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THE MOLECULAR BASIS OF THE DEVELOPMENT AND DIVERSITY OF PROPRIOCEPTIVE NEURONS

— A story of surviving and thriving

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THE MOLECULAR BASIS OF THE DEVELOPMENT AND DIVERSITY OF PROPRIOCEPTIVE NEURONS

—A story of surviving and thriving

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Haohao Wu

The thesis will be defended in public at Eva & Georg Klein lecture hall, Biomedicum, Solnavägen 9, Karolinska Institutet, at 9:30 on Friday, May 21st, 2021.

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Dedicated to my parents

致亲爱的爸爸妈妈

ABSTRACT

Proprioception, also known as the sixth sense, describes the sensation of our body position and movement. Its proper function is essential for our daily activities from coarse movements, e.g. locomotion, to precise movements, e.g. playing instruments. The key executors of proprioception are proprioceptive neurons (PNs), the peripheral sensory neurons which continuously monitor the status of muscles, and provide feedback to the central circuits to regulate motor outputs. This thesis aims to extend our current understanding of the development (**study I**) and functional organization (**study II**) of PNs. To contextualize the two studies, this thesis first reviews the relevant literatures in the chapter of *Introduction*, followed by the presentation of the major findings.

In **study I**, we revisit the long-standing neurotrophic hypothesis, which features the exclusive role of target-derived factors in controlling programmed cell death in developing nervous system. Using PNs as a model, we try to understand whether neurons themselves are actively engaged or passively selected during this competition to survive. We find that right before the cell death period, PNs exhibit diverse molecular profiles, which underlie their different responsiveness to target-derived factors and maturation states. The PNs with certain molecular signatures out compete others in this selection to survive, showing that the intrinsic properties of neurons endow some neurons with competitive advantages and are involved in the regulation of neuronal death together with environmental factors.

In **study II**, we use single-cell RNA sequencing to analyze the molecular profiles of adult PNs in mice. Through immunological, genetic and viral labeling, we identify three groups of PNs that correspond to the known functional subtypes (Ia, Ib and II) and provide long-awaited genetic markers to target them individually. We also unveil subtypes within Ia- and II-PNs ($Ia_{1/2/3}$-PNs and $II_{1/2/3/4}$-PNs) that have stereotyped distribution along the spinal cord, selective muscle targets, and unique molecular attributes, indicating an unanticipated and sophisticated organization of proprioceptive feedback. While all other subtypes are established neonatally before the onset of coordinated movements, Ia-PN subtypes emerge later along with the maturation of the animal's motor skills, suggesting the influence of sensory experience on the diversification of Ia-PN subtypes. This is supported by the experiment in which Ia-PN subtypes adjust their relative abundance (Ia_1-PNs switch to $Ia_{2/3}$-PNs) after sustained exercise training, showing the plasticity of the proprioceptive system to adapt to changing motor activity.

LIST OF SCIENTIFIC PAPERS

I. Wang Y*, **Wu H***, Fontanet P, Codeluppi S, Akkuratova N, Petitpré C, Xue-Franzén Y, Niederreither K, Sharma A, Da Silva F, Comai G, Agirman G, Palumberi D, Linnarsson S, Adameyko I, Moqrich A, Schedl A, La Manno G, Hadjab S, Lallemend F.
A cell fitness selection model for neuronal survival during development.
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*Co-first authors

II. **Wu H**, Petitpré C, Fontanet P, Sharma A, Bellardita C, Quadros RM, Jannig PR, Wang Y, Heimel JA, Cheung KKY, Wanderoy S, Xuan Y, Meletis K, Ruas J, Gurumurthy CB, Kiehn O, Hadjab S, Lallemend F.
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Nature Communications. 2021 Feb 15;12(1):1026.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Faure L, Wang Y, Kastriti ME, Fontanet P, Cheung KKY, Petitpré C, **Wu H**, Sun LL, Runge K, Croci L, Landy MA, Lai HC, Consalez GG, de Chevigny A, Lallemend F, Adameyko I, Hadjab S.
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II. Wang Y, **Wu H**, Zelenin P, Fontanet P, Wanderoy S, Petitpré C, Comai G, Bellardita C, Xue-Franzén Y, Huettl RE, Huber AB, Tajbakhsh S, Kiehn O, Ernfors P, Deliagina TG, Lallemend F, Hadjab S.
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III. Bartesaghi L*, Wang Y*, Fontanet P, Wanderoy S, Berger F, **Wu H**, Akkuratova N, Bouçanova F, Médard JJ, Petitpré C, Landy MA, Zhang MD, Harrer P, Stendel C, Stucka R, Dusl M, Kastriti ME, Croci L, Lai HC, Consalez GG, Pattyn A, Ernfors P, Senderek J, Adameyko I, Lallemend F, Hadjab S, Chrast R.
PRDM12 Is Required for Initiation of the Nociceptive Neuron Lineage during Neurogenesis.
Cell Reports. 2019 Mar 26;26(13):3484-3492.e4.

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REVIEW ARTICLE

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*Co-second authors

CONTENTS

LIST OF ABBREVIATIONS

PN(s)	Proprioceptive neuron(s)
DRG	Dorsal root ganglia
NCCs	Neural crest cells
NGF	Nerve growth factor
TrkA	Tropomyosin receptor kinase A
BDNF	Brain-derived neurotrophic factor
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
NSM	Neurosecretory motoneurons
MS(s)	Muscle spindle(s)
GTO(s)	Golgi tendon organ(s)
α-MN(s)	Alpha motor neuron(s)
FACS	Fluorescence-activated cell sorting
LTMRs	Low-threshold mechanoreceptors
scRNA-seq	Single-cell RNA sequencing
FeCO	Femoral chordotonal organ
E	Embryonic stage
TRKC	Tropomyosin kinase receptor C
siRNA	Small interfering RNA
4-OHT	4-Hydroxytamoxifen
P	Postnatal stage

The aspects of things that are most important for us are hidden because of their simplicity and familiarity. (One is unable to notice something — because it is always before one's eyes.) The real foundations of his enquiry do not strike a man at all.

Ludwig Wittgenstein — Philosophical investigations, 1953

1 INTRODUCTION

1.1 PROPRIOCEPTION

All motile animals communicate with the external world through a variety of behaviors, which could be eventually disassembled into a sequence of purposeful movements. To ensure its precision, efficiency and robustness, a movement is closely monitored by the sensory system and streamed to the central nervous system. One essential sensory system for movement control is muscle proprioception, which was termed by Charles Sherrington over a hundred years ago and defined as the internal perception of body position and movement (Sherrington, 1952).

1.1.1 Proprioceptive pathways

Almost all animals have evolved similar mechanisms of proprioception, and subsequently, the sensory receptors of proprioception, namely proprioceptive neurons (PNs, also called proprioceptors), have been detected in different species ranging from invertebrates to mammals (Tuthill and Azim, 2018). In mammals, PNs are pseudounipolar neurons whose cell bodies reside in a series of dorsal root ganglia (DRG) flanking the spinal cord while axons connect muscles and the central nervous system. The status of muscles (position, motion and load) is thereafter relayed by PNs collaterals through three major pathways (Figure 1).

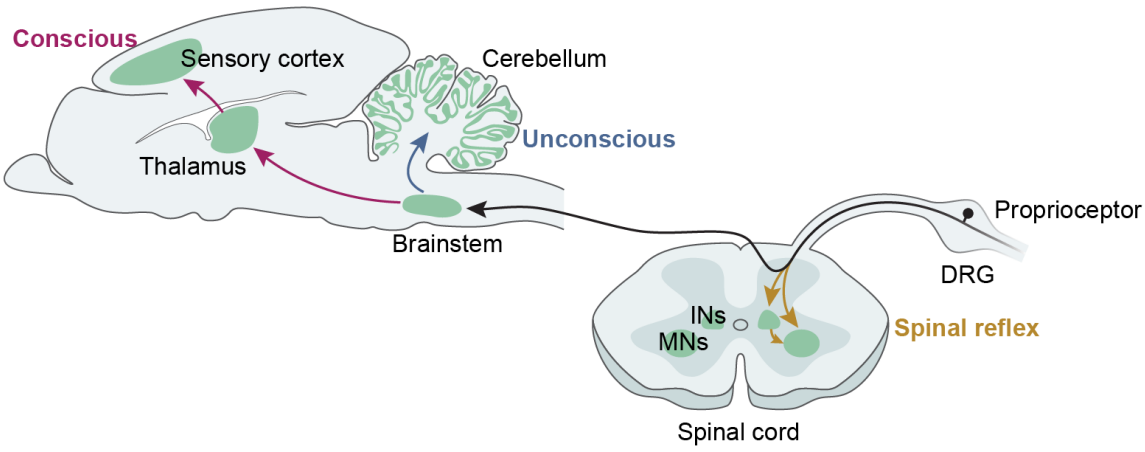

Figure 1. Descending and ascending proprioceptive pathways. *The illustration delineates proprioceptive pathways initiating from the PNs at cervical level. PNs relay sensory information from muscles, and upon entering the spinal cord, they form reflex pathways which directly control motor activities and send collaterals to various brain centers via the conscious and unconscious pathways. At and below thoracic level, the PNs first synapse on neurons in the Clarke's column in the spinal cord, which is not illustrated here. INs: interneurons; MNs: motor neurons; DRG: dorsal root ganglion. Design of the illustration is adapted from Kiehn, 2016.*

The best-characterized proprioceptive pathways are spinal reflexes, in which PNs synapse onto motor neurons directly or indirectly through local interneurons to modulate muscle activity independently from the brain (Figure 1) (Eccles et al., 1957; Liddell and Sherrington, 1924; Tuthill and Azim, 2018). The spinal reflexes respond rapidly to unexpected perturbations, and thus can stabilize the body (e.g. standing in a swaying bus) and protect muscles from excess stretch or tension. Besides, reflex pathways are integrated in other circuits for movement control.

Compared with the "stereotyped" spinal reflexes, ascending proprioceptive pathways and their corresponding neuronal substrates in the supraspinal centers are less understood. It is proposed that proprioceptive information ascends to the brain through two major routes, namely the unconscious and conscious pathways. At cervical level, central afferents of PNs travel along the spinal cord and synapse on second-order neurons in the dorsal column nuclei of the brainstem. The second-order neurons then give off collaterals which either enter into the cerebellum (unconscious pathway) or carry on the information to the sensory cortex through relay neurons in the thalamus (conscious pathway) (Figure 1). At and below thoracic level, PNs first form synapses on neurons in the Clarke's column in the spinal cord, which in turn ascend the proprioceptive information to the brain.

