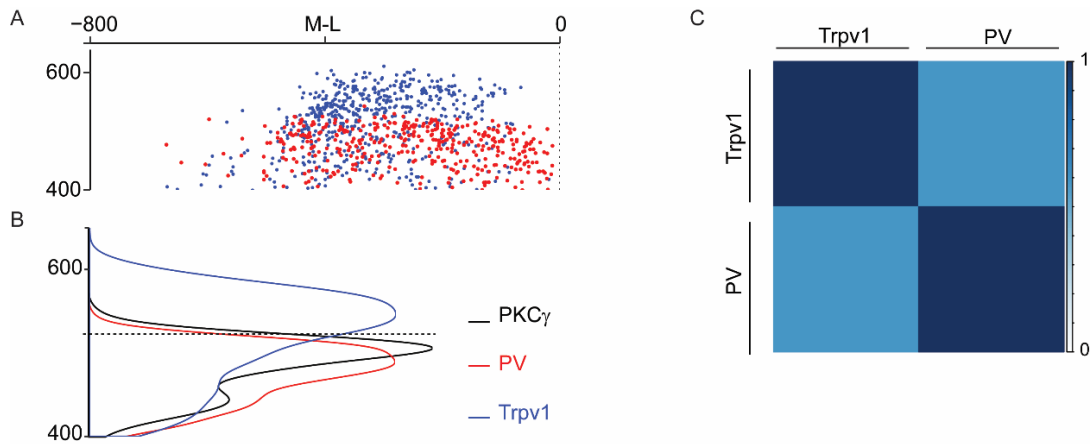
Figure 25. Distribution of labeled INs in the first two laminae of the dorsal horn. (A) Digitally reconstruction of RV+ INS involved in proprioception (red) and noxious/heat sensation (blue) in the first two laminae of the dorsal horn. (B) Dorso-ventral distribution of RV+ INs in the first two laminae of the dorsal horn. (C) Cross-correlation analysis of RV+ INs involved in proprioception and noxious/heat sensation located in the first two laminae of the dorsal horn.

In summary, the comparison of the INs distribution in the dorsal horn suggested the possibility of the existence of different populations of INs involved in decoding specific sensory modality in particular proprioception and noxious/heat sensation.

6. Discussion

The spinal cord plays an essential role in sensory processing. However little is known about the neuronal circuits that process sensory information, mainly due to the heterogeneity of the neural components that participate in those circuits (Sathyamurty et al., 2018). Sensory afferents synapse into spinal INs that send information to the MNs, which control motor behavior and to the brain where perception is formed. The precise logic of the sensory modalities in the periphery to encode distinct stimuli, implies an organized network of INs in the CNS. Yet, the underpinnings of such neural organization remain unclear. In this study, we depicted a strategy for defining INs in the spinal cord linked to a specific sensory modality.

6.1. Sensory projections in the spinal cord

Sensory neurons within the DRGs are highly heterogeneous with different physiological properties detecting distinct sensory information in the periphery (muscle and skin). The advent of the lineage tracing techniques has facilitated the characterization of SNs in the DRGs and their projection patterns in the CNS. The location of the interneurons contacted by different types of sensory fibers can be estimated from the area of projection of these fibers in the spinal cord. In order to elucidate how the somatosensory circuit is organized in the spinal cord, we first checked the reliability of our Cre mouse lines. We crossed *PV* and *Trpv1-cre* mouse lines with a reporter *Rosa-lsl-tdTomato* mouse line and checked the tdTomato expression of SNs in the DRGs and their projection pattern in the spinal cord. Our results, according with other studies, showed that proprioceptive sensory afferents enter the dorsal horn and terminate in the intermediate and ventral spinal cord, while the noxious/thermal afferents terminate in the dorsal laminae. Since very little overlapping in the projection pattern in the dorsal horn was seen, we conclude that these two cre mouse lines can be useful to characterize the sensory circuits in the spinal cord involved in proprioception and noxious/thermal sensation.

