

## Review article

## Exploring propriospinal neuron-mediated neural circuit plasticity using recombinant viruses after spinal cord injury

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## ABSTRACT

Propriospinal neurons (PSNs) play a crucial role in motor control and sensory processing and contribute to plastic reorganization of spinal circuits responsible for recovery from spinal cord injury (SCI). Due to their scattered distribution and various intersegmental projection patterns, it is challenging to dissect the function of PSNs within the neuronal network. New genetically encoded tools, particularly cell-type-specific transgene expression methods using recombinant viral vectors combined with other genetic, pharmacologic, and optogenetic approaches, have enormous potential for visualizing PSNs in the neuronal circuits and monitoring and manipulating their activity. Furthermore, recombinant viral tools have been utilized to promote the intrinsic regenerative capacities of PSNs, towards manipulating the 'hostile' microenvironment for improving functional regeneration of PSNs. Here we summarize the latest development in this fast-moving field and provide a perspective for using this technology to dissect PSN physiological role in contributing to recovery of function after SCI.

## 1. Introduction

It is now well known that the spinal cord includes a population of interneurons with vast diversity, complexity and morphologic heterogeneity known as the propriospinal system, which serves to integrate and modulate ascending and descending signals (Alstermark et al.,

2007; Sherrington, 1885; Jankowska, 1992). Propriospinal neurons (PSNs) are involved in diverse physiological functions, ranging from respiratory and gait control (Dobbins and Feldman, 1994; Grillner, 2003) to the general synchronization of motor circuits, evidenced by electrophysiological results (Lipski and Duffin, 1986). Neural plasticity mediates spontaneous functional recovery after spinal cord injury (SCI),

**Abbreviations:** AAV, adeno-associated virus; AAV-6-G, AAV serotype 6; BF, biceps femoris; AC, adenylate cyclase; ATP, adenosine triphosphate; cAMP, Cyclic Adenosine Monophosphate; CAV, canine adenovirus; ChR2, Channelrhodopsin-2; CLARITY, a tissue-clearing protocol; CMV, cytomegalovirus; CNS, central nervous system; CNTF, ciliary-derived neurotrophic factor; CPGs, central pattern generators; CREB, cAMP-response element-binding protein; CST, corticospinal tract; DREADDs, Designer receptors exclusively activated by designer drugs; DTR, diphtheria toxin receptor; DTX, diphtheria toxin; eGFP, enhanced green fluorescent protein; eIN, excitatory interneurons; EMG, Electromyography; EnvA, envelope pseudotype; eTeNT, enhanced tetanus neurotoxin light chain; GCaMP3, a synthetic fusion of green fluorescent protein (GFP) calmodulin (CaM) and M13 a peptide sequence from myosin light-chain kinase; GDNF, Glial cell line-derived neurotrophic factor; GFP, Green fluorescence protein; GL, glutens; G protein, glycoprotein; GS, gastrocnemius; HiRet, highly efficient retrograde gene transfer; HIV, human immunodeficiency virus; hM3Dq, an engineered human muscarinic receptor; hM4Di, a modified form of the human M4 muscarinic (hM4) receptor; HRP, horseradish peroxidase; IGF1, insulin-like growth factor 1; IL, iliopsoas; KCC2, the potassium-chloride co-transporter; KLF7, Kruppel-like factor 7; LRN, lateral reticular nucleus; LAPN, long ascending projecting PSNs; LDPT, long descending propriospinal tract; MRF, medullary reticular formation; NGF, nerve growth factor; NPCs, neural progenitor cells; NSCs, neural stem cells; OIC, osteopontin, insulin-like growth factor 1, ciliary-derived neurotrophic factor; OPN, osteopontin; PD, progenitor domain; PMNs, phrenic motor neurons; PRV, pseudorabies virus; PSNs, Propriospinal neurons; PTEN, phosphatase and tensin homologue; RABV, rabies virus; RABVΔG, a glycoprotein gene-deleted pseudotyped recombinant rabies virus; RAGs, regeneration-associated genes; rtTA, reverse tetracycline-controlled transactivator; SCI, spinal cord injury; sTPSN, short thoracic propriospinal neuron; TA, tibialis anterior; TetON, an inducible tetracycline sequence; TFs, transcription factors; TRE, tetracycline-responsive element; TrkA, tropomyosin receptor kinase A; TVA, receptor of the avian sarcoma leucosis virus subgroup A; VL, vastus lateralis; VSV-G, vesicular stomatitis virus glycoprotein.

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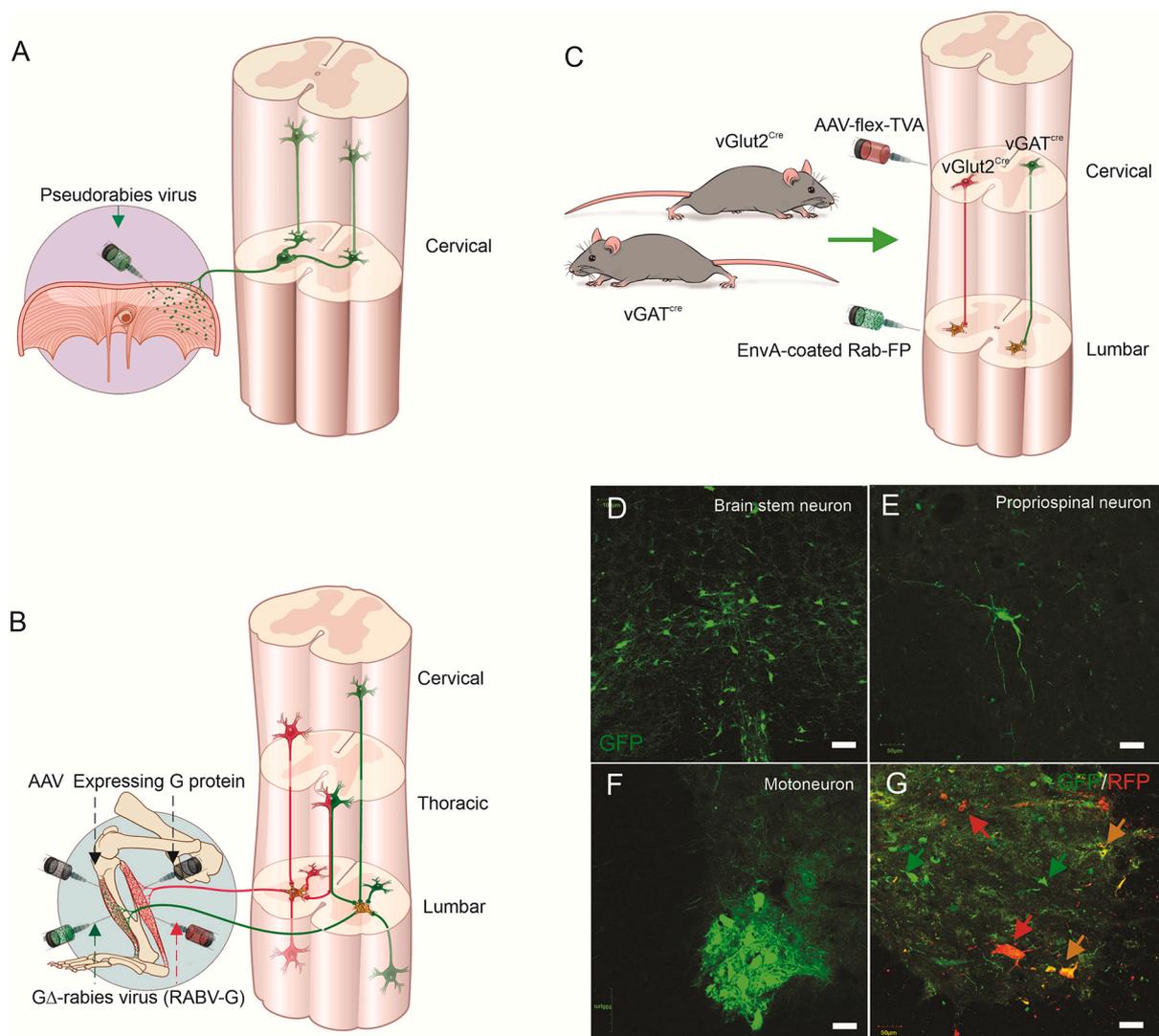
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and recent findings suggest that PSNs significantly contribute to this functional recovery (Filli and Schwab, 2015; Cote et al., 2012; Filli et al., 2014; Hou et al., 2008; Courtine et al., 2008; Bareyre et al., 2004). However, for multiple reasons it is challenging to functionally dissect the PSNs in the circuits upon which they act. Firstly, the spinal cord contains many types of PSNs, which can be categorized into various classes according to anatomical, physiological, and molecular criteria (Flynn et al., 2011). This diversity is heavily driven by the complex flux of embryonic transcription factors and neurotransmitters (Jensen et al., 2019; Zhouludeva et al., 2018). In addition, PSNs are arranged into complex projection patterns while scatteredly distributed within a hierarchical neural circuit, and the somata can be far away from the targeted axons (Szentagothai, 1964). Anatomically, PSNs are classified as either “short” or “long” PSNs based on the distances of their axonal projections (Cowley et al., 2010). In this review, we consider short PSN axons to be those spanning less than six spinal segments, whereas long PSN axons project farther than six spinal segments (Flynn et al., 2011).

