**INTRODUCTION**

Schwann cells (SCs) are the most abundant glial cells of the peripheral nervous system (PNS) (Reed and Feltri, 2021). They were named in honor of the German physiologist Theodor Schwann. Leveraging on his "cell theory" that animals (and plants) are composed by small individual units (i.e., the cells), in the 19th century he described for the first time that peripheral nerves are morphologically complex and composed of many cell types other than neurons. Follow-up studies by Ramon y Cajal, Waller, and Ranvier began to highlight SCs as among the largest and most structurally complex cells in the body, essential during PNS development and capable of remarkably rapid transformations upon nerve injury (Grahame, 2013; Jessen & Mirsky, 2016; Monk et al., 2015).

Building up on these landmark observations, in the last 20 years SCs have been at the center of an intense research that revealed them as fundamental actors in PNS pathophysiology of all vertebrates. One main function of myelinating SCs is to ensheath peripheral axons with myelin, a specialized plasma membrane, called myelin, which enhances the conduction of electric impulses; (2) non-myelinating SCs, including Remak SCs, which wrap bundles of multiple axons of small caliber, and perysinaptic SCs (PSCs), associated with motor axon terminals at the neuromuscular junction (NMJ). All types of SCs contribute to PNS regeneration through striking morphological and functional changes in response to nerve injury, are affected in peripheral neuropathies and show abnormalities and a diminished plasticity during aging. Therefore, methodological approaches to study and manipulate SCs in physiological and pathophysiological conditions are crucial to expand the present knowledge on SC biology and to devise new therapeutic strategies to counteract neurodegenerative conditions and age-derived denervation. We present here an updated overview of traditional and emerging methodologies for the study of SCs for scientists approaching this research field.
neurological conditions compromising PNS activity further support the relevance of these cells for peripheral nerve functionality (Kamil et al., 2019; Valentine, 2020).

SCs derive from precursors (SCPs) that originate from the neural crest during embryonic development (Jessen et al., 1994). SCPs are multipotent progenitors generating many cell types with different anatomical locations including SCs around peripheral axons, endoneurial fibroblasts, enteric neurons, melanoblasts, spleen glia, adrenal medulla chromaffin cells, mesenchymal stromal cells of dental pulp, and parasympathetic neurons (Aquino & Sierra, 2018).

During embryonic development, SCPs migrate along with nascent peripheral nerves, sort individual axons, and chaperone their growth. Their maturation continues during embryonic life, they become immature SCs and, following a well-defined sequence of events, they eventually ensheathe axons. At the end of this process, two distinct populations of myelinating and non-myelinating SCs, the latter referred to as Remak SCs, are generated, which eventually associate to either myelinated fast-conducting or non-myelinated slow-conducting axons (Jessen & Mirsky, 2005, 2019a; Reed & Feltri, 2021) (Figure 1a,b). Of note, myelination is not dictated by intrinsic programs of SC differentiation, rather it occurs as a consequence of the relationship established by SCs with the type of axons they contact, their caliber and the protein repertoire they express (Feltri et al., 2016; Taveggia et al., 2005; Voyvodic, 1989).

Myelinating SCs establish a unitary relationship with larger axons in a process called radial sorting, and spirally wrap myelin membranes around each axon, leaving gaps in the myelin sheaths (Nodes of Ranvier). This myelination pattern allows saltatory conduction to be established along the nerve, which is essential for fast transmission along the axons, as it occurs in α, γ, and β motor neurons (MNs) innervating the muscle fibers, in intrafusal fibers and muscle spindles controlling muscle contraction, and in some afferent fibers transmitting sensory information from the periphery to the CNS.

Conversely, Remak SCs enclose multiple axons of smaller caliber. Although they do not generate myelin, they are indispensable for PNS development and function (Harty & Monk, 2017). Examples of Remak SCs include those associated with C-fibers, which include post-ganglionic autonomic efferents and some afferents of the dorsal roots carrying sensory information (e.g., Pacinian and Meissner’s corpuscles) (Griffin & Thompson, 2008).

Additional non-myelinating SCs are perisynaptic SCs (PSCs), also called terminal SCs, associated with motor axon terminals at the neuromuscular junction (NMJ) (Figure 1c). Indeed, although motor axons are ensheathed by myelinating SCs along their entire length,
a carpet of non-myelinating SCs (PSCs) covers the axonal terminal branches, which form the functional connections with the muscle fiber. The differentiation process of non-myelinating PSCs from SCPs is still poorly understood (Feng & Ko, 2007; Ko & Robitaille, 2015; Sugiura & Lin, 2011). PSCs are strictly juxtaposed to motor nerve terminals, with no extracellular matrix between them (ECM). A specialized ECM, the basal lamina, surrounds the muscle fiber and extends contiguously to both the ECM covering the surface of PSCs and that surrounding myelinating SCs on the pre-terminal axon (Griffin & Thompson, 2008) (Figure 2).

PSCs play a key role in regulating NMJ structural organization and function. Although dispensable for the initial stages of NMJ synaptogenesis (Lin et al., 2000), when the first axon-muscle contacts are attained, PSCs become major drivers in the process of presynaptic structural specialization thereby contributing to the maturation of the NMJ. Notably, PSCs guide motor axons approaching the myofibers and manage synaptic competition by pruning less functional nerve terminals (Darabid et al., 2013a, 2014). In adult muscles, PSCs monitor and assist NMJ maintenance and function, for example, they are capable of modulating neurotransmitter release and, upon nerve injury, are main actors in regeneration (Alvarez-Suarez et al., 2020; Fitzsimonds & Poo, 1998; Ko & Robitaille, 2015; Sanes & Lichtman, 1999; Tintignac et al., 2015).

2 | WHY STUDY SCHWANN CELLS?

SCs play pivotal roles in nerve development, maturation, stability, and regeneration of both sensory and motor neurons of the PNS. Indeed, SC-autonomous dysfunction can impair the complex interactions between axons and SCs at the Ranvier’s nodes that ensures the rapid and saltatory propagation of the action potentials in the PNS, thus causing motor deficits (Chang et al., 2021). In addition, emerging evidence is demonstrating an active role for SCs in the regulation of neuropathic pain, which is known to be a serious consequence of nerve injury (Wei et al., 2019).

Thus, understanding their properties and function has helped to elucidate the molecular basis of nerve physiology on one hand, and is expected to further highlight their contribution to nerve pathology on the other.

Then, the identification of SC mediators and pathways orchestrating nerve re-growth after injury is crucial to devise novel therapeutic approaches to counteract nerve degeneration and support motor and sensory neurons survival in many neurodegenerative contexts.

In addition, the recent discovery that SCs involved in bridge formation upon nerve transection have a transcriptional profile reminiscent of cancer cells (Clements et al., 2017) has paved the way to the identification of signals and pathways activated in SCs and
driving wound repair that may be relevant also to the field of cancer biology.

### 2.1 | SCs in nerve physiology

Given their essential role in axonal myelination, SCs represent a valuable model system to dissect the process of myelin synthesis and deposition in the PNS. In this respect, SCs indeed offer a term of comparison for the study of oligodendrocytes, the glial cells in the central nervous system (CNS) devoted to myelination of central axons. Notably, SCs and oligodendrocytes display different potentials and patterns of axonal myelination. Indeed, in the PNS, one SC myelinates one tract of a single axon, while in the CNS one oligodendrocyte extends multiple cytoplasmic sprouts through which multiple segments of several axons are myelinated (Salzer & Zalc, 2016).

SCs also actively interact with neurons and modulate their activity in many regions of the nervous system. They regulate the formation and contribute to the structure of the nodes of Ranvier, critical for rapid saltatory nerve conduction, and influence several aspects of axonal structure, especially axonal diameter (Eichel et al., 2020; Rasband & Peles, 2021). PSCs also influence nerve physiology. In particular, they can decode and modulate synaptic activity and play a key role in synaptic competition (Auld et al., 2003; Darabid et al., 2013b).