6.2. Rabies virus technique allows tracing INs in the spinal cord

Our work is based on the evidence that rabies virus infects primary sensory neurons from the peripheral axon and moves to the CNS via anterograde axonal transport. For this reason, we decided to use a different approach and instead to inject the RV in the periphery (Zampieri et al., 2014) we injected it directly in the spinal cord, where sensory afferents terminate. Others and we have used this approach to identify second order neurons (INs) in the spinal cord that receive input from specific sensory neurons. A comprehensive characterization of connections between DRG neurons and spinal neurons is crucial for our understanding of how different sensory modalities are processed. Rabies virus is considered as a useful tool in neuroscience for its natural ability of spreading between synaptically- connected neurons. Although, the mechanism of transsynaptic spread remains unclear. Apparently, in the CNS the rabies spread exclusively in a retrograde direction, whereas in the primary SNs it appears to be bidirectional (Wickersham et al., 2007). Here we established a reliable and efficient strategy to identify the connection of specific INs with sensory afferents in the CNS to study the organization of neuronal network decoding different stimuli. First, we have sought to address the question if the spinal cord injection is a suitable method to identify INs directly connected with sensory afferents compared to other strategies used. The (direct) injection into DRGs or in the periphery compared to our approach has many disadvantages; the surgery is more complicated and has a low success rate due to the fact that DRGs will be destroyed by the injection procedure and the RV would not infect the SNs. Another reason why we decided to proceed in this direction was the idea to cover more than one segments of the spinal cord to better understand how the somatosensory circuits are organized. Indeed, our results showed that performing one spinal injection leads to SNs labelling in 3 lumbar/sacral DRGs covering two segments in the spinal cord, avoiding multiple injections in different DRGs. Numerous INs were observed on the ipsilateral side of the point of the injection. Therefore, our current approach is very efficient in tracing INS within somatosensory circuits in the spinal cord. Furthermore, this tracing approach could prove to be useful in defining the identity

of a wide range of INs along the rostro caudal axis of the spinal cord, which are directly connected with sensory neurons defining a specific sensory modality.

6.3. Technical concerns: specificity and efficiency of RV primary infection in DRGs

To trace specifically from SNs of interest, we took advantage of the ability of the rabies virus to spread monosynaptically from sensory neurons to INs in the spinal cord. To allow RV to infect specific SNs in the DRGs and jump to INs in the spinal cord, we used mouse genetics to conditionally express histone-tagged GFP, TVA receptor and rabies glycoprotein G (Rosa-HTB: Yan Li et al., 2013) under the control of Cre-recombinase activity. We showed that in our tracing, the primary infection specificity from both SNs on interest was 92% (Pv) and 95% (TRPV1). However, the efficiency value varied from 12% to 20%. These results, suggest that the ability suggest that the ability of the rabies virus to infect is common to almost all sensory neurons independently from their developmental provenience. Therefore, the infection efficiency varies only and most likely due to different properties of SNs. It has been shown (Albisetti et al.,2017) that two classes of SNs, involved in nociception, display resistance to retrograde infection by rabies virus injected in the spinal cord. Despite this, it is known that *TRPV1::cre* mouse line not only represent thermosensitive SNs in the DRG but also cover a portion of SNs involved in nociception. Thus, taking in consideration the total number of TRPV1+ SNs in the DRG (almost the 80% of all SNs/DRG), the low efficiency infection is due to the inability of the rabies virus to infect those nociceptive SNs in *TRPV1::cre* mouse line. Nevertheless, comparing our results with previous work (Zhang et al., 2015), where they injected the RV in the plantar skin of P1 mice, we got higher efficiency of primary SNs infection in the DRGs by performing the injection directly into the spinal cord. This finding suggests that RV spinal injection do not limit the infection of sensory neurons as it happens with focal RV injection into skin or muscle (Zhang et al., 2015, Zampieri et al., 2014). Surprisingly, the value that caught our attention in our tracing experiments was the connectivity index. Why was it dramatically decreased in *TRPV1::cre; Rosa-lsl-HTB* mouse line compare to the *PV::cre, Rosa-lsl-HTB*? Interstingly, Albisetti et al., 2017, in the same work