Finally, different subpopulations of PSNs may respond differently to the lesion environment and therapeutic manipulations. In Siebert’s 2010 study, long descending cervical propriospinal tract (LDPT) neurons differ from short thoracic propriospinal neurons (sTPSN) in response to low thoracic spinal injury (Siebert et al., 2010a). LDPT neurons lacked a post-axotomy retrograde cell death, while sTPSN neurons displayed a strong early cell death response. The more significant post-injury response observed in the TPSN was likely the result of the axotomy occurring closer to the cell body of the neuron. However, this study did not exclude the possibility that the distinctive protective effect was due to the phenotypic differences. The different genetic expression related to apoptosis, neuroprotection, and generation exists between uninjured LDPT and sTPSN neurons (Siebert et al., 2010a).

Recently, genetically encoded molecular tools for visualizing and perturbing neuronal circuits, in combination with cell-type-selective gene delivery approaches, have been dramatically expanding the capabilities for analysis of neural circuits, especially with the advent of viral



**Fig. 1. Three-dimensional topography of propriospinal neurons.** (A) Pseudorabies virus was injected into the diaphragm to trans-multi-synaptically label a phrenic motoneuron and the sequential orders of PSNs. (B) Intramuscular injections of  $\Delta$ G protein Rab-GFP and AAV-G protein into tibialis anterior muscle and  $\Delta$ G protein Rab-mCherry and AAV-G protein into right soleus muscle (two antagonist muscles) to label the premotor PSN. (C) To assess relative abundance and position of cervico-lumbar projection neurons with different neurotransmitters, AAV-flex-TVA and AAV-flex-G was injected in the cervical spinal cord of vGlut2<sup>Cre</sup> and vGAT<sup>Cre</sup> mice, followed by lumbar axonal infection with EnvA-coated rabies virus expressing fluorescence proteins (Rab-FP) to specifically target neurons with lumbar projections. (D-F) Pseudorabies virus transfected brainstem neurons (D), propriospinal neuron (E) and motoneurons (F) that express GFP. (G) PSNs with unilateral axonal projections were labeled by single color (red, green arrows); PSNs with bilateral axonal projections were labeled by dual colors (brown arrows). Scale bars: D, F 100  $\mu$ m; E, G 50  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tools (Kobayashi et al., 2018; Ginger et al., 2013; Parr-Brownlie et al., 2015). This methodology of using recombinant viruses, such as rabies virus (RABV), adeno-associated virus (AAV), and lentivirus, can be readily combined with the expression of a variety of genetic, pharmacologic, and optogenetic methods to functionally dissect and map propriospinal circuits, before and after injury, thus contributing to the ongoing, integrative studies of intraspinal neuronal circuitry (Sparta et al., 2013). In this review, we summarize some of the most recent studies utilizing recombinant viral tools to functionally dissect and map the PSNs that are responsible for different physiological functions and the mediation of functional recovery after SCI.

## 2. Three-dimensional topography of propriospinal neurons

### 2.1. Revealing propriospinal synaptic connectivity in a hierarchical neuronal pathway

Both pseudorabies virus (PRV) and rabies virus (RABV) can cross synaptic terminals in a retrograde direction and thereby spread efficiently within the peripheral nervous and the central nervous system (CNS) (Fig. 1 A, D, E, F). This synaptic transmissibility provides the possibility of using PRV- or RABV-based viral tools to trace neuronal circuitry (Lane et al., 2008). The limitation of PRV is that it is a polysynaptic tracer, and the widely labeled PSN network cannot be accurately distinguished by their hierarchical orders in the circuit (Fig. 1 A) (Callaway, 2008). After PRV is injected into the terminal area of the neuronal circuit, such as the respectively innervated muscle, the order of neurons labeled in a hierarchical neuronal pathway is roughly estimated by the post-injection time window. It usually took 40 h to label the ipsilateral, premotor interneurons after the initial virus injection to the muscle (Dobbins and Feldman, 1994; Jovanovic et al., 2010).

RABV-derived vectors have a broad range of host species making them suitable for use in a wide range of animal model systems (Ginger et al., 2013). Yet there are some limitations for RABV. The RABV genome never encompasses a DNA form and is confined to the cytoplasm which cannot integrate into the host genome and activate the transcription of host genes. The most important factor limiting its cloning capacity is probably the number of transcription initiation start sites introduced with the exogenous genes (Ghanem et al., 2012). DNA viruses, such as PRV, can readily express the encoded genetic tools such as lox P sites, tet-regulatory sequences, or cell-specific promoters through conditionally manipulating the interaction of the viral genome and the recombinase expressed in transgenic mouse lines. RABV, however, as a negative-sense single-stranded RNA does not have a double-stranded DNA binding site which limits its application of these genetic tools (Osakada et al., 2011; Wu and Rupprecht, 2008). Recent study has optimized the recovery condition to develop new RABV variants expressing useful neuroscience tools, including the Ca<sup>2+</sup> indicator GCaMP3 for monitoring activity; Channelrhodopsin-2 for photo-activation; allatostatin receptor for inactivation by ligand application; and rtTA, ERT2CreERT2, or FLPo, for control of gene expression (Osakada et al., 2011). Neurotoxicity is another limitation for the application of PRV and RABV. The basic properties of infected neurons are not altered by infection after 7 days but that over ninety nine percentages of infected neurons are killed by 3 weeks (Wickersham et al., 2007a). One should consider this working time window when functional studies of infected neurons are feasible. Besides, the PRV and RABV infection can cause some unwanted effects such as reduced expression of genes from the infected cells (Weible et al., 2010). It is therefore crucial to test for any adverse effects of rabies virus infection.