1.1.2 Proprioception impairment

Unlike the sensations elicited by external stimuli, e.g. vision and hearing, proprioception occurs constantly and often seamlessly, thus its importance hardly reaches awareness until it malfunctions. A pathological condition with complete deprivation of proprioception is observed in patients as a result of rare autoimmune response to virus infection (Cole, 1995; Rothwell et al., 1982; Sacks, 1995). A renowned case study came from Ian Waterman, who lost all proprioception below the neck after a gastric flu at the age of 19. Immediately after the trauma, while his muscle strengths remained unimpaired, Ian lost the ability to move. Only after years of rehabilitation, Ian relearnt to coordinate his body to some extent and solely depending on visual guidance (Cole, 1995; McNeill et al., 2010).

Temporary impairment of proprioception is reported in people experiencing drastic changes in body weight, e.g. growth in adolescence and bodybuilding activity. Progressive deterioration of proprioception occurs along with physiological aging, partially as a consequence of undergoing atrophy of PNs (Kim et al., 2007), contributing to the frequent falls in the elderly (Proske and Gandevia, 2012). Besides, patients with Parkinson's disease or

diabetes might show decreased proprioception (Konczak et al., 2009; Proske and Gandevia, 2012), whether the cause of which has a peripheral or central origin is yet to be answered.

1.2 THE EARLY DEVELOPMENT OF PROPRIOCEPTIVE NEURONS

During development, multipotent neural crest cells (NCCs) delaminate from the neural tube and a sensory lineage migrates ventrally to form the prospective DRG (Figure 2) (Marmigere and Ernfors, 2007). Subsequently, NCCs are differentiated into various sensory neuron types during three waves of neurogenesis: the 1st wave of NCCs generates only large diameter mechanoreceptors and proprioceptors; the 2nd wave of NCCs generates a mixed population of mechanoreceptors, proprioceptors and small diameter nociceptors; the 3rd wave of NCCs, the boundary cap cells, generates only nociceptors (Marmigere and Ernfors, 2007). In vertebrates, a significant amount of newly generated sensory neurons die upon reaching their synaptic targets, this striking process is known as programmed cell death (Figure 2).

1.2.1 Programmed cell death

The phenomenon of massive cell death in non-pathologic conditions has been observed in both neurons and non-neuronal cells, and it can serve different functions (Elmore, 2007). In animals which go through metamorphosis, the cell death acts as a way to remove tissues or organs that are no longer needed, e.g. the tail of tadpole, during the transition to a mature stage. This is named metamorphic cell death (Oppenheim, 1991). Some other organs which were present in the ancestors but have been phased out during evolution are removed by cell death during embryonic development, e.g. the tail of human embryo, which is called phylogenetic cell death (Oppenheim, 1991). Cell death can also occur in some structures to shape their stereotypical morphology, such as the interdigital tissue of the hand, and this is termed morphological cell death (Oppenheim, 1991).

In the nervous system, the occurrence of another kind of cell death, which is characterized by its massiveness (up to 80% of a cell type) and rapidness (within a few hours), distinguishes itself from the pre-mentioned types (Buss et al., 2006; Oppenheim, 1991). Neuronal death is detected in various types of neurons in both central and peripheral nervous system and takes place in almost all animals, serving to control the number of neurons in relation to the size of their targets (Buss et al., 2006; Oppenheim, 1991). Usually, this rapid degeneration of neurons occurs soon after they reach their target field, thus its control mechanism was initially thought to depend on target-derived factors.

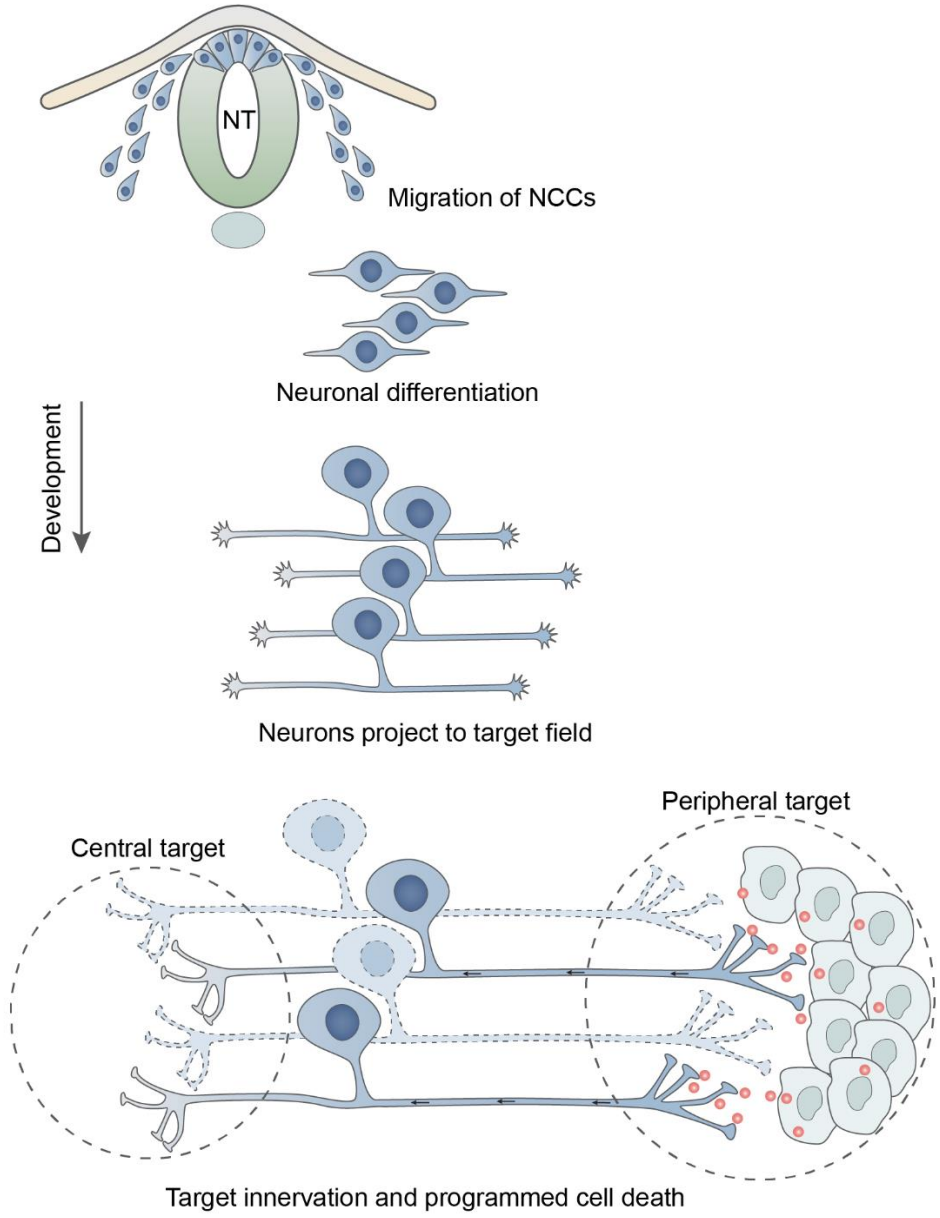

Figure 2. The development of vertebrate sensory neurons. *The neural crest cells migrate ventrally and differentiate into various sensory neuron types to form the DRG. Differentiated neurons then extend axons towards their central and peripheral targets. While neurons reach their target fields, they compete with each other for limited amount of target-derived neurotrophic factors. The neurons that receive enough neurotrophic factors survive, the others enter into apoptosis. NT: neural tube; NCCs: Neural crest cells.*

1.2.2 The influence of target-derived factors on neuronal death

The sympathetic, sensory and motor neurons represent the best-characterized systems for neuronal death. The evidences that neuronal death depends on their peripheral targets mainly come from the limb bud extirpation and transplantation studies in chick embryos (Cowan, 2001). In developing chick embryos, surgical removal of limb bud leaded to marked reduction of both motor neurons in the spinal cord and sensory neurons in the DRG (Cowan, 2001; Hamburger and Levi-Montalcini, 1949). In contrast, transplantation of additional limb bud to the trunk of a host chick embryo rescued both motor and sensory neurons, with visible

size increase of the lateral motor column and DRG (Cowan, 2001; Hamburger and Levi-Montalcini, 1949). Interestingly, under normal physiological condition, neuronal death is more profound at upper-cervical and thoracic levels that don't supply limb muscles (Cowan, 2001; Hamburger and Levi-Montalcini, 1949). All these studies pointed to the dependence of neuronal survival on the target field that they innervate and implied the presence of some functional elements in the target which could be retrogradely transmitted to the neurons by nerve fibers.

It was later discovered that the functional elements which support the neuronal survival are neurotrophic factors. The first and the most prototypical neurotrophic factor, nerve growth factor (NGF), was identified by Levi-Montalcini and Cohen who were awarded the Nobel Prize in 1986 for this discovery. *In vitro* experiments showed that adding NGF to mouse cell culture prevented the neuronal death of sympathetic neurons and of some sensory neurons while promoting their neurite outgrowth (Davies, 2003; Ellis et al., 1991; Levi-Montalcini, 1987). *In vivo* administration of anti-NGF antibody to neonatal mice resulted in neuronal death of nearly all sympathetic neurons, while administration of exogenous NGF could rescue the neurons which would otherwise die (Davies, 2003; Ellis et al., 1991; Levi-Montalcini, 1987). These experiments gave a possible explanation how the number of neurons might be regulated by target-derived neurotrophic factors, widely recognized as the neurotrophic hypothesis.

1.2.2.1 The neurotrophic hypothesis

The central doctrine of the neurotrophic hypothesis states that developing neurons compete for limited amount of neurotrophic factors that are secreted by their targets (Figure 2) (Davies, 1996). The receptors on the neurons and the neurotrophic factors released by the targets form high-affinity binding to induce the survival responsiveness in the developing neurons, thus the neurons which receive enough neurotrophic factors could survive, and their not-so-fortunate neighbours would die (Figure 2). Embedded in this hypothesis is the assumption that the selection between survival and death is entirely stochastic.

Though the most important evidences for the neurotrophic hypothesis arose from the experiments on NGF, the later discovery of other neurotrophic factors extended its generality. These neurotrophic factors include the brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), which were also shown to bind to different neurotrophic receptors and have the NGF-like survival-promoting effect to specific neuronal populations (Davies, 1994, 1996).