previously mentioned, conclude that the capability of the rabies virus to infect specific SNs depend on their activity. Focusing on our SNs, we know that they have different degrees of myelination that effect the axonal velocity (PV+ SNs are myelinated while TRPV1 + SNs are unmyelinated) and they exhibit different levels of physiological activity. If Trpv1+ SNs are activated within a specific temperature range, in contrast PV+ SNs display a constant activity due to their role in sensing body position and contribution to locomotion. Based on that, we hypothesized that in our tracing experiments the main problem was not the capability of the rabies to infect the TRPV1+ SN but the capability to spread monosynaptically to INs in the spinal cord due by their activation "status". This hypothesis is strongly suggested by the idea that the rabies virus spreads via synaptic mechanism. All together, these findings raise the issue whether the RV spread differently from distinct SNs and if this capability is dictated by differences in their activation level. To address this matter and to better understand if all these aspects are involved in the capability of the rabies to spread across synapses, an option would be to activate continuously the SN of interest, in this case TRPV1 and check if the connectivity index value would change. All these observations indicate that the RV is not efficiently transferred transsynaptically from small diameter C and Aδ fibers. Nevertheless, the rabies transsynaptic tracing remains a powerful tool for identifying direct input-output to defined subsets of neurons.

6.4. Sensory-recipient interneurons involved in different sensory modalities revealed by RV transsynaptic spread

The projections of afferents in defined layers of the spinal cord provide access to distinct neuronal pathway in the CNS, where spinal interneurons represent the first relay station controlling the coding of sensory stimuli. Spinal circuits, involved in processing sensory information, contain a heterogeneous population of excitatory and inhibitory interneurons (Andrew J. Todd Nature Review 2010; Haring et al., 2018). Despite the importance of the spinal cord, as intermediator between the periphery and the brain, little is known about the organization of spinal circuits involved in the integration of distinct sensory information. In particular, two main theories have been popular for explaining how sensory information is encoded

and processed by the CSN. One, based on the observation that primary neurons in the periphery are specialized to detect specific sensory stimuli, implies that different sensory modalities are processed in parallel on fixed neural circuits, termed labeled lines (Ma Q. J Clin Invest. 2010, Knowlton WM et al., 2013). However, a second theory based on physiological studies states that the generation of somatosensory sensation involves a cross-talk between sensory lines at the level of the CNS. Nonetheless, we still know little about the anatomical and functional organization of neural circuits that link incoming primary afferents to second-order neurons in the spinal cord. To better understand which theory holds true for sensory perception and how networks of neurons mediate behaviour in reponse to incoming sensory stimuli, it is necessary to classify the various cell types in the spinal cord and identify which population of cells are involved in processing specific stimuli. Recent studies have indicated transsynaptic RV tracing as a general method for delineating the neuronal network in the CSN. Using quantitative methods (Dewitz et al., 2018), we assessed the 3D distribution of labeled interneurons. As proof of principle we focused, our initial analysis, on proprioception using the *PV::cre* mouse line, already well described in the literature, to target proprioceptive sensory neurons (Hippenmeyer et al., 2005) and we performed stereotactic rabies injections (RVDG-mCherry/EnvA) into the spinal cord of *PV::cre +/- ; Rosa-HTB f/f* mice. We obtained highly reproducible distribution patterns consistent with the known trajectory and termination of proprioceptive afferents in the spinal cord. We observed a subset of virally labeled interneurons in lamina II, which are involved in modulation and transmission of nociceptive and mechanosensory information. Moreover, we identified interneurons in intermediated areas and motor neurons in the ventral areas of the spinal cord directly connected with the proprioceptive afferents. In contrast when we analyzed the position of the INs involved in noxious/thermal sensation we observed that the majority of them where located in the superficial laminae of the dorsal horn. The comparison between those sensory modalities showed a distinct laminar distribution pattern of second order neurons involved in proprioception and noxious/thermal sensation reflecting the specific termination pattern of the sensory afferents in the spinal cord. Interestingly, we observed that a specific population