Several strain variants of RABV have different biological characteristics, such as synaptic spreading and cytotoxicity, mainly due to amino acid mutations in RABV glycoprotein. RABVs widely used in neuroscience research are coated with CVS or SAD. The Rabies Virus CVS Strain exhibits stronger neurotropism and reduced cytotoxicity, and enhanced retrograde transsynaptic transfer than the Rabies virus SAD strain

(Schnell et al., 1994; Mori and Morimoto, 2014; Reardon et al., 2016). Since the SAD variant produces higher protein levels, it is more suitable for use as an acute vector when fast expression of the transgene is important (Wickersham et al., 2007a). The higher SAD- directed protein expression levels permit visualization of fine neuronal morphologies (Wickersham et al., 2007a). The generation of viral particles in the laboratory is currently easier with SAD than with CVS (Reardon et al., 2016).

### 2.2. Mapping the last order premotor propriospinal neurons

Premotor neurons are categorized as “last-order” since they are the ones that establish the direct synaptic connections onto the motor neurons. Uncovering the connectivity pattern of defined premotor PSNs with functionally distinct motoneuron pools is essential for understanding the organization of motor circuits within the spinal cord. Recently, the development of a glycoprotein gene-deleted, pseudotyped recombinant rabies virus (RABVΔG)-based method has enabled the trans-mono-synaptic tracing and functional investigation of mono-synaptic connections with a defined target cell population (Wickersham et al., 2007b). At the heart of this technology is the key role the rabies glycoprotein plays in the infection and exclusively retrograde trans-synaptic spread of rabies. Deletion of the glycoprotein gene renders the virus incapable of spreading from one neuron to another but does not affect its capacity for replication, meaning that rabies variants become trapped inside infected neurons. The ability to trans-synaptically migrate can be transiently restored by expression of the rabies glycoprotein in infected neurons in trans (Marshall et al., 2010). For example, when motoneurons are induced to express rabies glycoprotein, subsequent infection of those neurons by intramuscular injection of RABVΔG results in trans-synaptic retrograde infection of the neurons in the spinal cord and brainstem with monosynaptic input to the motoneurons (Fig. 1 B). These connections are “mono-synaptic” because the trans-synaptic travel of the virions is limited to one synaptic step between the primarily infected cells and their immediate, presynaptic partners. To elaborate, cDNA of G protein can be cloned into an AAV vector, and then when RABVΔG is complemented by equal volumes of AAV injected into the innervated target muscles, both viruses retrogradely co-infect the respective motor neurons. In this way, the trans-synaptic infection of RABVΔG to premotor PSNs only occurs within the motor neurons containing G-protein produced by AAV. This method can selectively map the 3D distribution of last-order premotor PSNs pools with high reproducibility. With RABVΔG expressing a different color of fluorescence protein, it is possible to study collateralized connections. Such a strategy may transfect one target tissue with RABVΔG expressing a red fluorescent protein, while also transfecting another target tissue with RABVΔG expressing a green fluorescent protein; then any neurons that project to both targets will be co-infected and labeled yellow (from the combination of red and green fluorescence) (Fig. 1 B, G).

Stepien's study applied that dual-color RABVΔG method and revealed that there are populations of unilaterally and bilaterally projecting premotor PSNs projecting onto the quadriceps muscle motor neurons, and both with widespread, rostrocaudal segmental origins, and the bilateral projections with connectivity to equivalent motor neuron pools bilaterally. Ipsilateral PSNs made up the majority of all premotor PSNs and they were more broadly distributed along the mediolateral and dorsoventral axes than contralateral premotor neurons. The majority of ipsilateral PSNs were found in Rexed laminae VI, VII, and X, while contralateral PSNs were mainly confined to Rexed lamina VIII and ventromedial lamina VII (Stepien et al., 2010). Antagonistic muscles coordinate extension and flexion to actualize motor functions, which are partially orchestrated by different premotor PSNs acting on the appropriate motor neurons. The correlation of muscle function with the spatial organization of premotor PSNs supports the existence of a distinct, topographic distribution of functionally different premotor PSN

pools. In Tripodi's study, Rabies-mCherry and Rabies-eGFP were separately injected into the following antagonistic muscles: the ankle extensor gastrocnemius (GS) & the ankle flexor tibialis anterior (TA), the knee extensor vastus lateralis (VL) & the knee flexor posterior biceps femoris (BF), and the hip extensor gluteus (GL) & the hip flexor iliopsoas (IL). The results revealed an apparent mediolateral segregation of extensors (GS/VL/GL) versus flexors (TA/BF/IL) within the premotor PSN pools of the ipsilateral, dorsal spinal cord, and these segregations were maintained across several rostral and caudal segments (Tripodi et al., 2011).

Many PSNs are located far away from their motor neuron targets of innervation, and so they have long projections across multiple spinal segments (Kostyuk and Vasilenko, 1979). Due to the ubiquity of long projections with PSNs, it was not feasible to analyze their distributions, morphologies, and integrations with conventional histological methods. In 2014, Ni et al. took the strategy of combining a single-color RABV $\Delta$ G injection into the TA muscle with a tissue-clearing protocol (CLARITY), that can image the rabies-mediated GFP fluorescence of transfected PSNs throughout the entire spinal cord (Ni et al., 2014). Interestingly, the distribution patterns of the PSNs were different between the cervical and thoracic levels. The majority of the labeled PSNs were seen in the lower thoracic levels, while within the upper thoracic and cervical segments, fewer PSNs were observed, and considerably fewer PSNs were labeled on the contralateral side. On the cervical level, all labeled PSNs were seen in Rexed lamina VII and VIII, both contralaterally and ipsilaterally (Ni et al., 2014), and these regions are known to be vital in mediating motor instructions; these results suggest that these cervical PSNs might play a role in interlimb motor coordination (Ruder et al., 2016). However, most of the thoracic PSNs were detected within the intermediate laminae of the spinal cord, ranging from lamina IV to VIII, suggesting that these thoracic PSNs may integrate both motor and sensory relays along the trunk region and then regulate the activity of motor neurons in the lumbar spinal cord (Ni et al., 2014). In Michael Lane's 2008 study, ipsilateral-projection-biased distribution was reported where a dual-color pseudorabies virus strategy was used to target the premotor neurons connected with phrenic motor neurons. He described that most of the premotor PSNs were found to be ipsilateral to the infected phrenic nucleus. The peak number of premotor PSNs labeled by PRV appeared to be at 64 h after PRV delivery. Regionally, the number of labeled interneurons in laminae VII and X was greater on the ipsilateral side ( $110.5 \pm 23.9$ ) than the contralateral side ( $48.1 \pm 14.2$ ). The number of labeled interneurons in dorsal horn was also greater on the ipsilateral side ( $38.2 \pm 14.7$ ) than the contralateral side ( $6.2 \pm 2.7$ ) (Lane et al., 2008). Collectively, these results support the notion that the ipsilateral-projection bias distribution pattern of premotor PSNs is conserved across functional-distinct muscles. Unfortunately, the successful retrograde transfection of motor neurons by intramuscular rabies injection can only be achieved in the animal pups of early postnatal development (approximately day p0 to p10), after which the efficiency of transfection declined dramatically (Stepien et al., 2010; Ni et al., 2014). The efficiency of transfection was also dependent on when the local, functional connections to motor neurons were established, so that time window needed to be considered as well. The peak numbers of labeled neurons were observed at p8 (Stepien et al., 2010; Tripodi et al., 2011; Ni et al., 2014; Dougherty et al., 2013).