1.2.3 Evidences for genetic predetermination of programmed cell death

Among all neurons that are produced in excess and compete for the chance to survive, who is the winner in the end? The well-accepted neurotrophic hypothesis proposes that the selection of survivors solely depends on the availability of environmental (extracellular) factors, in other words, all neurons have equal competitiveness. However, evidences from the studies of simpler organisms, e.g. the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, have shown that this might not be the whole truth, and the fate choice of survival-versus-death for some neurons is already determined at birth.

During the development of *C. elegans*, a total of 1090 cells are generated, all with a stereotypical pattern of division, differentiation and death, making it a perfect model system to study cell death at single-cell resolution (Conradt, 2009; Ellis and Horvitz, 1986). 131 cells, all with a neuronal origin, subsequently die at fixed time and place (Conradt, 2009; Ellis and Horvitz, 1986). One example is the death of the two neurosecretory motoneuron (NSM) sister cells. Two NSMs (larger, ventral-medially located) and two NSM sister cells (smaller, dorsal-laterally located) are born from asymmetrical division of two NSM neuroblasts. About 20 to 30 min after birth, the two NSM sister cells start to degenerate while the two NSMs survive and differentiate into serotonergic neurons. The invariant selection of NSMs to survive is genetically controlled by their specific expression of the transcription factor CES-1 which represses the pro-apoptotic protein EGL-1 (Conradt, 2009). And the lack of CES-1 expression in the NSM sister cells leads to the activation of EGL-1 and inevitable cell death (Conradt, 2009). Thus, the survival-versus-death fate choice is already genetically determined between the NSMs and NSM sister cells at birth and seems not to be affected by environmental factors.

The studies in *C. elegans* raises the question whether similar genetic predetermination plays a role in the control of programmed cell death in the mammalian nervous system. It is particularly challenging to address this question due to the large number of neurons for a given neuronal subtype in mammals, making it impossible to trace the fate of individual neurons. Secondly, owing to the complex nature of target innervation, e.g. some neurons innervate or being innervated by dozens of neuronal or non-neuronal targets, it is difficult to rule out the influence from targets completely. Thus, the neurotrophic hypothesis is still widely applied to date to explain the phenomenon of massive cell death in the developing mammalian nervous system.

1.3 THE CELLULAR DIVERSITY OF PROPRIOCEPTIVE NEURONS

Owing to the complex nature of our movements, it is conceivable that sensory processing of muscle activities demands the integration of diverse neuronal populations. Since Sherrington's initial demonstration in the early 20s century, decades of work on the anatomical and physiological properties of the proprioceptive neurons (PNs) have laid down a schema of the rudimentary organization of proprioceptive feedback. In mammals, three subtypes of PNs (Ia, Ib and II) have been classified and extensively studied. They differ morphologically and physiologically, innervate different sensory end organs, respond to specific stimuli and form stereotyped spinal reflex circuits (Tuthill and Azim, 2018; Vincent et al., 2017). Similarly, different subtypes of PNs, encoding distinct kinematic features, have been described in insects (Mamiya et al., 2018; Tuthill and Azim, 2018). Because PNs are the first relay in the proprioceptive pathways, a comprehensive characterization of PN subtypes is thus essential for the understanding of sensory encoding of proprioception.

1.3.1 Anatomical and physiological attributes of PN subtypes

Historically, PN subtypes have been characterized on the basis of their anatomical and physiological features. According to the Erlanger-Gasser classification of nerve fibers, PNs could be classified into large diameter primary receptors (Ia- and Ib-PNs) and medium diameter secondary receptor (II-PNs). The diameter of a nerve fiber is tightly correlated with its conduction velocity (Gasser, 1941). On account of this, Ia- and Ib-PNs have conduction velocity between 80 and 120 m/s, the fastest among all sensory neurons, while II-PNs are slower (33-75 m/s) (Gasser, 1941).

PNs could also be classified based on their specific innervation of sensory end organs. PNs receive sensory signals through muscle spindles (MSs) and Golgi tendon organs (GTOs), which are deeply embedded in the skeletal muscles throughout the body. MSs are innervated by both Ia- and II-PNs, while GTOs are innervated only by Ib-PNs (Figure 3a-c).

1.3.1.1 Muscle spindle and its sensory innervation

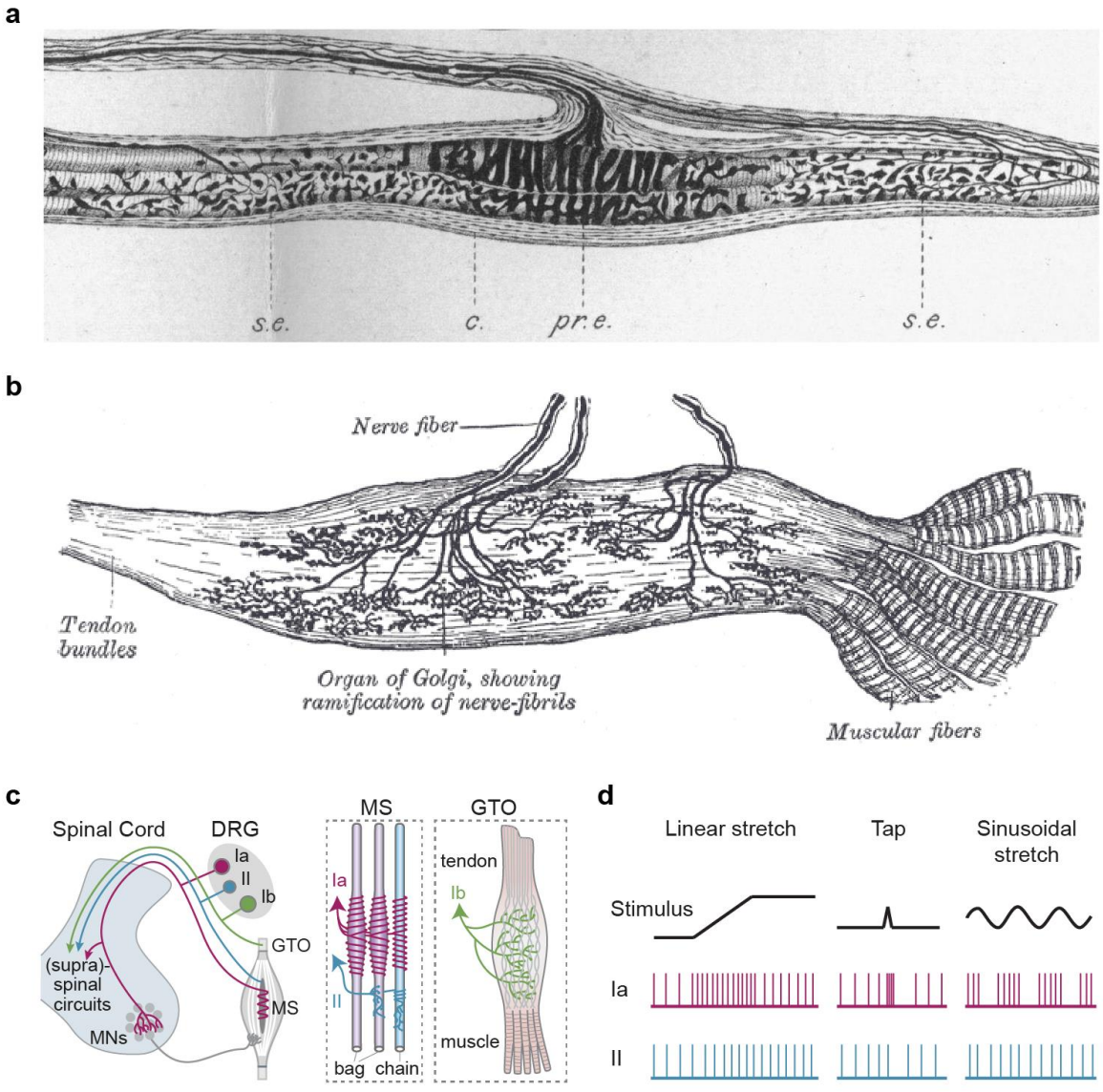

Figure 3. Distinct anatomical and physiological features of PN subtypes. ***a***. *Longitudinal view of a muscle spindle and its sensory innervation in a cat muscle illustrated by Italian histologist Angelo Ruffini in 1898. s.e.: secondary ending; c.: capsule; pr.e.: primary ending. Adapted from Ruffini, 1898.* ***b***. *Drawing of Golgi tendon organ and its sensory innervation by English anatomist Henry Vandyke Carter in 1858. Adapted from Carter and Gray, 1918, Anatomy of the Human Body.* ***c***. *Schematic depiction of central and peripheral innervation patterns of the three PN subtypes. MNs: motor neurons; DRG: dorsal root ganglion; MS: muscle spindle; GTO: Golgi tendon organ. The scheme is adapted from Wu et al., 2021.* ***d***. *Schematic depiction of "typical" responses of Ia- and II-PNs to different stimuli. Adapted from Matthews, 1964.*

MSs rank the most complex encapsulated sensory end organ and present in almost all skeletal muscles in mammals. The relative abundance of MSs varies in different muscles in accordance with their structural and functional properties (Banks, 2015; Banks et al., 2009). A MS is usually composed of multiple intrafusal muscle fibers, paralleling with the extrafusal muscle fibers, the contraction of which generates skeletal movements (Figure 3a). When a muscle is stretched, the MSs and intrafusal fibers within it are also stretched

accordingly, thus mechanically deforming the sensory endings of PNs, leading to the activation of PNs. There are two kinds of specialized intrafusal fibers: nuclear bag fibers that have higher dynamic sensitivity; and nuclear chain fibers that are static (Boyd, 1962; Matthews, 1964).

Early histological work by Ruffini showed stereotyped innervation patterns of Ia- and II-PNs in MSs (Figure 3a, c) (Ruffini, 1898). Ia-PNs innervate the central region of both nuclear bag and chain fibers, forming the distinguishable "annulo-spiral" morphology (Figure 3a, c) (Boyd, 1962; Matthews, 1964; Ruffini, 1898). II-PNs innervate mainly the two ends of the nuclear chain fibers and occasionally the nuclear bag fibers, exhibiting the irregular "flower-spray" structure (Figure 3a, c) (Boyd, 1962; Matthews, 1964; Ruffini, 1898). One MS is innervated by one Ia-PN and various numbers of II-PNs, hence could be classified into simple (no II-PN), intermediate (one II-PN) and complex (two or more II-PNs) types (Matthews, 1964; Ruffini, 1898).