of INs located in lamina I do not encode proprioceptive information. However, regarding this statement further experiments need to be done on the electrophysiological level to prove firmly that those INs respond only after specific stimulation e.g. by using capsaicin, a compound that specifically activate only TRPV1+ SN+.

6.5. Characterization of classes of INs involved in proprioception upon viral injection into the spinal cord

Following successful tracing from sensory neurons involved in proprioception, we started to characterize the molecular identity of the INs directly connected with this sensory modality in the spinal cord. Previous studies (Fink et al., 2014; Betley et al., 2009; Koch et al., 2017; Hilde et al., 2016; Bourane et al., 2015; Bui et al., 2013) have genetically identified already classes of INs in the spinal cord involved proprioception such as: GADA2+, RoRbeta+, Satb2+, RoRalpha+ INs and dl3 INs. For example, RoR beta+ inhibitory INs are required to suppress the sensory transmission pathway that activate flexor motor reflexes, a mechanism controlled by proprioceptive afferents (Koch et al., 2017) . Satb2+ interneurons receiving monosynaptic input from proprioceptive neurons (Hilde et al., 2016) and are involved in specific motor and sensory-evoked behaviors including proper limb position during runway walking. First, we used immunoistochemistry and known markers for already described IN populations (V2a, V0 and V1) to confirm our rabies tracing from proprioceptive SNs (Zampieri et al., 2014). We were able to detect positive V2a, V0 and V1 FoxP1+ INs and MNs. The positions of identified INs were in alignment with labelled afferents of a *PV::cre-Rosa-lsl-tdTom*ato in the spinal cord suggesting that we successfully traced INs involved in proprioception. Furthermore, we identified other classes (Lbx1+ and CB+ INs) of interneurons with a biased approach using different antibodies for specific transcription factors, based on the positional analysis. However, it cannot be used to have a complete overview of all INs due to lack of knowledge of neuronal cell types in adult spinal cord. Therefore, to understand how neural circuits generate behavior, it is necessary to identify the neuronal cell types within a circuit using a different approach that provides higher resolution of cellular differences. Neuronal cell

types reflect a combination of parameters including cell body location, dendritic morphology, axonal projection, gene expression pattern and function. Ideally, these parameters should define a unique entity. An important advance is the development of single-cell RNA sequencing techniques, where messenger RNAs expressed by individual cells can be quantified. This method published the first time in 2009 (Tang et al. 2009) measures the distribution of expression level of each gene across a population of cells and offers unique opportunities to dissect the differences between cell and their environment or neighboring cells. Hence, comparison of gene expression profiles between neurons should identify key molecular components that specify their distinct functions. Therefore, single-cell RNA sequencing is suitable to provide unbiased characterization of single cell. Recently, 43 neural populations that contribute to spinal sensory-motor circuits have been identified (Sathyamurthy et al., 2018) using a parallel single nucleus RNA-seq approach. Although, one major limitation in this study was the detection of a low number of genes due to the low amount of RNA in the nucleus compared with the whole cell. Taking in consideration this aspect, we plan to perform the single-cell RNA seq instead to use the nucleus RNA seq approach to identify at the molecular level INs receiving mono-synaptic input from specific sensory modality. Single cells, sorted from a dissociated cell suspension using fluorescence activated cell sorting, will be placed into individual wells containing barcodes and transcriptome profiling of individual cells will be achieved following the sequencing steps (Fig. 26). The key is to minimize debris because the amount of "junk" in a sample can often severely limit the purity, efficiency, and even viability of a sort. Based on that we will take advance of the property of different rabies virus: RVH2BdGmCherry/EnvA. This virus has the same ability to infect the SNs in the DRGs and move to the CNS with the only difference that H2B-mCherry, the fluorescent protein is transported into the nucleus instead to be present in the whole cell. However, we still do not know the consequence of the rabies infection on the transcriptome of the cell and if could represent a limitation in our experiments. Rabies viral vectors used for retrograde targeting and monosynaptic tracing have a single gene "G", encoding the viral glycoprotein, deleted from its genome with all the other viral genes left intact. This deletion prevents the virus