### 2.2 | SCs in aging

Myelinating and non-myelinating SCs play an important role even during normal aging, a process accompanied by deterioration of myelin sheaths, appearance of axonal functional deficits, and a general decline in nerve regeneration capability. Noticeably, the impairment in axonal recovery is not attributed to the neuronal dysfunction, rather to a decrease in plasticity of aged SCs (both myelinating and non-myelinating SCs), which fail to activate repair mechanisms (Painter, 2017; Painter et al., 2014; Wagstaff et al., 2021). Also, the global NMJ functionality declines during aging, but here the contribution of aged PSCs is less understood (Fuertes-Alvarez & Izeta, 2021; Taetzsch & Valdez, 2018; Willadt et al., 2018). It is currently known that this general decline is accompanied by morphological abnormalities in PSCs that decrease in number and fail to cover entirely the motor axon (Chai et al., 2011; Snyder-Warwick et al., 2018). Impaired and disorganized PSC sprouting in aged muscles has also been described (Kawabuchi et al., 2001).

### 2.3 | SCs in nerve regeneration

One of the most remarkable biological properties of SCs is their great plasticity after nerve degeneration. The notion that PNS axons are capable of regeneration to a certain extent, at variance from the majority of central ones, is long known. Cajal and others, including Waller and Ranvier, demonstrated that axonal regrowth occurs from the proximal to distal stump, and that axonal recovery in the PNS is the result of a “symbiotic” interaction between axons and SCs (Grahame et al., 2013).

Indeed, after peripheral nerve damage, SCs support the intrinsic regenerative potential of peripheral nerves, and contribute to the setting-up of a favorable cellular environment for nerve growth, finely orchestrated by a cross-talk of pro-regenerative signals exchanged by SCs, macrophages, fibroblasts, and endothelial cells (Cattin & Lloyd, 2016; Jessen & Arthur-Farraj, 2019). SCs distal to the damaged area mount an adaptive reaction in response to the loss of axonal contacts, with both Remak and myelin SCs converting to a repair-promoting phenotype, thus referred to as repair Schwann cells (rSCs) (Jessen & Mirsky, 2016). By this transition, rSCs start clearing axon and myelin debris (predominantly by myelinophagy and only partially by phagocytosis), and robustly activate a number of intracellular signaling pathways to downregulate genes involved in myelination and enable the expression of developmental growth-promoting genes. Growth factors and cytokines produced by rSCs recruit macrophages and help coordinating the response of nearby fibroblasts and endothelial cells. Moreover, rSCs also release neurotrophins, which target the axonal stump and activate pro-survival signals delivered from the axolemma to the cell soma (Jessen & Arthur-Farraj, 2019; Rigoni & Negro, 2020).

Collectively, rSCs contribute to the generation of a favorable microenvironment at the injury site that supports both motor and sensory neurons survival and helps the damaged nerve to repair and re-grow (Cattin & Lloyd, 2016; Rigoni & Negro, 2020). Interestingly, despite the activation of these mechanisms, the regenerative outcome of nerve injury remains poor, especially in humans, and strongly depends on the type and duration of injury (Hoke, 2006). For example, in aging and chronic denervation the regenerative capability of nerves is gradually lost and this seems related to a dysfunction of SCs in activating repair mechanisms (Wagstaff et al., 2021; Wilcox et al., 2020).

The remarkable plasticity of axonal SCs upon nerve damage is also shared by PSCs at the NMJ (Griffin & Thompson, 2008). The NMJ is the preferential target of toxins of bacterial and animal origin that affect the presynaptic element causing paralysis, with or without nerve terminal degeneration (Rigoni & Montecucco, 2017; Rossetto et al., 2004; Schiavo et al., 2000). Beside neurotoxins, the NMJ can also be targeted by autoantibodies against presynaptic antigens like polysialogangliosides, which cause an autoimmune, complement-mediated, degeneration of the presynaptic membrane. Together with the ensuing neuroparalysis, these are the hallmarks of some subtypes of the Guillain-Barré syndrome (Buchwald et al., 2007; Jacobs et al., 2002; Rupp et al., 2012; Yuki & Hartung, 2012). In these scenarios, PSCs undergo a number of morphological and functional changes, dictated by a transcriptional reprogramming, to assure an efficient motor axon regeneration. Among them, they...
extend sprouts from denervated endplates to reach innervated endplates (through which they guide the nerve terminal to the denervated endplate) (Kang et al., 2003; Son & Thompson, 1995a, 1995b) and they remove neuronal debris by phagocytosis (Duregotti et al., 2015a; Rigoni & Negro, 2020).

2.4 | SCs in nerve pathology

SCs and PSCs are both directly and indirectly involved in many PNS pathological contexts, for example, the Charcot–Marie–Tooth disease, a heterogeneous group of genetic neuropathies characterized by dysfunctions involving either SCs or peripheral axons (or both), which cause muscle atrophy and degeneration of peripheral sensory-motor nerves. Namely, mutations in genes responsible for myelin synthesis, composition or degradation and in gap junction organization cause demyelination, abnormalities in nerve conduction, axonal degeneration, and eventually PNS dysfunction (Berger et al., 2006).

SCs are also implicated in the pathogenesis of acquired autoimmune inflammatory neuropathies, that is, some axonal forms of Guillain–Barre syndrome and chronic inflammatory demyelinating polyneuropathies, where autoantibodies against SC antigens lead to a complement-mediated axonal demyelination and nerve degeneration, sensorimotor dysfunctions, and paralysis. In Miller Fisher syndrome, on the other hand, the pathogenetic mechanism caused by autoantibodies reactive against the neuronal gangliosides can cause the death of PSCs (Halstead et al., 2005).

Alterations in PSCs functionality have been reported also in experimental models of neurodegenerative diseases affecting the NMJ, such as Amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), or mouse model of Duchenne muscular dystrophy (Carrasco et al., 2016a; Hunter et al., 2014; Santosa et al., 2018; Voigt et al., 2010).

Several studies have shown alterations in PSCs as hallmarks of ALS pathophysiology with, for example, altered ability to detect synaptic activity, morphological abnormalities, and changes in protein expression involved in axon pathfinding such as Semaphorin 3A (Arbour et al., 2015; Carrasco et al., 2016b; De Winter et al., 2006).

Moreover, it is possible that PSCs have a crucial influence in ALS considering that motor neurons retain, for some time, a certain degree of regeneration capability, which may be then overwhelmed by degenerative events during disease progression (Martineau et al., 2018). Therefore, it is not surprising that SCs and PSCs, which are critical for axon and NMJ maintenance and repair, have attracted great attention for their possible involvement in disease pathogenesis on one hand, and as putative disease progression modifiers on the other.

2.5 | SCs in cancer research

SCs can undergo neoplastic transformation and generate nerve-sheath tumors of cranial and spinal nerves (even at root levels). These tumors can be of benign nature, and rarely give rise to malignancies (as the case of schwannomas), or malignant (e.g., malignant peripheral nerve sheath tumors, MPNST). Schwannomas originate from SCs of the peripheral nerve sheath, are mostly non-invasive unless if growing in anatomical regions that are not easily accessible surgically, as in the case of acoustic schwannomas which are often associated with hearing loss. Intense research is under way to understand the pathogenesis of schwannomas, which remains largely unknown. A step forward has been gained with the discovery that loss of function mutations in NF2 or NF1 genes, which encode for the oncosuppressor protein Merlin (also known as schwannomin) and Neurofibromin, respectively, are major determinants of familial schwannomas (Fletcher et al., 2020; Hilton & Hanemann, 2014; Ratner & Miller, 2015).

MPNSTs are highly aggressive peripheral nerve-associated tumors of SC origin. They have a strong metastatic potential and form independently or in association with NF1 mutation (Farid et al., 2014). SC transformation and growth in this type of tumors appear to be associated with activation of ERK, mTOR, and HIPPO-YAP/TAZ pathways, and with increased expression of the RNA-binding protein HuR/ELAV1 (Palomo-Irigoyen et al., 2020; Wu et al., 2018).

The role of SCs in tumor development is an emerging field of research. Despite the long-known interactions occurring between tumors and peripheral nerves, the impact of innervation on tumor growth and its molecular basis is only recently emerging. Several studies have unraveled that within tumor mass SCs acquire a phenotype close to that adopted after nerve injury, whereby the expression of chemokines and growth factor contributes to the attainment of a tumor-favorable microenvironment (Bunimovich et al., 2017).

3 | METHODS TO STUDY SCHWANN CELLS

Given their major contribution in essentially all the aspects of peripheral nerve biology and pathology, SCs have been and will be the subject of an intense study.