The specific innervation patterns of Ia- and II-PNs in the MSs raise the question whether they serve specific functions. A series of physiological studies in various muscles of different species have shown that, indeed, Ia- and II-PNs respond differentially to mechanical stimuli applied to muscles (Matthews, 1964). Ia-PNs show a large response during the dynamic phase of a stimulus, while II-PNs don't (Figure 3d) (Matthews, 1964). For instance, during the application of a linear stretch on the muscle, Ia-PNs fire at high frequency immediately from the start of the stretch and ease when the stretch is maintained, while II-PNs reach the highest firing frequency during the maintained stretch (Figure 3d). In other words, though both Ia- and II-PNs are sensitive to muscle stretch, II-PNs only signal the instantaneous length of the muscle, while Ia-PNs respond to both the instantaneous length and the speed of the muscle stretch (Matthews, 1964).

The functions of MS feedback have been addressed in *Egr3* mutant mice, in which mutation of transcription factor *Egr3* causes early postnatal degeneration of MSs and impairs the functions of MS-innervating PNs (Chen et al., 2002; Tourtellotte and Milbrandt, 1998). The *Egr3* mutant mice are able to locomote proficiently and adjust to changing locomotion speeds on a treadmill, but exhibit gait ataxia (Akay et al., 2014; Takeoka et al., 2014; Tourtellotte and Milbrandt, 1998). During the precise locomotor task of walking on a horizontal ladder, *Egr3* mutant mice frequently fail to place their feet on the rungs, highlighting the importance of MS feedback in precise locomotor tasks (Akay et al., 2014; Takeoka et al., 2014). Moreover, during swimming, a reduced weight-bearing

task in which GTO feedback is weakened, *Egr3* mutant mice fail to keep the alteration of extensor/flexor activity and thus exhibit uncoordinated limb movements (Akay et al., 2014; Takeoka et al., 2014). These studies of *Egr3* mutant mice have demonstrated that MS feedback is essential for precise motor tasks and swimming. However, due to the attenuated MS feedback from early life in *Egr3* mutant mice, various compensation mechanisms could have developed over the time, thus more profound influence of MS feedback might be still masked by the adaptivity of the sensorimotor system and yet to be discovered.

1.3.1.2 Golgi tendon organ and its sensory innervation

In mammals, GTOs are encapsulated structures located in the musculotendinous junction, where extrafusal muscle fibers blend with tendon fascicles (Figure 3b) (Jami, 1992). GTOs are found in most of the limb muscles but usually more abundant in antigravity muscles that keep the upright posture of animals, such as the extensors of limbs in quadrupeds (Moore, 1984). A GTO contains 5-20 braided collagen bundles each attached to a muscle fiber. It is often innervated by a single Ib-PN which divides into two to three daughter branches before penetrating the capsule, and the daughter branches in turn ramify within the capsule (Figure 3b) (Jami, 1992). The terminals of Ib-PNs are interwoven among collagen bundles with their membranes tightly attached to increase the contact area (Figure 3b) (Jami, 1992). The contraction of a muscle would stretch collagen bundles that lie in series with muscle fibers, thus cause deformation of sensory terminals that are braided within collagen bundles through lateral compression or longitudinal stretching, eventually leading to the activation of Ib-PNs (Jami, 1992).

Compared with the MS-innervating Ia- and II-PNs which function as stretch receptors, GTO-innervating Ib-PNs show much lower sensitivity and higher threshold to muscle stretch, and do not activate unless the stretch exceeds the physiological length of the muscle (Houk and Henneman, 1967; Jami, 1992). Thus, it is proposed that Ib-PNs could function to protect muscles from damage (Houk and Henneman, 1967; Jami, 1992). In addition, Ib-PNs are extremely sensitive to muscle contraction and could be activated by the contraction of just one or two muscle fibers (Jami, 1992; Moore, 1984). The feedback from all Ib-PNs innervating a given muscle would together provide an accurate calculation of the force in the muscle. Although the activation mechanisms of Ib-PNs have been well understood, their functional role in motor control remains obscure, largely due to the lack of genetic tools to manipulate them separately during motor behavior.

1.3.2 Stereotyped reflex circuits of PN subtypes

The PN subtypes transmit distinct sensory information to the central nervous system, where they form specific circuit motifs in the spinal cord and send collaterals to different brain centers (Figure 1). Among those, spinal reflex circuits represent the best-characterized proprioceptive circuits in mammals.

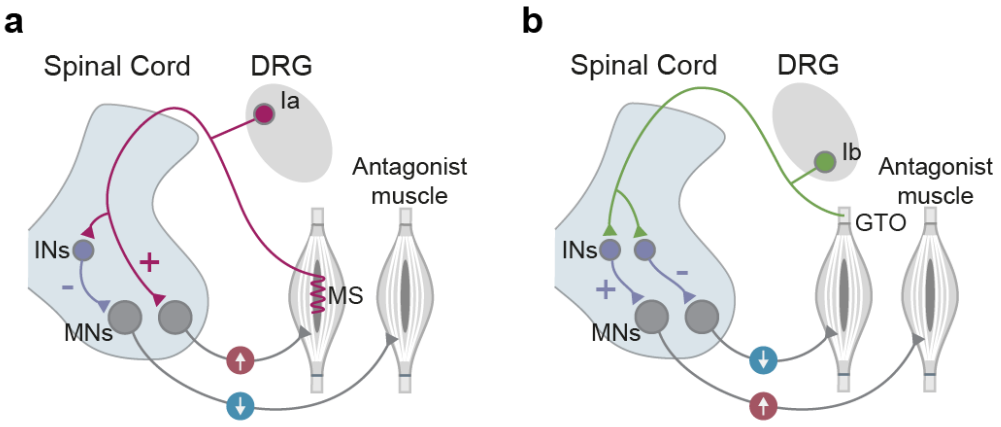

Figure 4. Spinal reflex circuits of PN subtypes. ***a***. *Schematic depiction of stretch reflex circuit. Ia-PNs form excitatory synapses on the MNs of the homonymous and synergist muscles, while inhibit the MNs of the antagonist muscles through inhibitory INs.* ***b***. *Schematic depiction of reverse stretch reflex circuit. Ib-PNs inhibit the MNs of the homonymous and synergist muscles through inhibitory INs, while activate the MNs innervating the antagonist muscles. DRG: dorsal root ganglion; MS: muscle spindle; GTO: Golgi tendon organ; INs: interneurons; MNs: motor neurons.*

One such reflex is the stretch reflex involving Ia-PNs. In the spinal cord, Ia-PNs form monosynaptic excitatory connections with the alpha motor neurons (α-MNs) innervating the homonymous and synergist muscles (Figure 4a) (Cote et al., 2018; Tuthill and Azim, 2018). The same Ia-PNs also inhibit the α-MNs innervating the antagonist muscles in a disynaptic connection through inhibitory interneurons (Figure 4a) (Cote et al., 2018; Tuthill and Azim, 2018). In response to muscle stretch, Ia-PNs would activate the α-MNs of the homonymous and synergist muscles, which causes their contraction, and inhibit the α-MNs of the antagonist muscles, which leads to their relaxation, thus rapidly adjust the muscle length.

Ib-PNs participate in another well-studied reflex circuit, the reverse stretch reflex. In contrast to the stretch reflex, Ib-PNs operate through a negative feedback by inhibiting the α-MNs innervating homonymous and synergist muscles through inhibitory interneurons, while exciting the α-MNs of the antagonist muscles (Figure 4b) (Cote et al., 2018; Tuthill and Azim, 2018). When muscles contract, the activation of Ib-PNs would reduce the activity of the α-MNs of the homonymous and synergist muscles, leading to their relaxation, thus efficiently regulate the muscle tension.

The characterization of the spinal reflex circuits has been possible largely thanks to the technical advances of electrophysiology in the past century. However, using the same approach to dissect the downstream proprioceptive pathways in the brain faces certain challenges due to the long-distance projections from the spinal cord to the brain and also the inevitable complexity of the brain. The recent development of trans-synaptic virus tracing technologies has enabled anatomical visualization of neuronal connectivity, providing promising tools to faithfully map the downstream proprioceptive pathways (Pimpinella and Zampieri, 2021; Zampieri et al., 2014). Nevertheless, these advances in neuronal connectivity have left the PN subtype-specific pathways largely unresolved, a problem that essentially stems from the lack of genetic accessibility to PN subtypes.

1.3.3 In search of genetic markers of PN subtypes

Advancing our understanding of the downstream circuits and functions of PN subtypes demands for their genetic accessibility. In recent years, numerous efforts have gone into the identification of genetic markers of PN subtypes along with the rapid development of molecular profiling technologies. To isolate the MS- and GTO-innervating PN subtypes, de Nooij et al. crossed the $Egr3^{WGA\text{-}mCherry}$ mouse line (in which MS-innervating subtypes are labeled with mCherry) with the Pv^{YFP} mouse line (in which all PN subtypes are labeled with YFP), allowing MS-innervating PNs ($mCherry^{+}/YFP^{+}$) and GTO-innervating PNs (YFP^{+}) to be distinguished by fluorescence-activated cell sorting (FACS) (de Nooij et al., 2015). Then the molecular profiles of isolated MS- and GTO-innervating PNs were analyzed through microarray, revealing a number of genes with enriched expression in MS-innervating PNs (de Nooij et al., 2015). However, the authors did not move further to validate those genes *in vivo*, leaving the conclusion ambiguous.

In another study using bulk RNA sequencing, Wu et al. compared the gene expression of PNs with other low-threshold mechanoreceptors (LTMRs) with similar developmental, morphological and functional characteristics, reasoning that the genetic markers of PN subtypes might be among those that distinguish them from other LTMRs (Wu et al., 2019). The authors have identified 24 PNs-specific genes, among which 9 are restricted to a subpopulation of PNs (Wu et al., 2019). 3 of those 9 genes, most notably *Heg1*, show significantly reduced expression in DRG neurons of *Egr3* mutant mice which lack MSs, suggesting those genes to be genetic markers of MS-specific PNs (Wu et al., 2019). Nevertheless, these speculative markers, though promising, have not been tested with regard to the anatomical and/or physiological features of their labeled PN populations, and are still awaiting for the direct verification.

The rapid progress of single-cell RNA sequencing(scRNA-seq) technologies has provided promising tools for molecular profiling of different cell types, including neurons. Several studies have used different scRNA-seq approaches to identify the sensory neuron subtypes in DRG (Li et al., 2016; Sharma et al., 2020; Usoskin et al., 2015; Zeisel et al., 2018). While these studies have demonstrated genetic distinctions among major sensory neuron subtypes, and also identified subtypes within nociceptors and mechanoreceptors, none of them has revealed molecularly distinct subtypes within PNs. The unsuccess to identify PN subtypes might be explained by: these studies sample all DRG neurons, among which PNs account for only 10% and are thus underrepresented in the data; these studies have modest gene coverage, which might not be sufficient to reveal the minor genetic differences among PN subtypes. Hence, increasing the cell number of PNs and improving the gene coverage would be the key to probe the molecular distinctions among PN subtypes using scRNA-seq.