from spreading beyond initially infected cells, but it does not prevent the replication of the virus. Therefore, in the spinal cord, the rabies virus can replicates its core to high copy number and express high level of fluorescent protein (mCherry). This property can be extremely useful for such application as imaging of neural circuits showing precisely the axons, dendrites and the soma of the cells but it also results in cytoxicity with death of infected neurons mainly after 2 weeks (Chatterjee et al., 2018). However, we do not know how all these aspects will affect the analysis of the transcriptome of single cells. In conclusion, single cell transcriptional profiling has the potential to reveal unprecedented knowledge about different cell types involved in decoding specific sensory modality and it may facilitate the understanding on how the somatosensory system is organized in the spinal cord.
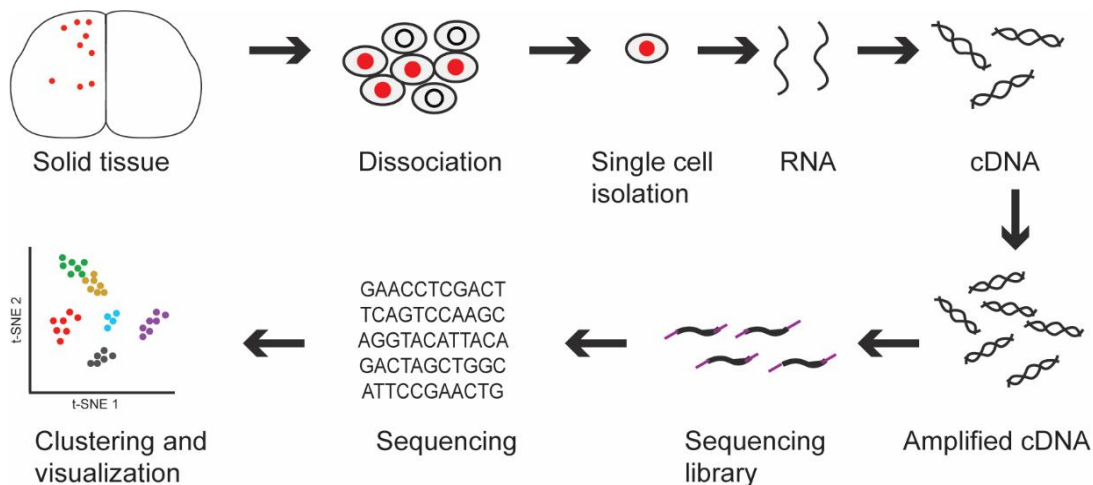
Figure 26.Single cell RNA –sequencing workflow. Solid tissue (injected spinal cord) is dissociated to obtain single cells. Single cells are sorted using fluorescence activated cell sorting. Amplification of the RNAs within each single cell. Library preparation. Analysis of the clusters.