Methods to study SCs have progressively evolved throughout the last decades and have been implemented with isolation, genetics, imaging, and –omics innovative approaches. Integration of these methods allows a sophisticated study of different SC populations residing in specific anatomical districts and biological contexts, whose accomplishment is leading to a comprehensive understanding of SC biology in different pathophysiological conditions. Combination of traditional and more recent technologies is expected to provide a significant contribution for extending the knowledge on these processes as well as for devising new therapeutic strategies and protocols to counteract neurodegenerative conditions and decay of PNS function in aging.
Here below, we present a concise description of the most widely used methodologies (Figure 3), with a particular emphasis on the recently developed ones, to provide researchers approaching the field of SC investigation with a general overview of the experimental possibilities currently available.

Due to space constraints, the present review will discuss methodologies dealing with murine and human models, and omit additional relevant models such as zebrafish, despite their great contribution (due to their striking regenerative abilities) to the identification of molecules and pathways involved in peripheral nerve repair and conserved through evolution. Detailed reviews on this topic can be found in (Ackerman & Monk, 2016; Rasmussen & Sagasti, 2017).

3.1  |  Methods to study SCs in vitro

3.1.1  |  SC primary cultures

Primary cultures are the most used and versatile research tool to study SC biology (Figure 4a), thanks to the simplicity and accessibility of cell isolation and culturing methods, the high yield and purity of the final culture, and the phenotypic and genetic stability of the cells, which allow expanding, propagating or freeze-storing the preparation. Indeed, primary SCs can be cryopreserved for a long time, and put back in culture with little, if any, loss of cell viability. In addition, SCs are very amenable to genetic manipulation via plasmid transfection or viral transduction.
The idea of culturing SCs dates back to the ’60s with the first organotypic cultures of dorsal root ganglia (DRG) explants, but it definitely took off in the late ’70s after the finding that cAMP is a potent stimulator of SC proliferation (Bunge et al., 1967; Salzer & Bunge, 1980a). This seminal discovery prompted the development of protocols to isolate SCs from whole nerves, plate them in culture dishes and induce proliferation by cAMP activators (Andersen et al., 2016; Ratner et al., 2006; Wei et al., 2009), settling the method of choice to reproducibly and reliably grow in culture these glial cells (Kim & Maurel, 2010; Tao, 2013).

Primary cultures of SCs can be prepared from almost any type of nerves, at any maturation stage (embryonic, postnatal, and adult), as well as from wild-type murine models and transgenic animals expressing gene reporters (Andersen et al., 2016; Ratner et al., 2006; Wei et al., 2009). Current protocols for SC cultures are robust, detailed, and well optimized (Monje, 2020a). In addition, SCs can be prepared from human subjects, although discrepancies in the molecular profiles of human and mouse cells revealed by transcriptomic and proteomic analyses argue against the direct translatability of results obtained from animal models (Monje, 2020b; Monje et al., 2018; Weiss et al., 2016).

Human SCs can be purified from peripheral nerves of (i) fetus, after pregnancy interruption (Scarpini et al., 1988), (ii) pediatric and adult autopsies and organ donors (Boyer et al., 1994; Morrissey et al., 1991), or (iii) patients who underwent nerve graft repair after injury (Morrissey et al., 1991; Rutkowski et al., 1992).

Alternatively, SCs can be obtained from human and mouse embryonic stem cells (ESC), adult and induced pluripotent stem cells (iPSC) (Huang et al., 2020; Lehmann & Hoke, 2016; Ziegler et al., 2011). Other than being a valuable tool to research basic aspects of SC biology, these cells have been used as: (1) a screening tool to develop new drugs to target dysfunctional SCs in PNS conditions,
such as in Charcot–Marie–Tooth disease, Guillain–Barré syndrome, and schwannomatosis; (2) in clinical trials for therapies based on the transplantation strategy of healthy cells (Ma et al., 2015). Noticeably, SCs transplantation alone or co-transplantation with other cell types improves remyelination following demyelination caused by either spinal cord injury or multiple sclerosis (Itoyama et al., 1983; Pearse et al., 2007; Vanevercooren et al., 1992).

Recently, it has been reported that SCs cultures can be prepared by reprogramming adult human skin cells, leading to a cell culture indistinguishable from that derived from human sciatic nerves (Stratton et al., 2017), which add a conveniently accessible method to investigate SC biology in cells from humans and patients carrying PNS disorders.

For all these reasons, primary SC cultures represent a versatile research model for standard procedures of cell biology, biochemistry, cytology, and imaging, as well as suitable tools to carry out more sophisticated and unbiased analyses, like the -omics technologies.

SC cultures have been widely used to elucidate basic aspects of SC biology, that is, mechanisms driving myelination and SC conversion into repair cells upon nerve injury, and those regulating cell proliferation, which is a key feature of rSCs in response to nerve damage.

For instance, by using rodent SC cultures, it was found that the intracellular level of cyclic AMP determines SC fate through activation of NRG1 signaling. Specifically, low levels of cyclic AMP stimulate SCs proliferation in vitro, while high levels promote myelin differentiation (Arthur-Farraj et al., 2011). Remarkably, this mechanism mimics what happens in vivo, where recently a new player in myelination, Laminin 211, has been added to this molecular pathway (Ghidinelli et al., 2017).

In addition, some of the mechanisms controlling myelination have also been characterized using SC primary cultures. These studies revealed that a long-term treatment with high doses of cAMP promotes myelination in both rat and mouse SCs, but not in human SCs (Arthur-Farraj et al., 2011; Morgan et al., 1991). Indeed, although human SCs respond to high doses of cAMP by changing morphology and cell cycle exit, they fail to reactivate the expression of key markers of myelination (Monje et al., 2018).

SC cultures have also helped to understand how mature SCs survive in the distal stump of transected nerves even in the absence of axonal contact. This is a crucial aspect of the regeneration process, which differentiates mature SCs from SC precursors whose survival strictly depends on axonal contact. These studies led to the identification of an autocrine signaling involving TGFβ (Transforming growth factor β), and of the pro-survival role of STAT3 (Benito et al., 2017b; Meier et al., 1999; Parkinson et al., 2001).

SC cultures are also very suitable for cell migration studies, a crucial process during PNS development but also in regeneration and remyelination after nerve injury. Indeed, several signaling molecules that promote SC motility and migration have been identified thanks to studies employing SC primary cultures, along with additional in vitro approaches such as the scratch assay (Figure 4d). For instance, it was found that TGF-β1 significantly increases SC migration and invasion through matrix metalloproteases MMP-2 and MMP-9 (Heermann & Schwab, 2013; Muscella et al., 2020).

A general advantage of cell cultures is the possibility to change the coating of the culture dishes to assess the adhesion to specific factors or matrices, and how the cells modulate their biological activity and responsiveness accordingly. This has been successfully adapted to SC primary cultures and most of the present knowledge on SC mechano-biology derives from these kind of in vitro studies.

By manipulating the stiffness of the adhesion milieu, recent studies highlighted the strong influence of mechanical stimuli on the activation of signaling pathways, that is, the Hippo pathway one, involved in the control of SC proliferation and myelination behavior (Belin et al., 2017, 2019; Poitelon et al., 2016). For example, a micro-patterned polydimethylsiloxane (PDMS) platform (where SCs can be cultured) was developed for tuning different ECM mechanical conditions, including the stiffness of the adhesion matrix. By this approach, it was shown that stiffer matrices impair the regenerative capacity of SCs through the inhibition of key regenerative markers by the engagement of RhoA and YAP/TAZ signaling pathways (Xu et al., 2021).

SC cultures represent a particularly convenient model to analyze the molecular determinants of host–pathogen interactions in which SCs represent a pathogenic target of the disease: for instance in SC cultures served as a platform to investigate how Mycobacterium Leprae binds and gets internalized by the SCs (Spierings et al., 2000). Notably, they were instrumental to identifying laminin and dystroglycan as the receptors exploited by the bacterium to adhere on SC membrane, and for the ensuing cell invasion and damage to peripheral nerves, which is a primary event in leprosy neuropathy (Mietto et al., 2020; Rambukkana et al., 1998).

Moreover, SC culture can be used as an easy model to screen for the presence of autoreactive antibodies in human sera from patients with inflammatory and autoimmune neuropathies as well as for the detection of novel non-myelin pathogenic epitopes of demyelinating neuropathies (Kwa et al., 2003).