1.3.4 PN subtypes in insects

While the search for genetic markers of PN subtypes in mammals is being carried out in full swing, considerable progress has been made in insect studies. In fruit fly *Drosophila*, proprioceptive sensory neurons of the legs reside in the femoral chordotonal organs (FeCO, functional analogous structure of MSs in vertebrate) in the femur (Mamiya et al., 2018; Tuthill and Azim, 2018). FeCO neurons send dendrites to detect the mechanical stimuli generated by the position and movement of the femur-tibia joint, and relay that information centrally to the ventral nerve cord (equivalent to spinal cord in vertebrate) and brain (Mamiya et al., 2018; Tuthill and Azim, 2018). Recent study from Mamiya et al. used *in vivo* calcium imaging to investigate the coding of proprioceptive information in FeCO neurons (Mamiya et al., 2018). The authors have identified genetically defined subtypes each encoding specific kinematic features of the tibia: claw neurons encode position; hook neurons encode directional movement; club neurons encode bidirectional movement and vibration (Mamiya et al., 2018). The cell bodies of FeCO neuron subtypes exhibit topographic organization in the FeCO, and their axons show stereotyped innervation pattern in the central nervous system, suggesting a parallel organization logic of the proprioceptive sensory processing in flies (Mamiya et al., 2018).

Subsequently, 3 genetically and functionally defined subtypes of second-order neurons, namely 9Aα, 10Bα and 13Bα neurons, were identified in the ventral nerve cord of *Drosophila* (Agrawal et al., 2020). The second-order neuron subtypes receive different synaptic inputs from FeCO neuron subtypes: 13Bα neurons receive unique input from claw neurons, while 9Aα neurons receive inputs from all 3 FeCO neuron subtypes (Agrawal et al.,

2020). Meanwhile, FeCO neuron subtypes also target different subtypes of second-order neurons: hook neurons target 9Aα neurons specifically, while claw neurons target all 3 subtypes of second-order neurons (Agrawal et al., 2020). Thus, already at the start of sensory processing, both convergence and divergence of proprioceptive information occur: signal of different kinematic features could converge to the same second-order neuron subtype; while one FeCO neuron subtype could diverge the signal to functionally distinct second-order neuron subtypes. It is apparent how the identification of genetically distinct FeCO neuron subtypes has facilitated the understanding of downstream proprioceptive circuits in the fly. It is of hope that similar progress will soon be achieved in mammals.

2 RESEARCH AIMS

This thesis aims to extend our understanding of the development and functional diversity of proprioceptive neurons. Using state-of-the-art genetic and molecular tools, two major aspects were investigated in study I and II respectively.

2.1 STUDY I

Programmed cell death in the vertebrate developing nervous system has long been thought to depend on target-released neurotrophic factors. While studies in invertebrate point to the cell-autonomous regulation of programmed cell death, this possibility has not yet been explored in vertebrates. The study I thus aims to use developing proprioceptive neurons as a model to explore this possibility using advanced genetic and molecular tools.

2.2 STUDY II

Extensive anatomical and physiological studies have identified three functional subtypes of proprioceptive neurons (Ia, Ib and II), whose molecular identities are still unclear, thus hampering the understanding of their downstream circuits and respective functions in motor control. The study II aims to explore the molecular diversity of proprioceptive neurons using up-to-date molecular tools and identify genetic markers to target them specifically, in order to probe the differential roles of proprioceptive neuron subtypes in normal physiological and disease conditions.

3 RESULTS AND DISCUSSIONS

3.1 BRIEF SUMMARY

3.1.1 Study I

Developing neurons encounter a period, known as programmed cell death, during which a massive number of neurons degenerate in a rapid fashion. This phenomenon has been explained as an environmental selection of the right number of neurons to match that of their targets (see chapter *1.2.2 The influence of target-derived factors on neuronal death* for details). In this study, we use PNs as a model to explore whether neurons had equal probability to survive in this selection. We found that, at both protein and RNA levels, PNs exhibited different molecular profiles before the cell death period. The PNs with certain molecular signatures, exampled by higher expression level of tropomyosin kinase receptor C (TRKC, receptor for neurotrophin-3), were shown to possess advantages to survive. This was backed up by single-cell RNA sequencing (scRNA-seq) analysis showing that PNs with high TRKC expression were more advanced in their maturation state. Thus, this study provides evidence that cell-autonomous mechanisms play a role in selecting the most fitted neurons to survive and integrate into functional neuronal networks.

3.1.2 Study II

Earlier anatomical and physiological studies have identified 3 subtypes of PNs (Ia, Ib and II) which are distinct in their axon diameters, conduction velocities, peripheral and central innervation patterns, and responses to mechanical stimuli (see chapter *1.3.1 Anatomical and physiological attributes of PN subtypes* for details). Yet, the molecular identities of PN subtypes remain obscure. In this study, we used scRNA-seq to explore the molecular heterogeneity among PNs, revealing 8 transcriptomically distinct subtypes, a diversity largely exceeding previous anatomical and physiological characterizations. Using immunological, genetic and viral strategies, we aligned these 8 subtypes with the known PN subtypes: 3 subtypes ($Ia_{1/2/3}$-PNs) were identified as Ia-PNs; 4 subtypes ($II_{1/2/3/4}$-PNs) were identified as II-PNs; 1 subtype was Ib-PNs. All PN subtypes exhibited specific spatial distribution along the spinal cord, muscle innervation selectivity and molecular profiles. During development, Ia-, Ib- and II-PNs emerged after innervating their peripheral targets. The II-PN subtypes were specified early postnatally, while the Ia-PN subtypes were specified later along with the maturation of motor skills. Lastly, in adult mice, sustained exercise training could convert Ia_1-PNs to $Ia_{2/3}$-PNs which have higher dynamic sensitivity, suggesting a high degree of plasticity of the proprioceptive system to adapt to changes in motor activities.

3.2 STUDY I

3.2.1 Differential TRKC expression in PNs

During embryogenesis, about 60% of PNs died within 12 hours between E12 and E12.5, after they reached the prospective muscles but before they formed any functional connections (Figure 5a). At E11.5, just before the cell death period started, we observed that PNs expressed various levels of TRKC, the receptor for neurotrophin-3 (NT3) (Figure 5b, c). The binding of NT3 to TRKC is known to induce pro-survival signaling, thus the heterogeneous expression of TRKC suggested that PNs might have different responsiveness to NT3.

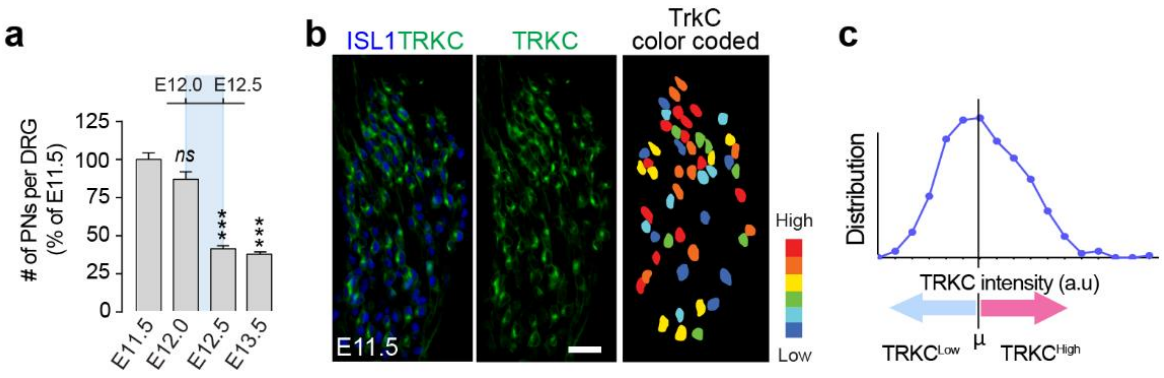

Figure 5. Heterogeneous expression of TrkC in PNs. ***a****. Quantification of number of PNs at C5 and C7 DRG (n = 2-3 embryos). One-way analysis of variance (ANOVA), ***p < 0.001.* ***b****. Immunostaining showing varied expression level of TRKC in E11.5 DRG. Right panel: different TRKC expression levels indicated in different color codes. Scale bar: 50 µm.* ***c****. Distribution of PNs expressing different levels of TRKC in E11.5 DRG.*

3.2.2 TRKC level predicts PNs survival probability

We observed that at E11.5, TRKC expression in PNs was positively correlated with the level of phosphorylated AKT (a serine/threonine-specific protein kinase), which acts at the hub of the pro-survival signaling pathway, suggesting that PNs with higher TRKC expression might have survival advantages (Figure 6a) (Segal, 2003). To directly test the role of TRKC in promoting PNs survival, we electroporated small interfering RNA (siRNA) targeting *Ntrk3* (coding for TRKC) together with GFP plasmid into chick embryos at E2, before the migration of neural crest cells (Figure 6b). This strategy enabled us to knock down *Ntrk3* in PNs and simultaneously trace those neurons with GFP (Figure 6c). The decreased expression of TRKC in PNs drastically reduced their survival rate as observed at E7 after the cell death period in chick embryos, indicating that PNs with lower TRKC level have lower survival probability (Figure 6c).