6.6. Outlook

Stimuli from different types of sensory neurons in the periphery that monitor the status of our body in a specific environment, converge onto spinal neurons, which are thought to serve as a local hub for integrating multiple sensory modalities. The organization of these local circuits and its cellular composition, capable to generate appropriate behavioral responses is not well understood. One great achievement in sensory biology was the identification of different receptors and

channels in the periphery that respond to specific sensory stimuli. However, there is an unsolved question: how sensory stimuli are encoded in the spinal cord? In the periphery, different stimuli are selectively processed along specific labeled lines. In contrast, in the spinal cord these labeled lines are not exclusively independent. However, the selectivity of sensory modality and precise responses to stimuli imply a precise logic to the engagement of second-order recipient neurons in the spinal cord. In this work, we want to point attention on the existence of specific laminar distribution of INs involved in distinct sensory modality without deny the presence of common classes of INs that encode the convergence of different stimuli as for example V2a Chx10+ INs. However, further experiments need to be done to confirm our hypothesis. We strongly believe that identifying INs on a molecular level after tracing from defined SNs will give the opportunity to better understand the organization of somatosensory circuits within the spinal cord.

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8.1. Acknowledgments

Here I am. Phew, this is probably the most difficult part for me, as I am not very good at expressing my feelings. However, let us start from the beginning: my PhD! I spent my last 5 years doing what I really want to do in my life: Science. I can be honest, there were days where I was sad, nights where I did not even sleep thinking about experiments but I can assure you that there were also glorious moments that wiped out all the rest! I enjoyed all of them but mostly the "sad" ones (don t call me crazy) because there I found the strength to keep going and those days remembered me why I started! Turning back, I can see all the people that supported me during this experience and now time arrived to thank all of them. First, I want to acknowledge all the people that have helped me in carrying out the research presented in this thesis. Therefore, my first thank you has to go to Dr. Niccolo Zampieri, who gave me the possibility to join his lab where I got the chance to grow both professionally and personally. I learnt from him that "hard work always pay off" and that is extremely true! Next, a big thank you goes to all the current members of the lab and in particular to Stephan, my "mate". He is an authentic person. He knows me better than all the others do. It was always nice talk with him about science, discuss and do experiments together. You, Stephan, made me always happy and I enjoyed all the days in the lab since you came, because we were a "team"! Thank you! I would like to thank Nino, Pierre-Louis, Carlos, Elias, Serena, Francesco, Alessandra, Luca, Claudia, Luigi, Pasquale, Halai and Alessandro for their support. I also want to thank an incredible person, a friend that I met during my PhD.He has a great mind, who genuinely loves what he is doing. You need only to look in his eyes when he is talking or even drawing scientific ideas to understand how much passionate he is about! I am glad to have met someone like him, because he changed completely my life and he teaching me that "you have to keep going"! I want to express my gratitude to my former mentors, Dr. Paul Heppenstall and of course Laura Batti. Paul, in your lab I felt as be home and you have shown me that curiosity and passion are always important if you want to be a scientist. No matter what, things can go wrong but in the end of the day, you are in "peace" with yourself because you have done everything in your

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8.2. Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit mit dem Titel "Characterization of neuronal circuits processing specific sensory modalities in the spinal cord" selbstständig und ohne Hilfe Dritter angefertigt habe. Sämtliche Hilfsmittel, Hilfen sowie Literaturquellen sind als solche kenntlich gemacht. Außerdem erkläre ich hiermit, dass ich mich nicht anderweitig um einen entsprechenden Doktorgrad beworben habe. Die Promotionsordnung des Fachbereichs Biologie, Chemie und Pharmazie der Freien Universität Berlin habe ich gelesen und akzeptiert.

Berlin, March 2020
Sofia Pimpinella

8.3. Curriculum Vitae

For reasons of data protection, the curriculum vitae is not included in the online version.

For reasons of data protection, the curriculum vitae is not included in the online version.

For reasons of data protection, the curriculum vitae is not included in the online version.

8.4. Publications

2018:

Nuclear Organization in the Spinal Cord Depends on Motor Neuron Lamination Orchestrated by Catenin and Afadin Function. Dewitz C.*, **Pimpinella S.***, Hackel P., Akalin A., Jessell T.M. and Niccolo Zampieri. (2018). *Cell Rep*.22, 1681-1694

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