### 2.3. Mapping defined neuronal subtype populations

A problem for manipulating and even distinguishing subtypes of neurons is that many different kinds are integrated within each anatomical level of the spinal cord. One approach to solve this is through using molecular signatures, which are specific to distinct subtypes of neurons, as the entry points and their corresponding promoter region to drive subtype-specific transgene expression. Such intersectional molecular/genetic approaches have been quite practical for subtype-selective targeting, with successful applications of fluorescent labeling,

optogenetics, and more (Intersectional Tools for Mouse Neuroscience, 2015).

Developmentally, PSNs and other interneuron pools of the spinal cord arise from different groups. Their differential expression of embryonic transcription factors has been utilized for transgenic manipulation, such as a Cre-based lineage-tracing approach. The Cre-system enables transcription factors to act as a mechanism for selective vector expression in a subpopulation of PSNs. For example, V2 interneurons arise from a progenitor pool in the ventral cord and express Lhx3. A dorsal interneuron, in the dl3 region expresses Isl1 (Lu et al., 2015). To target the V2 and dl3 interneurons at postnatal stages, Lhx3<sup>Cre</sup> and Isl1<sup>Cre</sup> mice can be crossed with mice of a conditional reporter strain. The presence of Cre-recombinase (Cre) activates the promoter, which tangentially enables the expression of the fluorescence protein in PSNs derived from Lhx3 and Isl1 progenitor cells. Meanwhile, target muscles were injected with a combination of RABV $\Delta$ G-mCherry and AAV-G (mentioned above). In this way, a subset of PSNs, which were derived from Lhx3- or Isl1-expressing progenitor cells, were co-labeled by mCherry due to their direct connection to motor neurons and by mGFP from their conditional Cre-lox expression. Using this method, Stepien et al. found the Lhx3- and Isl1-expressing PSNs ipsilaterally distributed across several anatomical levels, both rostral and caudal to the level of target motor neurons (Stepien et al., 2010). Tripodi et al. later identified Lbx1-expressing premotor PSNs, with a significantly unequal distribution bias within extensor populations (gastrocnemius (GS), 61%; tibialis anterior (TA), 31% of all dorsal derived interneurons) (Tripodi et al., 2011). However, Dougherty used a similar approach with Shox2 gene, and the labeled PSNs connected with both flexor and extensor motor neurons were also segregated but with a clear flexor bias. The percentage of Shox2 interneurons labeled from TA was three-fold greater than from GS. Whereas Shox2 interneurons constituted 5% of premotor neurons labeled from the TA motor neurons, they only made up 1.5% of GS premotor neurons (Dougherty et al., 2013). Thus, the anatomical segregation of premotor PSNs is more dependent on the extensor and flexor function, rather than on the developmental origin of the target muscle (Tripodi et al., 2011; Dougherty et al., 2013).

### 2.4. Morphological studies of long projecting propriospinal neurons

Identification of the dendritic morphological properties of normal and injured neurons can provide fundamental knowledge of the physiological conditions of the neurons and elucidate the mechanisms of their contribution to the spontaneous functional recovery after SCI and enhancements of that recovery with therapeutics. Non-viral small molecular dyes such as horseradish peroxidase (HRP), biocytin, and neurobiotin can display the dendritic morphology through chemical reaction or amplification with antibodies for better dendrite visualization (Lanciego and Wouterlood, 2020). At the end of the twentieth century, the recombinant viral based tracing had been developed based on the ability to switch on gene expression in infected cells (Chamberlin et al., 1998). In transfected neurons, fluorescent protein accumulates everywhere, most importantly in the dendrite and axon down to their terminals. The expression of EGFP in infected cells, made labeled, infected neurons directly visible under fluorescence (Chamberlin et al., 1998). Another unique advantage is that the viral tracer can target the specific type or selected order of neuron within the neural circuit (Callaway, 2008).

G protein-deleted rabies virus if without G protein complementation, or lentivirus, with modifications to the viral envelope (i.e. HiRet or NeuRet lentiviruses) can be injected into the region where the targeted neurons project and synapse; in this way, rabies virus or lentivirus can be transformed into a first-order, retrograde tracer and can only be retrogradely transported to immediate presynaptic neurons (Ni et al., 2014; Deng et al., 2016; Sheikh et al., 2018). Using this method, Deng et al. found that there is a dendritic pattern of intact thoracic PSNs with lumbar-reaching long projections characterized by a disproportionately

higher distribution along the mediolateral or ventrodorsal axes; however, following axotomy, this distribution pattern changed and displayed a ventrodorsal retraction and the lateral-medial expansion. The total dendritic length increased after injury, and glial cell line-derived neurotrophic factor (GDNF) treatment enhanced this effect. The increased density of spine-like structures indicated a more plastic state induced by the injury and GDNF treatment (Deng et al., 2016).

### 3. Functional dissection of propriospinal neurons by recombinant virus-mediated pharmacologic and optogenetic approaches

When considering the application of viral tools for evaluation of PSNs within a circuit, it is vital to consider the neuronal properties which determine their functions. These properties include neurotransmitters, the location of the neuronal soma, and the morphological properties of the dendrites and axonal projections. Since PSNs intensely integrate within neuronal circuits to maintain vital functions such as regulating the breath, hand/forelimb movements, locomotion, and postural support, it becomes apparent that precision is needed, both spatially and temporally, for proper functional dissection. Over the last decade, there has been an expansion in using divergent methods, including the use of genetically modified receptors that can be selectively activated with either light excitation (optogenetic) or with specific ligands (pharmacologic or chemogenetic).

#### 3.1. The functions of molecular subtypes in transgenic mice

As mentioned above, when the Cre-lox system of genetic manipulation is combined with viral tools, the PSN pools can be more effectively dissected into subtype pools, in which target genes can be overexpressed or under-expressed/deleted. In this way, functions of different subtypes of PSNs, within intact neural circuits, can be assessed, in addition to other modifications like injury or therapeutic treatment.

Channelrhodopsin-2 (ChR2) is an algae-derived ion channel that can be activated with blue light. This channel opens rapidly after absorption of photons to generate a large permeability for monovalent and divalent cations, thereby inducing neuronal depolarization and action potential generation (Deisseroth et al., 2006). Recently, novel opsins (VChR1) adapted from *Volvox carteri* have been found to be activated by red-shifted wavelengths (Zhang et al., 2008). An engineered chimeric opsin could enable control of two separate populations of circuits, which composed of the first two and one-half helices of ChR1 and the last four and one-half helices of VChR1. The resulting tool enable truly separable control of multiple populations of neurons when used in conjunction with ChR2 (Fenno et al., 2011). Complementing these depolarizing tools towards precise control of neural excitation, certain light-activated ion pumps may be used for inhibition. For instance, one inhibitory tool is a halorhodopsin called NpHR derived from the halobacterium *Natronomonas pharaonic*, which is a yellow light-activated electrogenic chloride pump that hyperpolarizes the targeted neuron upon activation (Zhang et al., 2007). Furthermore, manipulation of eukaryotic (type II) opsins has led to the precise control of well-defined GPCR signaling pathways (Deisseroth et al., 2006). With the selectivity of viral tools and Cre-lox manipulations, optogenetic manipulations can be isolated to specific subtypes of PSNs (Fenno et al., 2011). Chemogenetic techniques utilize a similar approach of inducing neuronal activation but by using exogenous molecules, which are typically biologically inert but redesigned as ligands for genetically modified receptors (Roth, 2016). Designer receptors exclusively activated by designer drugs (DREADDs) are the most commonly used chemogenetic technique for manipulation of neuronal activation, and their effects are mediated by G-protein-coupled receptors. These redesigned muscarinic acetylcholine receptors can be coupled to either Gi/o or Gs. The effector of both the Gs and Gi/o pathways is the cyclic-adenosine monophosphate (cAMP)-generating enzyme adenylate cyclase (AC). The Gs pathway can catalyze the

conversion of cytosolic adenosine triphosphate (ATP) to cAMP and activate cAMP-response element-binding protein (CREB), via the cAMP/ERK pathway, then induces firing of neurons (Alexander et al., 2009). The Gi/o pathways inhibit AC from generating cAMP leading to the inhibition of neurons (Yudin and Rohacs, 2018). Notably, the cascading effect of G-protein coupled receptors enables for signal amplification more than just ion channel activation. As mentioned with optogenetics, there are many experimental opportunities when neuron subtype specificity methods (like Cre-lox systems or other genetic manipulations) are combined with chemogenetic manipulations of neuronal activation.