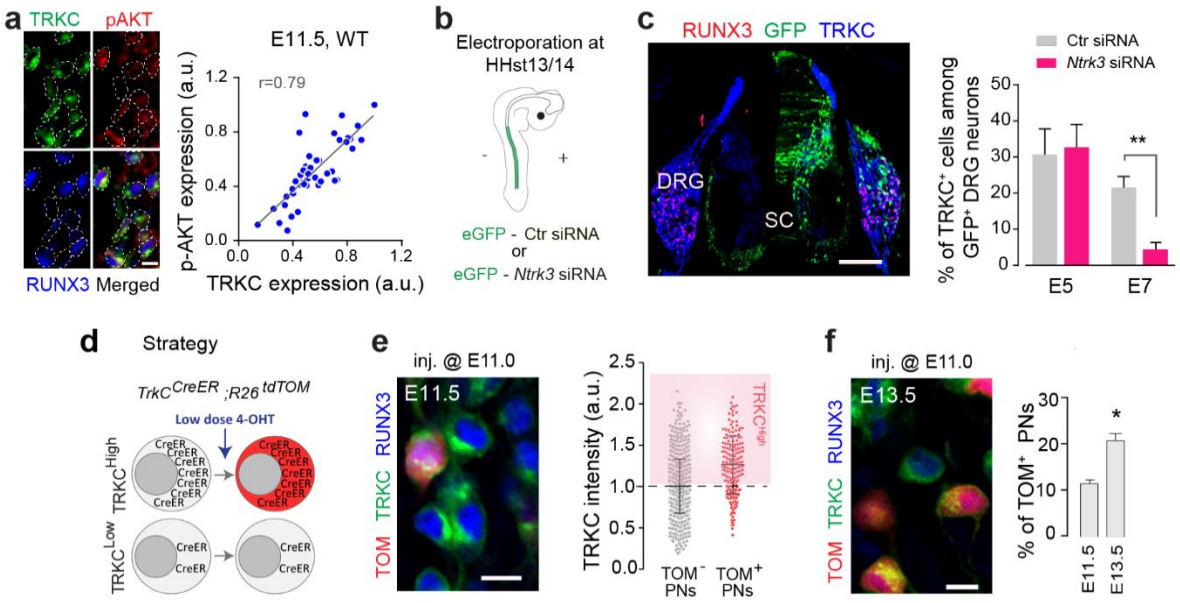

Figure 6. TRKC level predicts PNs survival probability. ***a***. *Immunostaining of TRKC and phosphorylated AKT (pAKT) in E11.5 DRG. Quantification showing the positive correlation between TRKC and pAKT.* ***b***. *Schematic representation of the electroporation experiment in chick embryo.* ***c***. *Spinal cord and DRG of E5 electroporated chick embryo showing transfected cells. Quantification showing percentage of PNs among transfected DRG neurons before (E5) and after (E7) cell death period (n = 4 embryos). Student's t-test, **p<0.01.* ***d***. *Schematic representation of the genetic strategy to trace the PNs with high TRKC expression.* ***e***. *DRG section of E11.5 TrkC^{CreER}; R26^{tdTOM} embryo injected with a low dose of 4-OHT at E11. Quantification showing that TOM preferentially trace PNs with high TRKC expression. Scale bar: 20 µm.* ***f***. *DRG section of E13.5 TrkC^{CreER}; R26^{tdTOM} embryo injected with a low dose of 4-OHT at E11. Quantification showing the percentage of TOM+ PNs among all PNs at E11.5 and E13.5 (n = 6 embryos for E11.5; n = 5 embryos for E13.5). Student's t-test, *p<0.05. Scale bar: 20 µm.*

To assess the survival rate of PNs with high TRKC expression, we developed a genetic strategy to fate trace them specifically using the *TrkC^{CreER}; R26^{tdTOM}* mouse line, in which administration of Tamoxifen or 4-Hydroxytamoxifen (4-OHT) induces cre-lox recombination and subsequent tdTomato fluorescent protein expression in TRKC^{+} cells (Figure 6d). We reasoned that PNs with higher TRKC expression should correlate with higher CreER expression, resulting in higher chance of recombination when given a limited dose of 4-OHT (Figure 9d). Indeed, when pregnant females were administrated with a low dose of 4-OHT (0.018 g/kg body weight) at E11, 11.6% recombination (TOM^{+}) was observed among PNs at E11.5 and the majority of them showed high TRKC expression (Figure 6e). At E13.5, after the cell death period, 20.1% PNs remained TOM^{+}, indicating a 69.3% survival rate of PNs with high TRKC expression, overperforming the 40% survival rate of the whole PNs population (Figure 6f). Altogether, these results provide evidence that the survival-versus-death selection for PNs is not a stochastic process, in fact, PNs with high TRKC expression possess higher survival probability over others.

The classic view of programmed cell death singles out the role of target-derived factors, and implies that the number of neurons to survive is determined by the target size (size-matching hypothesis). If this is the only mechanism, then doubling the target size would double the amount of survived neurons. In fact, evidences show that even at the most optimized experimental condition, increasing the target size never rescues neurons proportionally (Oppenheim, 1985). This implies that other factors are also involved in the decision making of survival-versus-death. Here we provide evidence that the intrinsic properties of PNs, exemplified by the TRKC expression, influence the neuronal survival, complementing the classic view in which neuronal survival is only determined by environmental factors.

3.2.3 scRNA-seq identifies PNs with distinct molecular profiles before cell death period

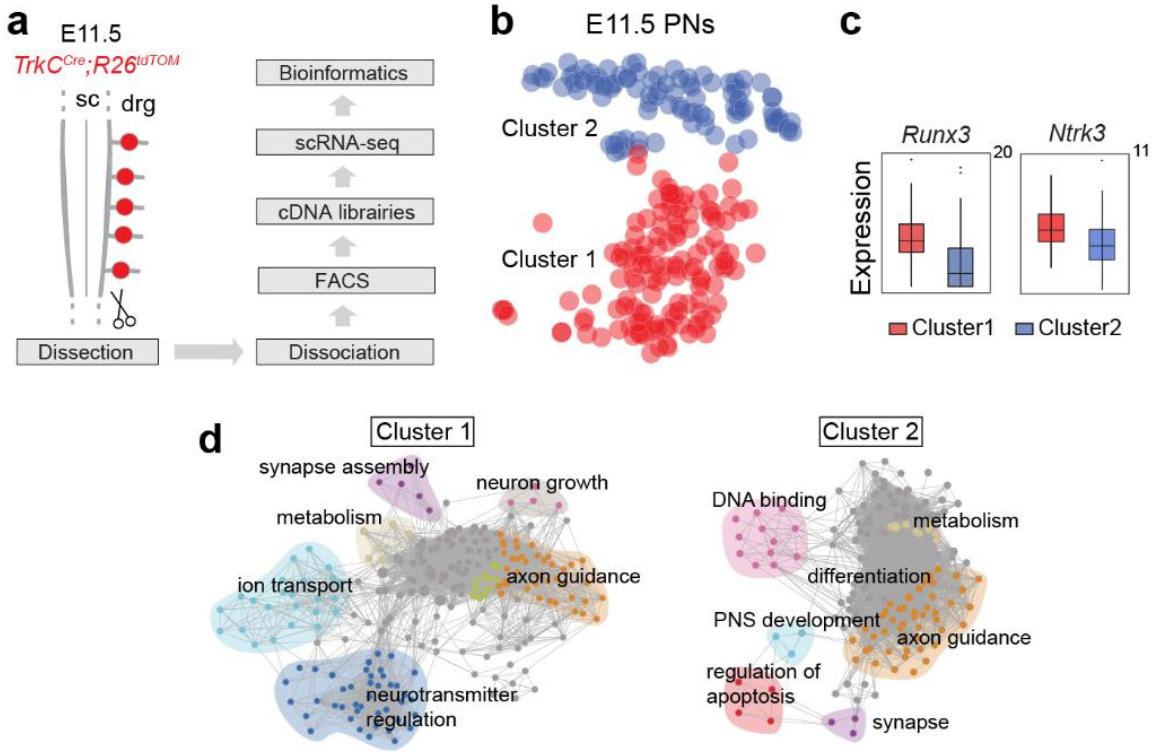

Figure 7. Molecular identification of PNs using scRNA-seq. ***a****. Schematic representation of the scRNA-seq experiment of E11.5 PNs.* ***b****. t-distributed stochastic neighbor embedding (tSNE) plot of E11.5 PNs single-cell transcriptomes, colored by the molecularly defined clusters.* ***c****. Expression of Runx3 and Ntrk3 in the 2 PNs clusters at E11.5* ***d****. Gene set enrichment analysis of the differentially expressed genes between the 2 PNs clusters visualized by network. Each node represents a Gene Ontology term, edges are drawn between the nodes with shared genes.*

We next asked what is the underlying molecular mechanism that endows some PNs with survival advantages? To acquire the full picture of the molecular profiles of PNs before they approach the cell death period, we collected PNs for deep scRNA-seq at E11.5 (Figure 7a). Unbiased clustering of the single PNs based on their overall transcriptomic similarity

revealed 2 molecularly distinct clusters (Figure 7b). The 2 clusters could be distinguished by numerous differentially expressed genes, among which *Ntrk3* and its transcription factor *Runx3*, suggesting that they represented the PNs expressing high and low TRKC, respectively (Figure 7c). Gene set enrichment analysis of the differentially expressed genes between the 2 clusters unveiled that only cluster 1 (high *Ntrk3*) exhibited characteristics for mature neurons, including gene ontology terms such as "ion transport" and "neurotransmitter regulation" (Figure 7d). These results suggest that the more advanced maturation state of PNs with high TRKC expression might endow them with competitive advantages during the cell death period. The existence of two molecularly distinct populations of PNs that have differential survival competitiveness could imply that there might be indeed a genetic predetermination of neuronal survival as observed in the nematode, to ensure that a fixed proportion of neurons would die (see chapter *1.2.3 Evidences for genetic predetermination of programmed cell death* for details). However, the observed genetic differences among PNs could also be the result of some other developmental events, e.g. faster and slower growth, and co-opted to contribute to the survival determination.