Very importantly, current protocols allow the elimination of contaminating cells during the preparation that allows obtaining highly pure primary SC cultures, very suitable to perform reliable ‘omics’ analyses like transcriptomics, proteomics, methylomics, translatomics, and seric etomics (Arthur-Farraj et al., 2017; Negro et al., 2018; Schira et al., 2019; Shen et al., 2012; Varela-Rey et al., 2014; Weiss et al., 2016). For example, they allowed the identification of some early steps of tumorigenesis in Neurofibromatosis type 1, a tumor that arises from SCs, and are contributing to shed light on the role played by these cells in the progression and survival of different tumor types (Deborde & Wong, 2017; Parrinello & Lloyd, 2009; Roger et al., 2019).

Also, SC lines are commercially available, the most used being RSC96 and IMS32. These cultures offer several advantages for the researcher, first and foremost the fact that they can expand indefinitively. Importantly, these cell lines have contributed to elucidate many aspects of PNS biology including nerve regeneration and SC migration (Chang et al., 2013; Hao et al., 2015; Muscella et al., 2020;
Torres-Mejia et al., 2020). Despite this, a number of limitations, among them the different proteomic profile with respect to primary SCs (Ji et al., 2012), limits the transferability of the results obtained with these experimental tools to the in vivo condition.

3.1.2 | Compartmentalized cell culture systems

An interesting and very useful culture platform is the Boyden chamber, a compartmentalized cell culture system where SCs are seeded in one of the two compartments separated by a microporous membrane (Figure 4c).

Boyden chambers are particularly suitable for the study of SC response to chemokines or putative chemotactic factors, as they allow monitor of SC movements across compartments through which the cells can migrate or extend processes when appropriately stimulated.

For example, they have been employed to examine the role of intracellular GTPases in SC migration after stimulation with chemotactic factors, and how SCs modulate the expression of specific membrane proteins in response to specific stimuli, for example, Prohibitin-2, a protein involved in SC-axon interaction, expressed in SC pseudopods after addition of neuronal membranes to the distal compartment (Poitelon et al., 2015; Yamauchi et al., 2004).

Another very useful cell culture system is microfluidic devices (Figure 4c), where cells are plated in two or more compartmentalized chambers that can be coated with specific adhesion substrates, filled with different culture media (e.g., containing specific growth factors), or seeded with different cell types. Chambers are separated by micrometers-sized connections, which impede the movement of whole cells across the compartments, but allow the passage of subcellular parts. Importantly, the chambers, which can be custom-designed with specific shapes and geometry, hold the great advantage to generate chemical gradients across the compartments.

These systems are especially suitable for studying the interplay between SCs and neurons, in particular for identifying biomolecules produced by either neurons or SCs to communicate with each other. Indeed, while the two cell types are plated separately, their axons/pseudopods can exit the cell body chamber and make contact. Therefore, this represents an amenable system to test the effects of putative chemotactic agents on SCs, and to monitor SC polarization in response to specific stimuli (Negro et al., 2018; Park et al., 2006). By these approaches, it was found that SCs undergo a profound cytoskeletal remodeling in response to environmental signals like hydrogen peroxide, and extend pseudopods toward the stimulus (Poitelon et al., 2015), which are sites of local protein synthesis (Negro et al., 2018; Poitelon et al., 2015).

In conclusion, microfluidic devices can be used for many purposes, and new platforms for cell culture and proliferation, whether for simple single-layer (2D) cell seeding processes or 3D configurations, will also be increasingly used. Furthermore, although primarily used to study the intercellular communication between neurons and glial cells, the devices can be implemented with additional cell types.

3.1.3 | Co-cultures of primary SCs with axons

Co-cultures of SCs and neurons in culture dishes or in other compatible devices are widely used in vitro approaches that allows studying several physiological and pathophysiological mechanisms of Schwann cells and how they interact with the neuronal component. (Chen et al., 2019c; Negro et al., 2016, 2018) (Figure 4b). For example, myelination has been extensively studied in normal and pathological conditions using co-cultures of DRG neurons and SCs (Chan et al., 2004; Trimarco et al., 2014; Zhang et al., 2009), or by co-culturing immortalized SC cell lines and adult rat DRG or MN-like cells (Eldridge et al., 1987; Sango et al., 2011; Takaku et al., 2018). In the first approach, SCs are plated to seeded DRG neurons. Specific myelination media is added to induce myelination, which is usually achieved within the next 12-28 days. By this approach many actors involved in the myelination process have been characterized (Bolis et al., 2009; Chen et al., 2019c; Su et al., 2016), as well as molecules localizing in internodes, nodes of Ranvier, and adjacent paranodal and juxtaparanodal regions involved in axon-glial communication (Eshed et al., 2005; Zhang et al., 2012). DRG-SCs co-cultures have been also successfully used to model in vitro Charcot Marie Tooth disease type 1A (CMT1A). MNs survived more and extended longer axons when co-cultured with SCs compared to MNs alone and, importantly, SCs differentiated and formed myelin sheaths within 3 weeks (Nobbyo et al., 2004). (Duregotti et al., 2015a; Hyung et al., 2015). The same co-cultures allowed the identification of a set of signals involved in the response of SCs to neuronal damage (Duregotti et al., 2015a; Negro et al., 2016; Rodella et al., 2016, 2017).

SC and neuron co-cultures are also extensively used to study the mechanisms driving SC response to PNS injury. For example, they allowed to study SC proliferation in vitro following neuronal damage, showing that this ability, as well as that of degrading myelin, is independent of the presence of macrophages (Fernandezzavalle et al., 1995; Salzer & Bunge, 1980b).

Studies employing compartmentalized cell culture systems such as Camperot and microfluidic chambers and SC-neuron co-cultures showed that injured axons signal to SCs and vice versa. For instance, axotomy activates the erbB2 signaling to initiate demyelination and promoting SC cytoskeletal remodelling, pivotal to clearing of the distal stump of the axon (Guertin et al., 2005; Vaquie et al., 2019). SCs in turn can even assist axon growth of CNS neurons such as retinal neurites (Fallon, 1985) and axon regeneration of axotomized DRG neurons in culture in a c-Jun dependent manner (Arthur-Farraj et al., 2012).

Co-cultures can also be set up using stem cells: a stable and long-lasting myelinating co-culture of sensory neurons derived from human induced pluripotent stem cells (iPSCs) and rat SCs has been recently established and used to study axoglial interaction (Clark et al., 2017).

Thus, co-cultures have greatly contributed to and improved our understanding of PNS development, regeneration and myelination, and will help deciphering the still unknown signaling mechanisms occurring between SCs and axons during the same processes (Wilson...
et al., 2021). A future, key target for the field is the generation of myelinating co-cultures composed of human SCs and human neurons.

### 3.2 Methods to study SCs in vivo

**In vitro** approaches fail to replicate all aspects of SC behavior within the context of a whole organism. In addition, primary SCs isolated from enzymatically and mechanically disrupted nerves resemble more the dedifferentiated SCs that are observed after nerve injury than the mature, differentiated ones, thus introducing a sort of "methodological bias" that might restrict the general value of *in vitro* results (Liu et al., 2015).

Accordingly, it is often required that *in vitro* experiments be complemented by analyses performed under conditions that better reproduce the environment and the complexity of SC interactions with neuronal axons, with other cell types and with the extracellular matrix.

The generation of mouse genetics significantly facilitated SC research as it allowed to study SC physiology and pathology *in vivo*, that is, respecting their natural anatomy and interactions with nearby cells.

While mice expressing fluorescent proteins in SCs have provided insights into SC morphology, plasticity, and relationship with motor axons, the knocking-out or overexpression of selective genes has allowed identification of specific gene signatures/molecular pathways defining SC identity in physiological and pathological contexts (Jang et al., 2016; Miyamoto et al., 2018; Schulz et al., 2014).

Alternative to transgenic models is the *in vivo* gene manipulation of SCs by injection of engineered viral vectors into the sciatic nerve (Gonzalez et al., 2014) or of DNA-encoding plasmids followed by nerve electroporation (Ino & Iino, 2016).