In 2014, Azim et al. applied a combined strategy of Cre-lox and optogenetics to study how cervical PSNs act on the cervical motoneurons that control the muscles of front limbs while also project rostrally to the lateral reticular nucleus (LRN). It was known that one subtype of PSNs belongs to the homeodomain transcription factor Chx10-expressing V2a interneuron class; so Chx10-cre mice were crossed with a Rosa-lsl-tdTomato reporter line to generate the Cre/lox-dependent PSN-subtype-specific reporter line, Chx10 Cre::tdT mice. Later they introduced the optogenetic aspect into those Chx10 Cre::tdT mice by transfecting the rostral end of the cervical spinal cord segments – more specifically, the V2a interneurons - with a unilateral injection of a Cre-dependent AAV vector which enabled channelrhodopsin (ChR2) expression in the Chx10-positive V2a PSNs across spinal cord levels C3 through T1. Photostimulation of ChR2 expressed in PSN terminals, within the LRN, allowed for modulation of the PSN internal copy pathway (an interpreted neural circuit involved in motor function). Optogenetic stimulation severely degraded reach success in the multi-reach task. When that particular subtype of PSN was conditionally targeted for elimination (using a selective viral vector encoding for diphtheria toxin receptor (DTR)); subsequent administration of diphtheria toxin (DTX) eliminated 80% of that subtype of PSN (C3-T1 V2a Chx10::tdT1-positive interneurons), and consequently, forelimb-reaching function was reduced (Azim et al., 2014). Reaching movements were also ablated in another study that utilized a similar approach (Fink et al., 2014). However, in that strategy, they focused on the subtype of interneurons that produce the inhibitory neurotransmitter, Gad2. They demonstrated that this subtype of PSNs made contacts on sensory terminals and that their selective ChR2-mediated activation-induced pre-synaptic inhibition, thus elucidating an aspect of a “genetically hardwired gain control system”. Concurrently, when this PSN subtype was eliminated with a DTR/DTX method, the motor feedback circuit was disrupted (Fink et al., 2014).

#### 3.2. The function of propriospinal neurons with unique axonal projection in specific neural pathways

For PSNs whose axons that traverse long distances from the cell body, functional dissection methods need to take both soma and axon into account. One method has been utilized to co-label those long-projecting PSNs by injecting one virus into areas of axon termination and injecting another virus into areas of the somata. Then, by using each of these viral tools as two halves of a whole key, the neurons, which are dual-transfected, can be exogenously manipulated or be made to express molecular targets, like fluorescent proteins or destructive ablation proteins, conditional and reversible synaptic transmission silencing, and conditionally block specific signaling cascades within specific neural pathways or population subtypes.

Within intact neural circuits Ruder et al. investigated the genetic identities of the subtypes of long-projection PSNs in cervical segments (C4–7) with axonal projections to lumbar regions by selective, genetic ablation of those subtypes utilizing the DTR/DTX method (Ruder et al., 2016). A genetic target-tethered Cre-recombinase expressing retrograde canine adenovirus (CAV) was injected into the lumbar spinal cord, which retrogradely infected PSNs that projected to the lumbar injection site; then only those PSNs that were expressing one of the genetic targets, such as V0-Dbx2, V2-Shox2, vGlut2, or vGAT, were induced to

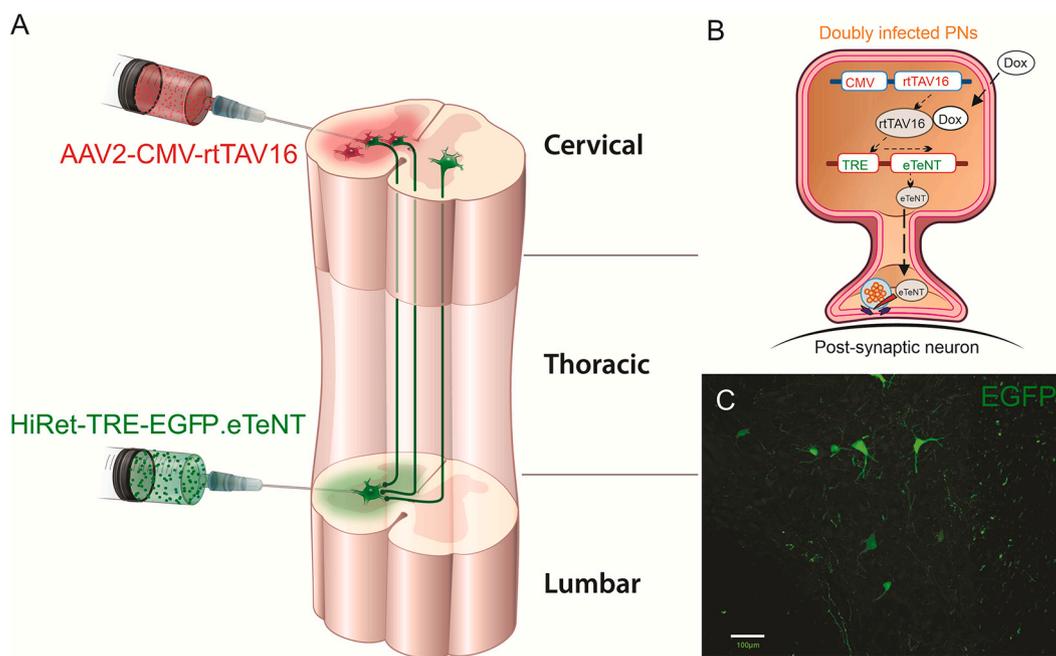
express Cre-recombinase. A Cre-dependent AAV was injected into the cervical spinal cord to express DTR. In this way, those co-infected cervical PSNs would be selectively killed by the administration of DTX. With this strategy, behavioral assessments were conducted before selective ablation and after, so that each subtype's impact on overall function could be determined. The ablation of cervico-lumbar projection neurons impairs proficiency of exploratory locomotion, notably leading to postural instability accompanied by decreases in speed, and inter-limb coordination in the high speed. These cervico-lumbar projection neurons do not only target neurons in the lumbar spinal cord but also broadcast synaptic output information much more widely such as to the lateral reticular nucleus (LRN) in the caudal brainstem. To separately find the sources for inputs to excitatory and inhibitory cervico-lumbar projection neurons, AAV-flex-TVA (a receptor of the avian sarcoma leucosis virus subgroup A) and AAV-flex-G injections were injected to the cervical spinal cord of vGlut2<sup>Cre</sup> or vGAT<sup>Cre</sup> mice, respectively. Two weeks later, the corresponding cervico-lumbar projection neurons from the lumbar spinal cord infected by EnvA-coated Rabies-fluorescence protein injections (EnvA, an envelope pseudotype which can only initially infect the cells expressing TVA). This strategy allowed mapping the origin of synaptic inputs to vGlut2<sup>ON</sup> or vGAT<sup>ON</sup> cervico-lumbar projection neurons or their presynaptic local (Fig. 1 C). Both excitatory and inhibitory cervico-lumbar projection neurons receive input from a broad range of supraspinal centers. The most abundant projection originated from the medullary reticular formation (MRF) and exhibited a bias towards vGlut2<sup>ON</sup> over vGAT<sup>ON</sup> neurons. The results also showed more pronounced input from primary motor and somatosensory cortices to excitatory compared to inhibitory cervico-lumbar projection neurons (Ruder et al., 2016).