3.3 STUDY II

3.3.1 Molecular identification of PN subtypes

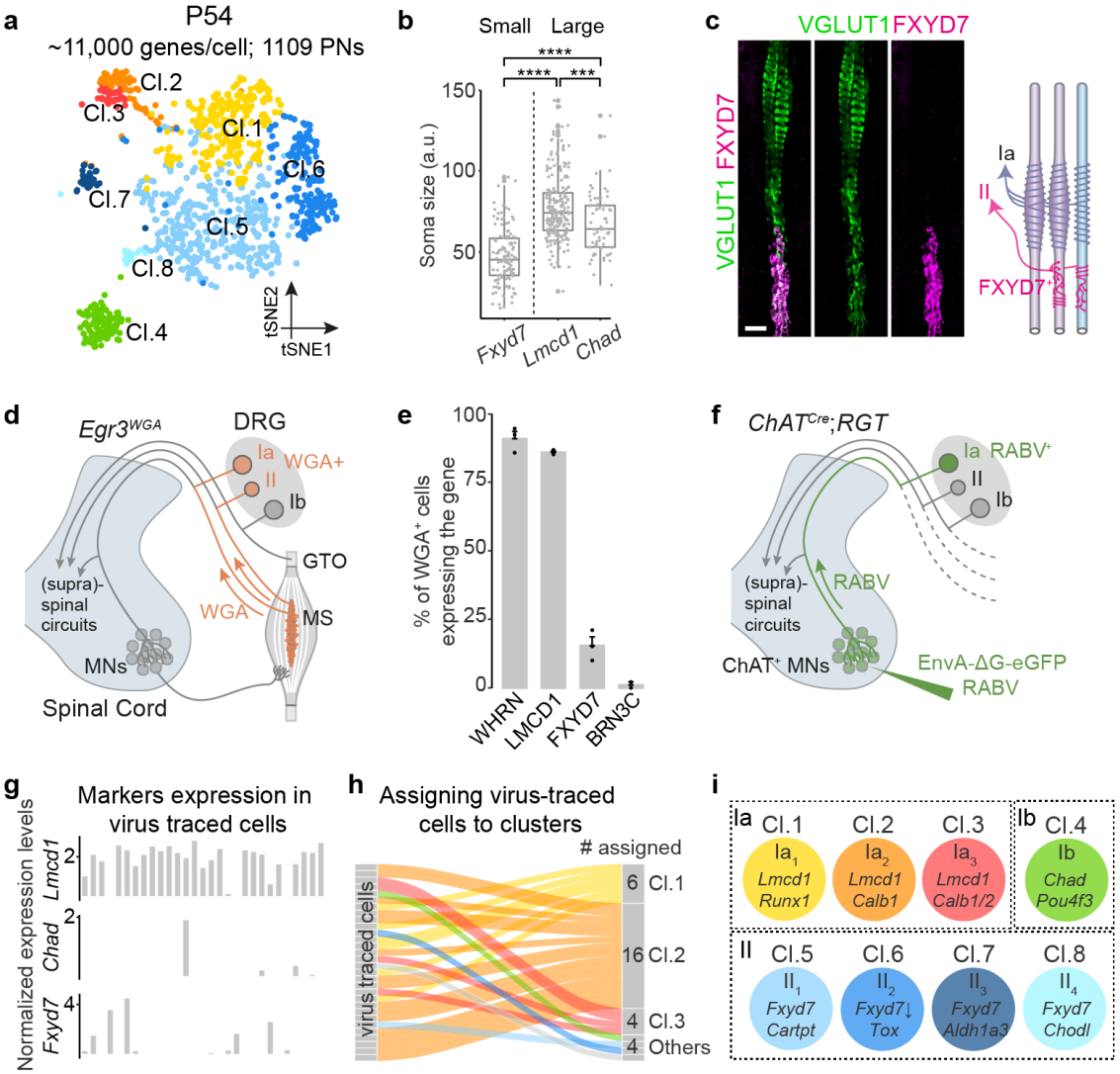

Figure 8. Molecular identification of PN subtypes. ***a****. t-distributed stochastic neighbor embedding (tSNE) plot of adult PNs single-cell transcriptomes, colored by the molecularly defined clusters.* ***b****. Comparison of soma sizes of the 3 major groups of PNs. Two-tailed t-test, ****p < 0.0001, ***p < 0.001.* ***c****. Innervation of FXYD7^{+} PNs in the muscle spindle, VGLUT1 labels sensory endings. Scale bar: 20 µm.* ***d****. Schematic representation of the genetic strategy to label Ia- and II-PNs using Egr3^{WGA} mice.* ***e****. Quantification of WGA^{+} cells expressing pan-PN marker WHRN, and PN subtype markers LMCD1, FXYD7 and BRN3C in P14 DRG (n = 3 animals).* ***f****. Schematic representation of the monosynaptic virus tracing strategy to target Ia-PNs specifically.* ***g****. The virus-traced DRG neurons (the majority are Ia-PNs) express Lmcd1 but not Chad or Fxyd7.* ***h****. Most virus-traced DRG neurons are assigned to Cl.1,2,3 using machine learning algorithm.* ***i****. A summary of the correspondence between the clusters identified by single-cell RNA sequencing and the known PN subtypes.*

To enrich PNs for scRNA-seq, we used the *Pv^{Cre};Ai14* mouse line in which tdTomato fluorescent protein labeled mostly PNs and some mechanoreceptors in DRG. This genetic strategy resulted in 1,109 deep-sequenced PNs with ~11,000 detected genes per cell,

capturing the genes usually with low RNA copies, e.g. receptors and ion channels, that would otherwise be dropped with a less targeted or sensitive strategy (Li et al., 2016; Sharma et al., 2020; Usoskin et al., 2015; Zeisel et al., 2018). The single PNs were subsequently classified into 8 clusters based on their transcriptomic similarity (Figure 8a), which at first seemed to be at odds with the previous anatomical and physiological classifications of PNs (Ia, Ib, II) (Tuthill and Azim, 2018). Interestingly, these 8 clusters further sorted into 3 groups that shared similar gene expression patterns, identifiable by the expression of *Lmcd1* (Cl.1,2,3), *Chad/Pou4f3* (Cl.4) and *Fxyd7* (Cl.5,6,7,8) respectively. We then sought to understand whether these 3 groups corresponded to the known PN classification.

We first assessed soma sizes of the 3 groups with newly identified markers and found the *Fxyd7*$^{+}$ PNs exhibited smaller soma sizes, suggestive of their II-PNs identity (Figure 8b). This is confirmed by the immunostaining of the nerve ending, showing that FXYD7^{+} PNs selectively innervated the polar ends of the muscle spindles (MSs), a typical feature of II-PNs (Figure 8c). To identify another 2 groups of PNs, we used the *Egr3*^{WGA} mouse line which specifically labels MS-innervating Ia- and II-PNs with wheat germ agglutinin (WGA) (Figure 8d). Co-labeling of the DRG with WGA and the newly identified markers showed that WGA^{+} neurons expressed LMCD1 or FXYD7 (labeling II-PNs) but not BRN3C (encoded by *Pou4f3*), suggesting LMCD1^{+} PNs to be Ia-PNs and BRN3C^{+} PNs to be Ib-PNs (Figure 8e). Lastly, to confirm the Ia-PN identity of LMCD1^{+} population, we used a monosynaptic virus tracing strategy to specifically target Ia-PNs for scRNA-seq (Figure 8f). This revealed that the majority of the infected cells expressed *Lmcd1* and were unbiasedly assigned to Cl.1,2,3, providing further evidence that *Lmcd1* marks Ia-PNs (Figure 8g, h). Taken together, the immunological, genetic and viral labeling experiments have identified the 3 groups of PNs labeled by *Lmcd1*, *Chad/Pou4f3* and *Fxyd7* to correspond to Ia-, Ib- and II-PNs respectively, offering, for the first time, genetic markers to label the PN subtypes (Figure 8i).

Oliver et al. approached to the molecular basis of PN subtypes with a similar PNs-focused strategy, albeit with fewer cells (193 PNs) and more modest sensitivity (~6,000 detected genes per cell), resulting in 5 molecularly distinct clusters (Oliver et al., 2021). Through immunological and genetic labeling, 2 clusters (expressing *Calb1* and *Chad*/*Pou4f3*) were identified as Ia- and Ib-PNs respectively, and 3 clusters were suggested to be II-PNs (Oliver et al., 2021). The authors, using genetic tracing, provided direct evidence that the *Chad*/*Pou4f3*$^{+}$ PNs innervated GTOs, thus were unequivocally identified as Ib-PNs, offering important complement to our study (Oliver et al., 2021). The discrepancy in number of

clusters between the two studies might be explained by the different number of cells acquired and the sequencing depth.

3.3.2 Functional subdivisions within PN subtypes

We then set to address the functional relevance of the subdivisions within Ia- and II-PNs by anatomical and molecular characterizations. Among Ia-PN subtypes, $Ia_{2/3}$-PNs were specifically enriched at brachial and lumbar levels (Figure 9a). Among II-PN subtypes, II_2-PNs were mostly located at thoracic level, while $II_{3/4}$-PNs were highly enriched at brachial and lumbar levels (Figure 9a). Knowing that DRG neurons at brachial and lumbar levels innervate both limb and trunk muscles, while DRG neurons at upper cervical and thoracic levels innervate only trunk muscles, the specificity of segmental locations thus suggests that some PN subtypes have limb-versus-trunk innervation bias. To further examine the muscle innervation selectivity of PN subtypes, we used the *Calb2*^{Cre}; *Ai14* mouse line in which *Calb2*$^{+}$ cells (only Ia_3-PNs among all PN subtypes) are labeled by tdTomato fluorescent protein, allowing to trace the nerve endings of Ia_3-PNs (Figure 9b-d). We observed that Ia_3-PNs frequently innervated the MSs in the distal-dorsal region of the forelimb (Figure 9b, c). Among the individual muscles examined, Ia_3-PNs innervated about 75% of the MSs in the extensor carpi radialis (extensor of the wrist and fingers), and none of the MSs in the triceps (extensor of the elbow) (Figure 9c). Within one MS, Ia_3-PNs innervated specifically the nuclear bag fibers but not the chain fibers, suggesting their higher dynamic sensitivity (Figure 9d). All these results point to Ia_3-PN as a functionally specialized subtype that innervates selective limb muscles and is endowed with higher dynamic sensitivity, possibly contributing to the skilled movements. The functional subdivisions within Ia- and II-PNs with specific muscle targets and different dynamic sensitivities also reveal a much more sophisticated organization of MS proprioceptive feedback than previously anticipated.

We next analyzed the differential gene expression in PN subtypes, reasoning that if the PN subtypes serve different functions, it would be reflected in the expression of genes for various neuronal functions. Indeed, numerous genes for mechanosensitive ion channels, neurotransmitter transporters, neurotransmitter receptors and voltage gated ion channels were differentially expressed in PN subtypes. For instance, *Kcna1* and *Kcna2* (coding for potassium channels $K_v1.1$ and $K_v1.2$ respectively) were highly expressed in $Ia_{2/3}$- and Ib-PNs, and pharmacological inhibition of these channels reverted phasic firing PNs to tonic firing, emphasizing the importance of these channels in shaping the physiological properties of PNs (Oliver et al., 2021). Thus, the different anatomical and molecular attributes of PN subtypes together indicate that they are functional subtypes.