#### 3.2.1 CRE recombinase-expressing mouse lines

Genetically modified mice are widely used to study genes of interest in a tissue-specific manner, among them those based on the Cre-loxP system based on the recombinase enzyme Cre, which is able to specifically cut DNA sequences that are flanked by two loxP sites (Kim et al., 2018). Two different genetically engineered mouse lines, Cre- and loxP-containing, respectively, are developed independently and then crossed to generate offspring with the tissue-specific gene deletion. The choice of the appropriate promoter to drive Cre recombinase expression is fundamental to properly target the cell population of interest. A number of SC Cre-driver mouse lines have been generated to date, and have been employed for studies of gene function and lineage tracing. An example is the PO-Cre transgenic mouse which expresses the Cre recombinase under the control of the myelin protein zero (P0) (Mpz) gene, which encodes for the SC glycoprotein P0. This transgenic mouse, first employed to inactivate adhesion molecules in SCs (Feltri et al., 1999), is currently used to knock out genes of interest (Benito et al., 2017a; Wagstaff et al., 2021). In another transgenic mouse, the Dhh-Cre, the Cre recombinase gene is under control of the mouse desert hedgehog (Dhh) promoter. In this mouse, the Cre recombinase is active predominantly during the embryonic phase in SC precursors (from E12.5) of the developing peripheral nerves, and in the testes (Jaegle et al., 2003).

To achieve a precise timing and a more accurate control of Cre activation, inducible transgenic mice lines have been developed. In these mice, Cre activation is triggered by i.p. injection of the exogenous inducer tamoxifen. An example is the mice line where the Cre-mediated recombination system is driven by the proteolipid protein 1 (Plp1) promoter, widely employed for gene function and lineage tracing studies in SCs (Gomez-Sanchez et al., 2017; Grove et al., 2017). Table 1 summarizes some of the most used SC-Cre mouse lines.

#### 3.2.2 Imaging

Given the cellular complexity of peripheral nerves, microscopy is one of the most convenient method to examine in situ SCs as well as all components of peripheral nerves, during development and degeneration/regeneration.

Although electron microscopy has been widely employed in early studies to understand myelin biogenesis and structure in healthy and pathological conditions, and it is still widely used (Friede & Martinez, 1970; Liu et al., 2019), the advent of novel staining protocols for light and fluorescence microscopy have made a step forward in the visualization of SCs under different conditions, and are currently the most used approaches. Moreover, the development/improvement of new microscopy technologies may further help to visualize SCs. One example is Correlative light and electron microscopy (CLEM), which is a combination of fluorescence and high-resolution microscopy. This method allows to gain information on both cellular function and ultrastructure on the same exact area, and has been employed to study SC morphology before and after nerve injury, and to evaluate functional and structural aspects of myelin in DRG-SCs co-cultures (Booth et al., 2019; Stierli et al., 2018).

Even though, in principle, any peripheral nerve could be used for light and fluorescence microscopy, practically all the studies are conducted on the sciatic nerve, which offers the advantage of an easy dissection and manipulation. The nerve can be fixed or frozen, paraffin embedded or even teased into single fibers.

The teasing out of individual nerve fibers offers a nice way to study, especially by fluorescence microscopy, the structure and size of myelin sheaths and the general organization and/or pathologic changes of distinct domains such as internodes, paranodal regions, and the Ranvier’s nodes (D’Este et al., 2017; Traka et al., 2002).

The whole frozen or paraffin-embedded nerve can be easily cut by a cryostat or a microtome, both longitudinally and transversely and then processed for light and immunofluorescence microscopy. Nerve sections are frequently employed to detect normal or degenerating myelin and may contribute to define a diagnosis of neurodegenerative disorders. In particular, given that myelin is primarily...
composed of lipids, freezing-cryosectioning protocols are preferable to paraffin-embedding ones (Carriel et al., 2017a; Kiernan, 2007).

For light microscopy studies, although nerve components can be efficiently stained and imaged by conventional histology techniques, SCs identification via classical methods like Hematoxylin and Eosin or Trichrome stainings is difficult. Toluidine blue staining of nerve cross sections is, however, one of the most reproducible methods for qualitative and quantitative assessments of myelination and to monitor the number and morphology of myelinated axons following degeneration and/or regeneration. Other histochemical methods for the staining of myelin such as Luxol Fast blue and osmium tetroxide methods have also been developed and are well described (Carriel et al., 2017a; Kiernan, 2007).

Differently from light microscopy, immunohistochemistry or immunofluorescence microscopy, thanks to the use of antibodies against specific markers, allow faster and more reproducible methods to identify SCs than light microscopy. For example, different SC developmental stages are usually associated with the expression of specific transcription factors and signaling molecules (Balakrishnan et al., 2021) that can be easily identified with these microscopy techniques. The most commonly used immunological marker of SCs in both mouse and human studies (Chen et al., 2021; Longo et al., 2021) is the calcium-binding protein S100β, which is not expressed by SC precursors but appears late during embryonic nerve development (Jessen & Milsky, 2019b). Notably, S100β decline in expression in response to denervation makes it unsuitable for a deep morphological investigation of SC changes upon nerve injury (Magill et al., 2007). On the contrary, the transcriptional activator Sox10 is the only known marker constitutively expressed in both immature and mature SCs (Jessen & Milsky, 2005; Kuhlbrodt et al., 1998). Sox10 is a key regulator of SC development, and controls homeostasis and myelin maintenance of mature SCs, therefore can be stained to identify SCs in adult tissues (Bremer et al., 2011; Grove et al., 2017; Kumar et al., 2016). In addition, Sox10 is sufficient to identify SCs both after chronic denervation and during aging (Wagstaff et al., 2021). Non-myelinating SCs constitutively express the gial fibrillary acid protein (GFAP), a cytoskeletal protein, while myelinating SCs express it upon the loss of contact with their axons, after nerve injury or in peripheral neuropathies with axonal degeneration (Jessen et al., 1990). Therefore, an increased staining of GFAP in the PNS is considered a hallmark of activation and enhanced reactivity of myelinating SCs.

No specific markers of aged SCs have been reported so far, rather the impairment of rSCs activation in aged mice in response to nerve damage results in a dysregulation of markers commonly used to identify this cell type (Painter, 2017).

A great improvement in the study of SCs in vivo via microscopy has been provided by the generation of transgenic mice expressing fluorescent proteins specifically in SCs.

The first, still used, transgenic mouse expresses a cytoplasmic enhanced green fluorescent protein (EGFP) driven by the mouse proteolipid protein promoter (PLP) (Mallon et al., 2002). In these mice, developed to label oligodendrocytes in the CNS, the EGFP is detectable in both myelinating and non-myelinating SCs of peripheral nerves. Given that EGFP expression is not influenced by the activation status of SCs, this transgenic mouse is ideal to monitor SC migration after injury to direct nerve re-growth, to visualize SC interactions with the axons and the cellular environment, and SC phagocytosis of nerve debris (Cattin et al., 2015; Chen et al., 2019a; Dun et al., 2019).

These transgenic mice have been used, in combination with markers specific for different cell types, to show that upon nerve damage, polarized blood vessels direct the migrating cords of SCs across the bridge (Cattin et al., 2015). Moreover, whole-mount staining of PLP-EGFP muscles provides a useful research model to follow the time course of peripheral nerve regeneration (Dun & Parkinson, 2015) (Figure 5a). Indeed, after nerve damage, sciatic nerves can be collected and stained with antibodies specific for axons (against neurofilaments) or growth marker such as GAP43 or SG10 to evaluate the rate of regeneration and how this rate correlates with SC activation. Thanks to this approach, it has been shown that after nerve transection, the proximal nerve stump starts re-growing alone, without SCs support. Indeed, SCs begin to migrate later on, when they

### Table 1: Schwann cell-Cre mouse lines

| Mouse Line     | Promoter                           | Expression                                      | Inducible     | Reference                  |
|----------------|------------------------------------|-------------------------------------------------|---------------|----------------------------|
| P0-Cre         | Myelin protein zero (P0) (Mpz)      | SCs Periventricular cells neural-crest derived progenitors in the heart | No            | Feltri et al., 1999        |
| Dhh-Cre        | Mouse desert hedgehog (Dhh)         | SC precursors Pre-Sertoli cells Endothelial cells Sertoli cells | No            | Jaegle et al., 2003        |
| Plp-CreERT2    | Proteolipid protein (myelin) 1 (Plp1) | SCs Oligodendrocyte | Tamoxifen inducible | Doerflinger et al., 2003   |
| SOX10-CreERT2  | SRY (sex determining region Y)-box 10 (Sox10) | Neural crest lineage (SCs and Oligodendrocyte included) | Tamoxifen inducible | McKenzie et al., 2014     |
| P0-CreERT2     | Myelin protein zero (P0) (Mpz)      | Developing and adult SCs and Oligodendrocyte lineage | Tamoxifen inducible | Leone et al., 2003        |
localize in front of the neuron allowing a polarized axonal extension (Chen et al., 2019a).