Instead of irreversibly genetically ablating the subtype population of PSN mentioned above, an alternative approach is to selectively depress their synaptic activity in a temporary and reversible way (Fig. 2). This strategy uses an AAV vector and a lentiviral vector with highly efficient retrograde transport (HiRet). HiRet lentiviral vectors are modified human immunodeficiency virus (HIV)-1 vectors with enhanced ability of retrograde transportation, enabled by a pseudotyped

modification with rabies virus G protein (Kato et al., 2011). The lentiviral vector was also modified to express genes encoding for enhanced tetanus neurotoxin light chain (eTeNT) downstream of a tetracycline-responsive element (TRE). The modified HiRet lentivirus was injected into the area where the axonal terminals of the target PSN were located. One week later, the somas of PSNs were directly transfected with the AAV-based vector. More specifically, the AAV vector was modified to contain an inducible tetracycline sequence (Tet<sup>ON</sup>) downstream of a strong cytomegaloviral (CMV) promoter. The end result was co-transfection of the long-projection PSNs by these two viruses (Kinoshita et al., 2012). A month later, the administration of doxycycline for 5 days activated the inducible eTeNT element, resulting in decreased synaptic activity in these PSNs, but only so long as the doxycycline remained. The clearance of doxycycline made this silencing strategy temporary and repeatable with 1-month intervals. A major appeal of this system is that it does not require any transgenic aspect, so it can be applied to understand the contributions of specific subtypes of long-projection PSNs in non-transgenic animal models, like rats, macaques, and more.

In an elegant 2012 study, Kinoshita and colleagues investigated the PSNs involved in the neural circuit between the corticospinal tract (CST) and motor neurons of the arms in monkeys (Kinoshita et al., 2012). Doxycycline-mediated eTeNT activation elicited overt functional deficits with reaching and grasping behaviors, thus supporting the notion that PSN-mediated, poly-synaptic transmission is required for complex coordinated behaviors like hand reaching/grasping. Interestingly, underlying this notion, there seemed to be an adaptive compensation mechanism because the arm and hand motor deficits gradually dissipated with consistent doxycycline administration for five days, showing that within intact neural systems, the direct cortico-motoneuronal pathway, or other indirect pathways, could compensate for the loss of control from the silenced PSNs. The inhibition in reaching and grasping movements by doxycycline can be reproduced months later in the same monkeys, thus indicating that the compensation by other pathways was only temporary (Kinoshita et al., 2012).

Pocratsky and colleagues demonstrated that selective synaptic



**Fig. 2. Pathway-specific and reversible silencing of synaptic transmission.** (A) Schematic diagrams of vector injections: HiRet-TRE-EGFP.eTeNT was injected into the lumbar spinal cord, and AAV2-CMV-rtTAV16 was injected into the cervical spinal cord. HiRet-TRE-EGFP.eTeNT retrogradely transfected the cervical PSNs. Some of the cervical PSNs were co-infected by both viruses. (B) Schematic diagrams of how HiRet-TRE-EGFP.eTeNT and AAV2-CMV-rtTAV16 interact in the double-infected cells. (C) Neurons co-infected by two viruses expressed EGFP upon the induction of doxycycline. Scale bar: C 100  $\mu$ m.

silencing of the L2 PSNs ipsi- and contralaterally projecting to interneurons located in the L5 intermediate gray matter (L2–L5 interneuron) resulted in disrupted left-right hindlimb alternation and produced a forelimb-leading and hindlimb-trailing quadrupedal stepping. The intralimb coordination still existed indicating that L2–L5 interneurons are likely not involved in intralimb, flexor–extensor coordination during overground locomotion. The L2–L5 interneurons that were silenced did not participate in hindlimb alternation during a task such as swimming where afferent feedback (such as proprioceptive and cutaneous feedback associated with stepping) was altered/removed, indicating a context-specific manner. Silencing L2–L5 interneurons alters hindlimb coordination independent of locomotor speed and step frequency. These perturbations to hindlimb alternation did not influence or “spread” to the forelimbs (Pocratsky et al., 2017). In another study, Pocratsky and colleagues simultaneously targeted ipsilateral and commissural long-projecting, ascending PSNs (LAPN) (L1–L3 to C6–C8), which integrate the central pattern generators (CPGs) of the cervical and lumbar levels. Histological analyses of the caudal cervical enlargement revealed that eTeNT.EGFP-expressing putative fibers co-localized with the synapse-related markers synaptophysin, vesicular glutamate transporter 2, and vesicular GABA transporter, indicating that the targeted ascending PSN included the excitatory and inhibitory neurons. Upon synaptic silencing of these PSNs, contralateral hindlimb-forelimb coordination was selectively disrupted. Conversely, ipsilateral hindlimb-forelimb coordination remained intact. The altered coordination between limb pairs did not affect the coordination of the intra-limb movement. Unlike long-projecting, descending PSNs (LDPNs), the pathway reciprocal to LAPNs, which play a key role in the supportive feature of locomotion such as balance/postural stability (Ruder et al., 2016), LAPN silencing did not affect overall postural control and spatiotemporal features of limb movement. Remarkably, Pocratsky’s results demonstrated a degree of context-dependence of the deficits such that the locomotor deficits were not apparent in paradigms involving swimming, treadmill stepping, exploratory locomotion, or locomotion on a slick surface (Pocratsky et al., 2020). Our study targeted to silence the descending, long-projecting PSNs, which originate in the T5–T7 levels, and project onto the motoneurons within the L2–L4 levels and found the functional deficits within the grid walking and Rotarod behavioral paradigms but not overground locomotion (Han et al., 2019).

### 3.3. Spontaneous plasticity of propriospinal neurons after spinal cord injury

It has been demonstrated that after SCI, the damaged supraspinal axons can form new synapses on surviving PSNs. These PSNs axons, particularly from long descending PSNs, travel in the ventral and lateral funiculi and are therefore spared by some incomplete injuries such as mid-thoracic dorsal hemisection or contusive injury. These spared axons serve as an “anatomical bridge” to connect the spinal levels above and below the lesion (Courtine et al., 2008; Bareyre et al., 2004; Asboth et al., 2018; Vavrek et al., 2006). Nevertheless, the heterogeneous PSNs are within the hierarchical neural circuit and intermingle with complicated intraspinal neural networks. Traditional chemical ablation or mechanic lesion cannot separate the contribution of PSNs to neural plasticity from other local neuronal components. However, recombinant virus-based transgenic strategies may provide a tool to accurately dissect the function of subpopulations of PSNs after SCI.