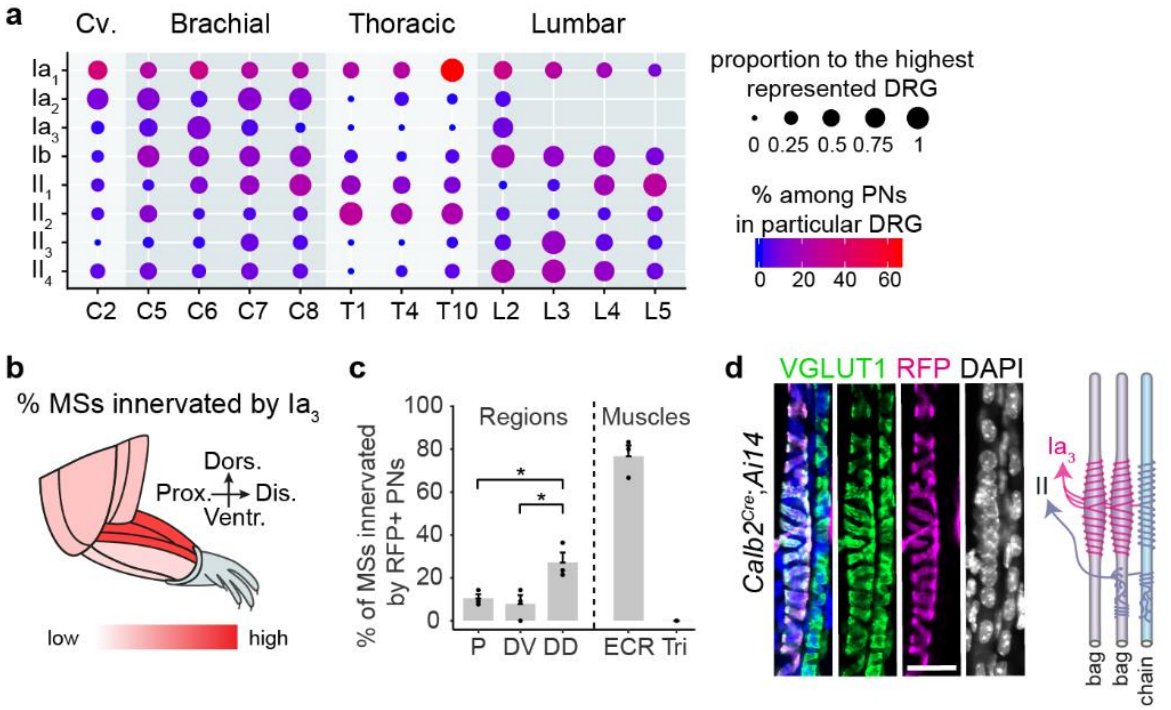

Figure 9. Functional subdivisions within PN subtypes. ***a****. The distribution of PN subtypes along the spinal cord.* ***b****. Schematic representation of Ia$_3$-PNs innervation in the forelimb.* ***c****. Percentage of MSs innervated by Ia$_3$-PNs in different forelimb regions and muscles (n = 3 animals). P: proximal; DV: Distal-ventral; DD: Distal-dorsal; ECR: extensor carpi radialis; Tri: Triceps.* ***d****. Selective innervation of Ia$_3$-PNs (RFP$^+$) to nuclear bag fibers. VGLUT1 labels sensory endings. Scale bar: 20 µm.*

3.3.3 Plasticity of Ia-PN subtypes in adult mice

Following single-cell transcriptomic analysis of PNs at E16.5 and postnatal stage (P) 5, and *in vivo* verification, it became clear that the diversification of the main PN subtypes (Ia, Ib, II) already occurred by E16.5 after PNs innervated their peripheral and central targets (Oliver et al., 2021; Wu et al., 2019). Further diversification of II-PN subtypes, but not of Ia-PN subtypes, could be observed at P5 before the onset of coordinated movements. The late diversification of Ia-PN subtypes along with the maturation of the animal's locomotion skills suggested the influence of sensorimotor experience on the diversification program of Ia-PN subtypes, and prompted us to ask whether the Ia-PN subtypes remained versatile into adulthood.

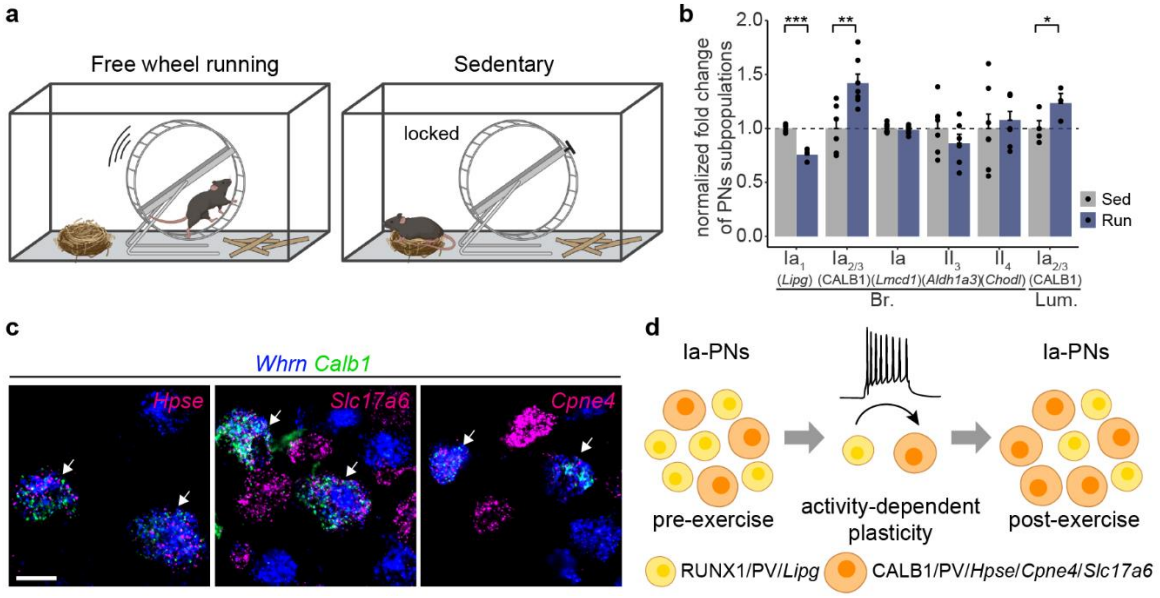

Figure 10. Motor activity induced plasticity of Ia-PN subtypes. ***a****. Schematic representation of the housing environment of free wheel running experiment.* ***b****. Percentage of PN subtypes among all PNs (running group is normalized to sedentary group) (n = 3-7 animals). One-tailed t-test, *p < 0.05, **p < 0.01, ***p < 0.001.* ***c****. RNAscope on DRG sections of running group showing the expression of other $Ia_{2/3}$-PNs markers in $Calb1^+$ PNs (including both existed and induced $Ia_{2/3}$-PNs). Scale bar: 20 µm.* ***d****. Schematic representation of motor activity induced plasticity of Ia-PN subtypes.*

To answer this, we assessed the composition of PN subtypes in mice after 4 weeks of free wheel running, which is known to cause experience-induced plasticity in the central nervous system (Figure 10a) (Li and Spitzer, 2020). While the proportion of Ia-PNs among all PNs did not differ in running and sedentary group, $Ia_{2/3}$-PNs increased significantly in the running group in contrast to the decrease of Ia_1-PNs, suggesting that Ia_1-PNs might convert, through transcriptional changes, to the more dynamic $Ia_{2/3}$-PNs after sustained running (Figure 10b). The II-PN subtypes did not vary between the running and the sedentary group, indicating that the observed plasticity was specific to Ia-PNs (Figure 10b). Moreover, virtually all $Calb1^+$ PNs (including the existed and induced $Ia_{2/3}$-PNs) expressed other $Ia_{2/3}$-PNs markers, e.g. *Hpse*, *Slc17a6* and *Cpne4*, confirming that the transcriptional changes were not restricted to single marker (Figure 10c). Together, these results suggest that the relative abundance of Ia-PN subtypes could be adjusted to adapt to the changing motor activity.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Our abilities to perceive the external world through sensations have fascinated generations of scientists. Much progress has been achieved in our understanding of sensory processing, especially worth mentioning the Nobel Prize-winning discoveries on the organization of the visual and olfactory systems (Grant, 2016). However, among all our sensations, proprioception remains the most enigmatic, with its molecular and cellular mechanisms largely unresolved to date. This thesis thus aims to extend our current knowledge of proprioception through its gate keepers, the proprioceptive neurons (PNs).

One important event during the development of PNs, the programmed cell death, was revisited using modern genetic and genomic tools in this thesis. We found that the selection of which neurons to survive is not a stochastic process, on the contrary, some PNs distinguished by specific molecular codes are more competitive to survive. This finding has challenged the classic view that neuronal survival is determined by target-derived factors, which has been known as the neurotrophic hypothesis and widely accepted for decades (Davies, 1996). Hence, the programmed cell death of developing PNs depends on both the intrinsic properties of the neurons and the environmental factors, which together select the most fitted neurons to suit the needs of a functional neuronal network. Further investigations will be needed to understand whether this mechanism of cell death is specific for only PNs or all peripheral sensory neurons or more generic for all neurons in both peripheral and central nervous system.

The second half of this thesis aims to unveil the molecular heterogeneity of adult PNs taking advantage of the rapid progress of single-cell RNA sequencing technologies. This is a particularly important gap to fill in the field of proprioception, because the anatomical and physiological studies have revealed the 3 PN subtypes (Ia, Ib and II) already a century ago, while the genetic tools to target them are still lacking. Thus, the genetic markers of PN types identified in this thesis finally make it possible to study their downstream circuits and respective functions in the motor control. In this regard, monosynaptic virus tracing techniques have enabled the tracing of the postsynaptic partners of the PNs (Pimpinella and Zampieri, 2021; Zampieri et al., 2014), now combining it with mouse genetic tools to target each PN subtype, it is just a matter of time to finally uncover the neuronal substrates that receive the sensory information from the PN subtypes. The mouse genetic tools to target a specific PN subtype will also enable the use of optogenetic or chemogenetic approaches to

transiently activate or inhibit these neurons to address their unique function in motor control (Deisseroth, 2015; Roth, 2016).

Previous anatomical and physiological analysis of PNs favor the view of modular organization of proprioceptive feedback, with Ia-, Ib- and II-PNs each responsible for the velocity, length and load of muscles. This thesis has revealed the unexpected subdivisions within the know PN subtypes, including 3 subtypes of Ia-PNs and 4 subtypes of II-PNs. Those subtypes have distinct molecular profiles, spatial location and muscle innervation patterns, indicating that the organization of the proprioceptive feedback is far more sophisticated than previously understood. Further studies are needed to explore the functional differences among the subtypes, such as their different dynamic sensitivities, their respective roles in coarse versus precise movements, and their participation in different physiological and disease conditions. As a touch of this, we accessed the relative proportions of PN subtypes in mice after sustained exercise training, revealing that $Ia_{2/3}$-PNs increased at the expense of Ia_1-PNs. This experiment showed that the relative abundance of Ia-PN subtypes could be adjusted to adapt to the changing motor activity. Further investigation will be needed to understand whether the observed plasticity of Ia-PN subtypes is regulated by neuronal activity-dependent program or target-derived factors, e.g. neurotrophic factors.

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