An innovative tool to monitor migration of individual cells is the Confetti mouse model, in which individual cells have been genetically labeled by a stochastic combination of fluorescent proteins (a combination of four fluorophores) in a cell type-specific manner, in order to track them in vivo (Snippert et al., 2010). These mice have been crossed with Tamoxifen inducible P0-Cre mice to induce the stochastic expression of fluorophores specifically in SCs, and then to follow their activation and migration upon injury. These studies revealed that both myelinating and non myelinating SCs undergo extensive remodeling after nerve damage and migrate through the bridge by forming cellular cords (Stierli et al., 2018).

Recently, the development of novel transgenic mice expressing fluorescent proteins in immature or myelinating SCs, in combination with advanced microscopy technologies, have provided for the first time a direct evidence for two basic assumptions in SC biology: that myelin and Remak cells generate the elongated cells that build the Bungner bands in injured nerves, and that the same cells remyelinate after regeneration. Furthermore, by the same approach we now know that rSCs are 2- to 3-fold longer than myelinating and Remak cells, and 7- to 10-fold longer than immature SCs. Remarkably, when rSCs transit back to myelinating cells they shorten ~7-fold, yet another example of extraordinary plasticity (Gomez-Sanchez et al., 2017).

Thus, the use of transgenic animals expressing fluorescent proteins specifically in SCs, together with live imaging techniques,
provide valuable information about different aspects of the SC differentiation and response to a neuronal damage.

3.2.3 Assessing SC roles in nerve regeneration

The role of SCs in nerve regeneration has been mainly elucidated in models of traumatic injuries of the sciatic nerve thanks to its favorable location, size, and easy manipulation (Florio et al., 2018; Gerber et al., 2019; Grove et al., 2020).

Compression and transection of the nerve, although characterized by different regenerative outcomes, elicit similar adaptive responses by SCs. Sciatic nerve compression interrupts all axons while preserving connective tissue sheaths and SCs, allowing regeneration to occur in 3–4 weeks. Conversely, the complete transection of the nerve disrupts axons, connective sheaths and basal lamina: also in this case regeneration takes place, but the process is less efficient and functional recovery is generally poor (Dun & Parkinson, 2015; Nguyen et al., 2002).

Nerve cut is the method of choice to study a structure called bridge, a newly formed tissue essential to re-connect the proximal and distal stumps to allow regenerating axons to cross the nerve gap. The bridge is composed mainly of ECM, neo-vessels, inflammatory cells, and fibroblasts: a favorable environment where migrating SCs, coming from both nerve stumps, form bundles of cells that guide axon regeneration (Cattin et al., 2015; Dun et al., 2019). Accordingly, sciatic nerve transection is widely used to monitor SCs migration in vivo after damage, their ability to drive and guide axonal growth, and their interaction with the nerve and the environment (Min et al., 2021).

Sciatic nerve injury performed in genetically modified mice with SC-specific gene knockout or SC-specific gene expression, in combination with in vitro/ex vivo assays (e.g., imaging and/or electrophysiology), have provided important clues about the specific role of individual genes in nerve repair, and characterized the molecular profile of activated SCs. For instance, functional assays such as SSI (sciatic static index) and SFI (sciatic functional index) measurements, the toe pinch test or the assessment of Rotarod performance (Arthur-Farraj et al., 2012; Baptista et al., 2007; Brugger et al., 2017) are commonly undertaken to assay the effect of a specific SC mutation on sensory–motor coordination in animals.

Beside them, the CMAP (compound muscle action potential) recordings allow the assessment of nerve conduction velocity and of the number of functional axons in mice after injury. In this test, the degree of functional recovery correlates with the efficiency of remyelination and axonal regeneration (Mindos et al., 2017).

Axonal regeneration can be evaluated also by imaging, employing antibodies specific for axon growth markers such as GAP43 and SG10, or anti-CGRP and Galanin that label regenerating DRG and motor neurons (Arthur-Farraj et al., 2012; Carriel et al., 2017b; Hervera et al., 2018).

Retrograde tracing is another well-characterized method which consists of the incubation or direct injection of a neuronal retrograde tracer (e.g., True Blue and Fluorogold) into the nerve at a certain distance from the injured area, or in the muscle. After retrograde labelling of neurons, the collection of spinal cord and/or DRG permits to evaluate the number, distribution and axonal projections of both regenerating motor and sensory neurons in the PNS (Arthur-Farraj et al., 2012; Wagstaff et al., 2021). For a comprehensive review of the methodologies applicable to the study of PNS regeneration, see (Navarro, 2016).

The combination of genetically engineered mice with functional assays allowed the identification of a set of rSC markers, for example, the transcription factors Jun, Sox2, Egr1, Olig1, and Pax3 (Arthur-Farraj et al., 2012; Blake & Ziman, 2013; Parrinello et al., 2010; Topilko et al., 1997).

In addition, by the same approaches, it was found that c-Jun, STAT3, histone H3, and the tumor suppressor protein Merlin (Arthur-Farraj et al., 2012; Benito et al., 2017b; Ma et al., 2016; Mindos et al., 2017) are exclusively engaged by rSCs, at variance from a number of factors/pathways that participate both in SC development and myelination (i.e., ERK 1/2, Sox2, Zeb2, and Gpr126) (Jessen & Mirsky, 2019b; Mogha et al., 2016; Napoli et al., 2012; Quintes et al., 2016; Roberts et al., 2017).

Beside mouse models, the role of SCs in regeneration can be assessed by classical pharmacological approaches, for example, by administration, after nerve injury, of inhibitors or agonists of specific receptors, or modulators of specific signaling pathways and evaluation of their impact on SCs activity and nerve regeneration via imaging and electrophysiology (Ko et al., 2018; Zanetti et al., 2019).

For instance, intraperitoneal administration of the neuro-hormone melatonin accelerates nerve recovery through stimulation of its G-coupled receptors expressed in SCs after sciatic nerve damage (Stazi et al., 2021).

Along the same lines, intraperitoneal stimulation with pharmacological agents can help to stimulate and support signaling pathways involving SCs that are important for PNS regeneration. For example, the CXCL12-CXCR4 signaling pathway is activated following sciatic nerve injury and plays a critical role in neuronal regeneration. But, the expression of the ligand CXCL12 by SCs, declines rapidly following injury resulting in the shutdown of this molecular axis. However, it has been shown that stimulation of the CXCL12-CXCR4 pathway with the CXCR4 receptor agonist, called NUCC-390, allows a prolonged stimulation of the signaling pathway, boosting the regenerative process (Negro et al., 2017; Zanetti et al., 2019).

Although the cut or crush of the sciatic nerve remains the most widely used experimental tool to induce PNS injury, additional models are currently available. One of them consists of the cross anastomosis of the peroneal-tibial (or viceversa) nerves that has been employed to study the role of SCs in regeneration after acute damage and immediate repair, or upon chronic denervation. The tibial nerve is cut and both proximal and distal stumps are sutured into muscle to prevent regeneration. In the first case (immediate repair) the distal tibial nerve stump is immediately detached from the muscle and sutured to the freshly transected peroneal nerve, while in
the case of chronic denervation the distal tibial stump, prior to suturing to the transected peroneal nerve, is left sutured to the muscle for weeks, in order to induce chronic denervation (Fu & Gordon, 1995; Sulaiman & Gordon, 2009; Wagstaff et al., 2021).

Another experimental approach to study SCs after severe injury and chronic denervation is the spinal root avulsion model. It consists of the tearing of the spinal roots from the spinal cord without removing the vertebra, usually at the level of the cervical and lumbar segments. As the damage is performed proximally, the complete denervation leads to a chronic injury with a limited degree of axonal regeneration and poor recovery of function, because of the long distance required for motor axons to regenerate and reach their target. This approach, although technically challenging, constitutes an excellent lesion model closely mimicking the clinical situation of patients with brachial plexus injury, where regeneration takes months or even years (Eggers et al., 2019, 2020).

### 3.2.4 Profiling SCs transcriptome

The definition of the transcriptional and proteomic changes in SCs occurring during nerve development, regeneration, or aging represents a key step toward the understanding of the molecular mechanisms governing cell plasticity.