Some researchers have already employed these tactics to study the PSN subtypes involved in phrenic and respiratory functional resilience following SCI. Respiration functions were only slightly affected following cervical SCI, despite a significant loss of local phrenic motor neurons (PMNs) (Lane et al., 2012). Zholudeva and colleagues used pseudorabies viral tools to demonstrate that the resilience of phrenic function was at least in part attributable to the Chx10 subtype of PSNs, which were recruited into the phrenic circuit two weeks after injury and were capable of modulating phrenic motor output and functional

plasticity after SCI (Zholudeva et al., 2017). A related study sought to determine the involvement of excitatory PSNs in the preservation of respiratory function in SCI. In this study, researchers utilized selective expression of Cre recombinase (targeting the vGLUT2-positive, excitatory interneurons (eIN)) combined with spatially specific injections of a Cre-dependent AAV encoding for an inhibitory receptor (chloride channel) or an excitatory receptor (hM3Dq) into the cervical spinal cord. This enabled target-specific co-transfection of eIN, and thus the subtype-selective upregulation of either inhibitory receptors or excitatory receptors. Respectively, these upregulations reduced or enhanced the activity of the transfected eINs when stimulated by the respective ligands, and the stimulation of eINs quickly induced recovery of the amplitude and area of the EMG waveforms of the bilateral hemidiaphragm muscle. Notably, the frequency of the EMG waveforms did not change with the treatment, indicating that respiration rhythm is controlled elsewhere – likely the supraspinal centers which were unaffected by eIN stimulation. This result was different from epidural stimulation of the whole mid-cervical spinal cord as previously reported (Satkunendrarajah et al., 2018). In a model of bilaterally staggered hemisection to explore the roles of various PSN subpopulations in relaying connections, Chen and colleagues employed a Cre-dependent AAV to upregulate the expression of a neural inhibitory receptor, the potassium-chloride co-transporter (KCC2), within inhibitory PSNs in the T5–T12 regions. The selective upregulation of inhibition within those PSNs resulted in improved functional locomotion following injury. Similarly, when hyperpolarizing DREADDs (Gi-coupled DREADD (hM4Di-mCherry)) were expressed in the same subtype of thoracic PSNs, functional recovery of locomotion was also observed upon the treatment with the respective ligand. However, while direct activation of inhibitory neurons reduced the overall activation patterns across the spinal cord, it failed to reestablish more physiological activation patterns to promote functional improvements. Neither did the direct excitation of spinal excitatory interneurons induce lasting functional recovery after SCI (Chen et al., 2018). A similar DREADD strategy was used in a follow-up study from the same group, but using a more thorough injury model (severe, complete crush SCI), they explored whether manipulating PSNs can still affect functional recovery, even without receiving the supraspinal input. The activation, but not inhibition, of non-selective thoracic PSNs, led to hindlimb locomotor improvements. Activating excitatory neurons preferentially improved standing ability but silencing inhibitory neurons preferentially promoted stepping. Importantly, activating the PSNs, which were caudal to the lesion and projected to the lumbar region, was enough to improve motor function (Brommer et al., 2021).

As previously mentioned, the HiRet/AAV viral system is excellent for animal models which are not usually transgenic, such as monkeys. In one study using monkeys, the relay function of PSNs was found to be vital for functional recovery of precision gripping (Tohyama et al., 2017). In that study, the injury to dorsolateral funiculus in the cervical region of the spinal cord was induced to transect the ipsilesional CST. The spontaneous recovery of grip function was observed, but that functional recovery could be removed when the viral tools selectively blocked activities of local PSNs. Moreover, if the viral blockade was instated throughout the entire period of postoperative observation, then the spontaneous recovery of grip function did not occur. Collectively, these data suggest that plastic adaptations of PSNs are responsible for mediating some of the functional recovery of SCI, although the extent of that recovery is limited (Tohyama et al., 2017). Sheikh and colleagues used a similar approach to map the lumbar-connecting, descending PSN subtypes which have long projections from the cervical segments or short projections from the thoracic regions. The results of non-injured animals showed a larger proportion of transfected, GFP-positive neurons in the cervical region than in the thoracic; and that discrepancy was even more pronounced in the animals with severe T10 contusive SCI, indicating more significant loss of GFP-labeled PSNs in the thoracic region after injury (Sheikh et al., 2018). Towards understanding the intricacies of spontaneous functional recovery, the functional

contributions of different PSN subpopulations need to be mapped and compared.

#### 4. Functional regeneration of propriospinal axons after spinal cord injury

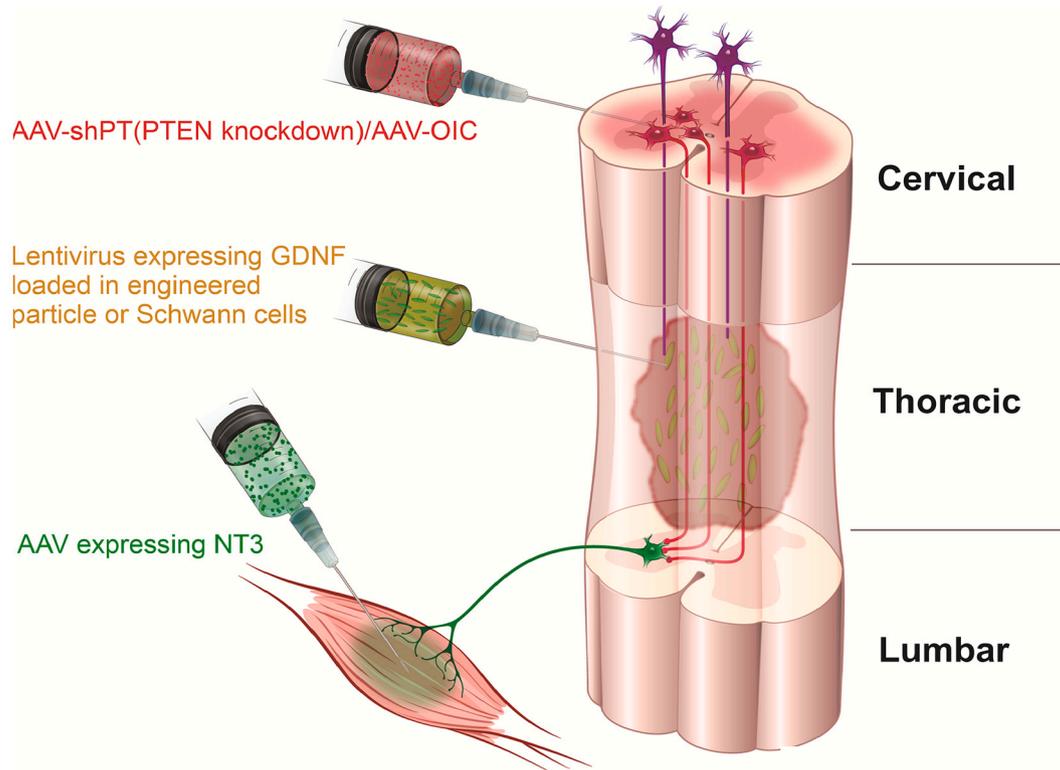
A major barrier for post-SCI functional recovery is the lack of regeneration of CNS axons in adult mammals. Compared to other CNS neurons, PSNs have demonstrated a high capacity for regeneration and sprouting, which may be related to their differing regulation of genes responsible for growth-factor receptors, cell survival, and neuroprotection (Siebert et al., 2010a; Siebert et al., 2010b). These regeneration-associated genes (RAGs) may be targeted within the injured CNS neurons by using specific transcription factors (TFs) to recapitulate the propensity of PSNs to regenerate (Fig. 3). Li and colleagues utilized AAV-mediated overexpression of the TF Kruppel-like factor 7 (KLF7) in PSNs above the spinal cord lesion site, and they observed enhanced neurite outgrowth as well as enhanced expression of downstream target genes: nerve growth factor (NGF) and tropomyosin receptor kinase A (TrkA) (Li et al., 2017). Similarly, Anderson and colleagues utilized AAV-mediated methods to either downregulate phosphatase and tensin homologue (PTEN) or to upregulate a combination of osteopontin (OPN), insulin-like growth factor 1 (IGF1), and ciliary-derived neurotrophic factor (CNTF) within the thoracic spinal cord and they observed significant regeneration of PSN axons, even across the astrocytic scar and lesion core (Anderson et al., 2018). However, functional contribution of regenerating axons of the PSNs could not be verified in either of the two studies because non-selective AAV transfection was used in their approaches.