Most genome sequencing studies have been performed on the entire sciatic nerve, since the vast majority of cells in this tissue are SCs (~70%) (Stierli et al., 2018). They have provided mRNA, miRNA, and long non-coding RNA profiles (Adilakshmi et al., 2012; Arthur-Farraj et al., 2017; Viader et al., 2011a), and assessed epigenomic changes such as histone modifications or DNA methylation (Arthur-Farraj et al., 2017; Hung et al., 2015; Varela-Rey et al., 2014).

Investigations starting from whole nerves cannot profile individual SCs, due to the cellular heterogeneity of the tissue (Arthur-Farraj et al., 2012; Frob et al., 2019; Jiang et al., 2014; Liu et al., 2018; Siems et al., 2020). This limitation has been overcome by the use of GFP-PLP mice that allowed the specific isolation via fluorescence-activated cell sorting (FACS) of SCPs and developing SCs from the trunk and sciatic nerves of different embryonic stages. Analysis of RNA extracted from sorted cells, amplified and subjected to array hybridization, identified genes involved in glial cell development, differentiation, and maturation (Buchstaller et al., 2004). Recently, the combination of a novel transgenic mice expressing the red fluorescent protein tdTomato in SCs under the control of the Myelin Protein Zero (Mpz) promoter and a FACS-based approach allowed to purify SCs from control adult sciatic nerves and from different regions of transected nerves. The following RNA sequencing provided the transcriptional profile of in vivo SCs from intact nerves, from the bridge and distal stumps of transected nerves, which differs depending on their location with respect to the injury site. This study revealed that SCs from nerve bridge are different from those of other regions of the injured nerve and are reminiscent of cancer cells given the high expression of genes involved in proliferation, metabolism, and epithelial to mesenchymal transition (Clements et al., 2017). Thus, the identification of signals and pathways activated in SCs, that drive wound repair, is relevant also to cancer research and could help the identification of therapeutic targets against cancer progression.

Recent technical advances have enabled unprecedented transcriptome studies at single-cell level, obtaining individual transcriptomes of thousands of cells. For example, using the single-cell transcriptomics approach, it was possible to identify the entire cellular composition of peripheral nerves, confirming previously identified cell types and characterizing two new distinct populations of myeloid cells (Wolbert et al., 2020). The authors, using transgenic mice expressing the reporter eYFP in SCs, independently harvested myelinating and non-myelinating SCs via FACS sorting, and then performed single-cell RNA sequencing (Gerber et al., 2021). This approach is the starting point for follow-up investigations on SC behavior and plasticity in other physiological and pathological contexts, that is, aging, injury, and peripheral chronic neuropathies. However, the single-cell RNA sequencing technique may result in gene expression artifacts. For example, tissue dissociation (particularly enzymatic incubation at 37°C) and FACS sorting protocols can lead to activation/up-regulation of typical injury genes including transcription factors such as c-Jun (Adam et al., 2017; van den Brink et al., 2017). In addition, tissue dissociation processes can interfere with the viability and structural integrity of cells, implying that the downstream analysis could include cells that are no longer viable, or parts of them, thus “contaminating” the final results. Finally, considering the very low amount of the starting material, the technical limitations of the approach, and the biological variability, single-cell RNA-seq data are noisier and more complex than bulk RNA-seq data (Chen et al., 2019b).

### 3.3 Methods to study Perisynaptic Schwann cells in vivo

PSCs play a critical role in several aspects of NMJ physiology and pathology including development, maintenance, and regeneration after denervation. In addition, their direct contribution to several neuromuscular and neurodegenerative disorders is currently under debate.

Methods to study specifically PSCs are very challenging: indeed, these cells are exclusive of the NMJ, which represents an infinitesimal part of the PNS, and are very few, usually 3-5 per synapse, thus constitute an extremely rare cell population compared to axonal SCs. This has limited the development of protocols and methods to selectively purify and culture PSCs. Therefore, PSCs have been mainly studied by in vivo and ex vivo approaches. In addition, no reliable protein markers allow the unambiguous identification of PSCs and their discrimination from axonal myelinating and non-myelinating SCs, which has further slowed down the study of PSCs by classical methodological approaches.
3.3.1 Imaging

Microscopy of in vivo or ex vivo nerve-muscle preparations is the main approach to examine the morphological changes of PSCs during development and in pathophysiological conditions. Most studies are conducted in murine models, although studies in Xenopus laevis (Herrera et al., 2000) and Drosophila Melanogaster (Danjo et al., 2011; Strauss et al., 2015) have greatly contributed to the present knowledge of PSCs physiology. For instance, PSCs ablation in frog muscle showed that these cells are essential for NMJ development and for its maintenance in adult life (Reddy et al., 2003).

At the NMJ, PSCs lay over the clusters of Acetylcholine receptors (AChR) on the myofibers, which are detectable using α-bungarotoxin (α-BTX) derivatives to identify the synapse. The most widely used antibody to label PSCs in mammals recognizes the calcium binding protein S100β (Jablonka-Shariff et al., 2020), whose expression declines in response to denervation making it not suitable for deep morphological investigations of SC changes upon nerve injury (Magill et al., 2007; Perez & Moore, 1968). Other protein markers, including LNX-1, the sodium channel Nav 1.6, and TrkC proteins have also been employed. The E3 ubiquitin ligase LNX1 is selectively expressed by PSCs and not by axonal SCs, early at developmental stages, but it is downregulated after NMJ denervation (Young et al., 2005). On the contrary, Nav 1.6 and TrkC are not exclusively expressed in PSCs but also in node of Ranvier and myelinating SCs, respectively (Hess et al., 2007; Musarella et al., 2006).

Because PSCs become reactive following neuronal damage, it is important to have the ability to differentiate them from quiescent ones. One of the most robust immunological markers of the "reactive" state of PSCs is the intermediate filament protein Nestin (Kang et al., 2007), which is upregulated within 1–2 days of denervation (Magill et al., 2007; Perez & Moore, 1968). Other protein markers, including LNX-1, the sodium channel Nav 1.6, and TrkC proteins have also been employed. The E3 ubiquitin ligase LNX1 is selectively expressed by PSCs and not by axonal SCs, early at developmental stages, but it is downregulated after NMJ denervation (Young et al., 2005). On the contrary, Nav 1.6 and TrkC are not exclusively expressed in PSCs but also in node of Ranvier and myelinating SCs, respectively (Hess et al., 2007; Musarella et al., 2006).

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It was recently reported that the T-Box transcription factor 21 (TBX21) is physiologically expressed by PSCs, making it a new specific candidate marker (Jablonka-Shariff et al.). Of note, even though PSCs do not synthesize myelin, they still express proteins involved in myelination, for example, the adhesion protein Gpr126 (Jablonka-Shariff et al., 2020), meaning that the expression of proteins linked to myelination does not allow discrimination between myelinating SCs and PSCs.

The best approach to visualize PSCs remains the use of transgenic mice expressing fluorescent proteins in these cells, for example, the PLP-EGFP mice (Figure 5b,c), although terminal SCs cannot be easily individually resolved by fluorescence microscopy, as they are in direct membrane contact. This limitation was overcome by single SC photo bleaching in PLP-EGFP mice that allowed reconstructing PSC morphology by image subtraction. This approach revealed that: (1) in adult NMJs, terminal and axonal SCs belong to distinct compartments, (2) immature PSCs are much more dynamic than adult ones, (3) PSCs are capable of intrasynaptic growth after nerve injury, and (4) they rapidly engulf debris even of non-neuronal origin (Brill et al., 2011, 2013).

PLP-EGFP mice are ideal to monitor the morpho-functional changes occurring in PSCs after an acute axon terminal injury, like that induced by presynaptic neurotoxin causing degeneration of nerve terminals. In this model, a sub-lethal amount of α-Latrotoxin (a pore-forming neurotoxin from the black widow spider) or SPANs (Snake PLA2 neurotoxins) locally injected in the mouse hind limb induces a rapid Ca2+-mediated degeneration of motor axon terminals. Strikingly, nerve terminal degeneration is fully reversible, and motor axon terminals completely recover both structurally and functionally in a few days (Duregotti et al., 2015b, 2015c; Negro et al., 2017; Stazi et al., 2020). By this toxin-based experimental model, one can monitor the time course of morphological changes of PSCs, the activation of specific intracellular signaling pathways, and the effects of their manipulation on the recovery of motor function. In this regard, soon after nerve terminal degeneration PSCs, activated by alarm molecules released by injured motor axons (such as hydrogen peroxide), undergo a profound morphological remodeling, begin engulfing neuronal debris (Figure 5c), and release the chemokine CXCL-12α to foster regeneration by promoting axon terminal regrowth (Duregotti et al., 2015b; Negro et al., 2017).

PLP-GFP mice are particularly convenient to monitor the expression of specific membrane receptors, for example, melatonin receptors expressed by PSCs throughout the degeneration-regeneration process (Stazi et al., 2021). Recently a new methodology called NMJ-morph, a Fiji plug in originally created for analysis of motor nerve terminal, has been modified to allow morphometric analysis of PSCs, after immunohistochemistry staining, both in human and mouse (Alhindi et al., 2021).

3.3.2 Ca++ imaging

An important feature of PSCs biology is their ability to sense NMJ activity. Changes in cytosolic Ca++ concentrations are commonly used to assess excitation, activation, and responsiveness to cell stimulation in all cell types, including PSCs. Ca++ indicators like Ca++ green or Fluo-4 can be selectively electroporated in PSCs in ex vivo nerve-muscle preparations from different animal models, including amphibians and mammals, to measure Ca++ transients by fluorescence microscopy in several experimental paradigms. This experimental set up allows to collect very accurate measurements of Ca++ responses at single PSCs depending on the frequency and strength of synaptic activity, as it occurs at poly-innervated NMJs during maturation (Darabid et al., 2013b, 2018) and at the adult NMJ (Jahromi et al., 1992; Robitaille, 1998). In addition, by the same approach one can assess the impact on Ca++ responses of different molecules that can be added to the muscle–nerve preparations, for example, activators of purinergic receptors (Darabid et al., 2018). Recently, in a SOD1 mouse model of ALS, PSCs were loaded with the fluorescent Ca++ indicator Rhod-3.
3.3.3 PSC transcriptional profiling

Laser capture microdissection (LCM) is a technique that allows the laser-assisted microdissection and subsequent collection of a microscopic area of a tissue thanks to its direct visualization under a microscope. Initially developed to collect specific parts of cryosliced tumor samples, this technique has been adjusted to dissect NMJs from muscle cryo-sections to profile the transcriptome of the neuromuscular synapses (Ketterer et al., 2010; Nazarian et al., 2005). This method has been recently used to microdissect NMJs from PLP-EGFP mice in order to obtain a transcriptome strongly enriched in PSCs transcripts during the time course of motor axon terminal degeneration and regeneration induced by a neurotoxin (Negro et al., 2017). This transcriptome revealed that PSCs express the chemokine CXCL12α in response to injury, which, by engaging the CXCR4 receptor on the motor nerve stump, promotes axonal re-growth and neurotransmission rescue. Of note, a chemical agonist of CXCR4 stimulates nerve regeneration in several conditions, recapitulating CXCL12α action (Negro et al., 2019; Zanetti et al., 2019; Stazi et al., 2020).

Hence, NMJ microdissection coupled to transcriptomics is a powerful source of candidate molecules with pro-regenerative potential. Nonetheless, LCM leads to the collection of all the cell types present at the NMJ, that is, the nerve terminal, a part of the myofiber and the PSCs, which all contribute to the transcripts of the dataset, therefore the additional investigation is required to assign a transcript to a specific cell type. To overcome this limitation, Castro and colleagues recently developed a novel method to specifically isolate PSCs from whole muscles. They found that PSCs can be identified by the combined expression of two protein markers, S100β and neuron-glial antigen-2 (NG2) protein. Double transgenic mice generated by crossing a NG2-dsRED mouse with a S100β-GFP mouse enabled the selective isolation of PSCs by FACs (Fluorescence-Activated Cell Sorting) from collagenase-digested skeletal muscles. Coupled with RNA sequencing, this approach led to the first cell-specific transcriptome of PSCs (Castro et al., 2020). The analysis revealed that PSCs express a battery of genes involved in the regulation of synaptic activity, synaptic pruning, and synaptic maintenance, a unique gene expression signature that distinguishes PSCs from all other SCs. The same authors also crossed the ALS mouse model SOD1G93A with the NG2-dsRED - S100β-GFP line, demonstrating that the labeling approach can be extended to several disease models to study the role of PSCs over disease progression (Castro et al., 2020).

With these tools, it is now possible to determine the cellular and molecular determinants of PSC differentiation, maturation, and function at the NMJ, as well as to ascertain the contribution of PSCs in: (i) NMJ repair following injury, (ii) NMJ degeneration during normal aging, and (iii) the progression of neuromuscular diseases (e.g., ALS and spinal muscular atrophy).

4 CONCLUSIONS

SCs play key roles in nerve development, myelination, regeneration following nerve injury, in neurodegenerative conditions and in aging. Expanding our knowledge on these cells and the way they interact with other PNS components will contribute to the understanding of the pathophysiology of many neurodegenerative diseases, of aging, as well as to devise new therapeutic strategies to counteract them. Recently, the emerging role played by PSCs in aging and in the progression of neuromuscular diseases, such as ALS and spinal muscular atrophy, has posited them as valuable therapeutic targets. Unfortunately, the study of these cells is complicated by their low number in muscle tissue, their localization restricted to the NMJ, the lack of specific markers, and the limitations of in vitro models.

It is clear, therefore, that the development of alternative or complementary methods to study SCs and PSCs is essential. To date, several experimental approaches have been developed, some of them displaying shortcomings. In vitro approaches, despite their wide use, cannot reproduce the complexity of the whole organism. Moreover, SCs associated with the nerve are quite different from the same cells in primary cultures. In fact, once detached from the axons during the isolation/purification procedure, they lose their characteristics of mature cells (e.g., they reduce the expression of myelin-associated proteins) and acquire a new phenotype that make them more similar to activated SCs. Moreover, SC cultures cannot reproduce in vitro all aspects of the complex intercellular interplay between neurons and SCs that drives myelination as well as axonal regeneration.

To overcome these issues, co-cultures of SCs and different types of neurons have been developed, with DRG sensory neuron-SCs co-cultures successfully used as in vitro models of Charcot Marie Tooth disease type 1A (CMT1A) (Nobbio et al., 2004). Nonetheless, these co-cultures cannot always entirely mimic the complex cellular architecture of native tissue. For example, some protocols for preparing DRGs select only small-caliber sensory neurons, and the extent of myelin formed in the co-cultures fails to reproduce in vivo myelination, compromising the study of the axonal sorting mechanism (Taveggia & Bolino, 2018). In addition, most studies performed on static cultures do not reproduce the dynamic flow conditions encountered in biological systems.

On the other hand, studies with transgenic mice in combination with biochemical techniques and/or electron, optical, or fluorescence microscopy, have helped to address and overcome many of the major limitations of in vitro studies. Notably, they allow a better characterization of the role played by SCs and PSCs during development, in pathophysiological conditions and nerve regeneration. For example, the use of transgenic mice expressing fluorescent proteins specifically in SCs has significantly improved the evaluation of their morphological reaction in response to nerve damage and of their interaction with the cellular...
environment. At the same time, they also stimulated the development of dissection and purification techniques that allowed the specific study of the molecular profile of SCs, avoiding contamination with other cell types. A further improvement is given by the combination of -omics technologies with novel methods to: (1) harvest and separate SCs via FACS sorting, (2) microdissect NMJs from muscle cryoslices to enrich biological samples in PSCs, and (3) purify PSCs via identification of specific markers. By these technological advancements it has been possible, for the first time, to determine SCs and PSCs transcriptomes at the single-cell level.

In conclusion, although many experimental methods have allowed and still allow the study of SCs, the improvement of existing techniques and the development of new methodological approaches to study and manipulate them is crucial. Moreover, given the increasing interest around PSCs, due to their role in many neurodegenerative contexts and in aging, the development of new tools to identify, isolate and manipulate them would contribute towards a better understanding of PSCs biology, and the development of therapies of potential medical relevance.

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CONFLICT OF INTEREST
The authors of this manuscript declare no conflict of interest.

AUTHOR CONTRIBUTION
S.N., M.P., and M.R. conceived the manuscripts, reviewed the literature, and wrote the paper.

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