Another effective method to stimulate axonal regeneration is to supplement the lesion with additional growth factors, and this can be

achieved by exogenous administration, such as via a biomaterial or implanted cellular components, or this can be achieved by upregulating endogenous production through viral infection of glial cell or neurons (Fig. 3). Glial cell line-derived neurotrophic factor (GDNF) has demonstrated a broad range of effects on recovery after injury, including neuronal protection and survival, axonal regeneration, remyelination, and synaptic formation. Within descending PSNs that had enhanced expression of GDNF receptors, a robust regenerative effect was observed following axotomy. Recombinant-virus-embedded biomaterials have been applied to enhance the expression of GDNF within the lesion and caudal to the lesion, and both significantly enhanced the axonal regeneration (Anderson et al., 2018; Blesch and Tuszynski, 2003; Deng et al., 2013).

To be functionally effective, regenerating propriospinal axons need to develop synaptic connections with motoneurons below the lesion site. In a 2018 report, our lab demonstrated that a retrograde AAV vector mediated an increased expression of neurotrophic factor NT-3 in lumbar motoneurons, which acted as a chemoattractant signal for either regenerating or sprouting (Wang et al., 2018). In this study, the descending PSNs axons formed close opposition to motor neurons (Fig. 3). However, functional synaptic connections remain to be verified by viral silencing technique, electron microscopy, or a transsynaptic virus (such as rabies). A subsequent study from our lab reported that the observed functional recovery following a contusive SCI was at least in part attributed to the plasticity of the descending PSN pathway, verified by a silencing strategy using a combination of HiRet-TRE-eTeNT with AAV-2-rtTAV16 (Han et al., 2019).

Enhanced functional recovery has also been observed by transplanting neural stem cells (NSCs) or neural progenitor cells (NPCs) to the injured spinal cord. It is believed that integration of the graft with the host tissue is critical to the recovery of function; however, information



**Fig. 3.** A combinatorial strategy designed to promote axonal regeneration of PSNs across the lesion to innervate motoneurons caudal to a SCI. Three strategies include: 1) AAV mediate the downregulation of phosphatase and tensin homologue (AAV-shPT PTEN knockdown), or upregulation of a combination of osteopontin, insulin-like growth factor 1, and ciliary-derived neurotrophic factor (AAV-OIC); 2) Supplement the lesion with additional growth factors by grafting lentivirus-infected glial cells overexpressing GDNF; and 3) AAV vector injection into a leg muscle that retrogradely infects the motoneurons to express neurotrophic factors such as NT-3 which acts as chemoattractant signal for either regenerated or sprouted descending PSN axons to form synapses.

related to the synaptogenesis of regenerating axons with the grafted neurons is fragmental and unintegrated. By using engineered rabies viral tools, the synaptic maps of regenerating axons, particularly the mono-synaptic connections, can be accurately imaged (Osakada and Callaway, 2013). In the study by Adler et al., grafted cells were infected to co-express G protein and TVA. Another rabies virus engineered for G-protein deletion and expressing EnvA which can only initially infect the grafted cells expressing TVA, and then it is only capable of trans-synaptic transport in the cells co-expressing rabies G protein. In this way, the rabies virus becomes a trans-monosynaptic tracer and can label all descending and ascending PSNs rostral and caudal to the grafting site, which also regenerated and directly formed the synaptic connections with the NSCs or NPCs of the grafts (Adler et al., 2017).

## 5. Limitation and future direction

The current applications of viral tools for neural circuit research are mainly within genetically modified animals. Future advances for viral vectors in neural circuit research will likely be using new approaches to assess functional neuronal populations in genetically unmodified animals. To that end, an attractive approach would be to engineer the method to allow for selective viral tropism into specific cell types or isolated populations. For rabies virus and lentivirus, the pseudotyped viral vector approaches employing modified glycoproteins of the viral envelope could recognize unique cell-surface receptors to induce sequential entry into targeted cell type. However, selection of viral tropism for subpopulations of PSNs distributed in complicated hierarchical orders in the neuronal system can be difficult. Vesicular Stomatitis Virus Glycoprotein (VSV-G) is very common protein used for pseudotyping lentivirus which help lentivirus to target almost all mammalian cells. Avian sarcoma leukosis virus envelope proteins (Env A, Env B, Env C, and Env E) and their paired avian derived receptors (TVA, TVB, etc.) target lentiviruses to specific neurons within a population, in which the avian receptor is often expressed conditionally (e.g., recombinase dependent) on the surface of target neurons (Matsuyama et al., 2015).

For the non-pseudotyped virus such as AAV whose surface is coated by capsid proteins, the interaction between AAV capsid and the host cell surface proteins determines what type of cells are infected by AAVs. Serotypes are variations in the amino acid sequences of capsid proteins which dictate viral avidity and tropism. Variations on AAV serotypes can be deliberately introduced by engineered mutations in capsid proteins (Kotterman and Schaffer, 2015). The engineering design of capsid, such as changing surface residues ligand modification (Wang and Zhang, 2021), peptide insertion (Teruo et al., 2016), etc., can regulate the retrograde transport capacity of AAV. AAVs are typically delivered by stereotactic injections into the central nervous system. Engineered serotypes such as PHP.eB and PHP-S can be administrated intravenously and cross the blood-brain barrier to transduce the entire central or peripheral nervous system, respectively (Chan et al., 2017).

Although both AAV (retroAAV) and lentivirus (HiRet lentivirus) can be modified to retrogradely transduce neurons, their efficiencies are different. One study applied these two viruses to retrogradely transfect long ascending propriospinal neurons (LAPN) in the L2–3 spinal cord which projected to the C5–6 spinal cord. The number of neurons labeled by retroAAV was 76%, less than that labeled by HiRet lentivirus (Brown et al., 2021). This phenomenon may result from poor viral uptake at LAPN axon terminals due to little expression of the requisite receptor and/or co-receptor for internalization of retroAAV virions (Brown et al., 2021). Another reason may be that the VP1 region of the AAV2 capsid was mutated to create the ability of retrograde transportation (Teruo et al., 2016), which caused increased phosphorylation of viral particles, subsequent ubiquitination, and proteasomal degradation of retroAAV in propriospinal neurons (Buning and Srivastava, 2019). Brown et al. also found that the lamina in which the labeled LAPNs were located displayed a virus-based difference (Brown et al., 2021). The neurons

labeled by HiRet lentivirus were mainly located in lamina X while those labeled by retroAAV were mainly in lamina VI–VII. Since LAPNs are a heterogeneous population of neurons with different projection patterns (ipsi- and contralaterally) (Pocratsky et al., 2020) and neurotransmitter capabilities (Ruder et al., 2016), it is necessary to explore whether these differences in the laminar distributions are due to preferential or random labeling. Lastly, it would be interesting to see how other advanced technologies in neuroscience, such as tissue-computer interfaces, complex biomaterials, and remote-controlled systems, couple with viral methods towards functionally dissecting the PSN-mediated regeneration following SCI.

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## Declaration of Competing Interest

All authors have declared no conflict of interest.

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