Schwann cells are axo-protective after injury irrespective of myelination status in mouse Schwann cell/neuron cocultures

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Running title

Schwann cells protect injured axons.

Summary Statement

We describe a novel method for compartmentalised mouse myelinating Schwann cell-DRG neuron cocultures and show that Schwann cell myelination status does not influence their axon protective effect after injury.

Keywords:

Mouse, Schwann cell, Dorsal root ganglion neuron, Myelination, Coculture, Axon degeneration, Wallerian degeneration

Abstract

Myelinating Schwann cell (SC)– dorsal root ganglion (DRG) neuron cocultures are an important technique for understanding cell-cell signalling and interactions during peripheral nervous system (PNS) myelination, injury, and regeneration. While methods using rat SCs and neurons or mouse DRG explants are commonplace, there are no established protocols for compartmentalised myelinating cocultures with dissociated mouse cells. There consequently is a need for a coculture protocol that allows separate genetic manipulation of mouse SCs or neurons, or use of cells from different transgenic animals to complement *in vivo* mouse experiments. However, inducing myelination of dissociated mouse SCs in culture is challenging. Here we describe a new method to coculture dissociated mouse SCs and DRG neurons in microfluidic chambers and induce robust myelination. Cocultures can be axotomised to study injury, used for drug treatments, and cells can be lentivirally transduced for live imaging. We used this model to investigate axon degeneration after traumatic axotomy and find that SCs, irrespective of myelination status, are axo-protective. At later timepoints after injury, live imaging of cocultures shows that SCs break up, ingest, and clear axonal debris.

Introduction

Dissociated myelinating SC-DRG cocultures from rats were first developed by the Bunge laboratory in the 1980's to investigate PNS myelination in a more dynamic way (Bunge et al., 1989; Eldridge et al., 1987). These cultures have been used to make seminal discoveries in uncovering the cellular and molecular mechanisms of SC myelination alongside *in vivo* investigation. These include how the inner SC membrane (mesaxon) advances to myelinate axons, and the role of β-neuregulin-1 (βNRG1) and polarity proteins in SC myelination (Bunge et al., 1989; Chan et al., 2006; Shen et al., 2014; Taveggia et al., 2005). Similarly, SC-DRG cocultures have been useful in demonstrating how SCs proliferate after axon injury, transfer metabolites, such as pyruvate, to delay axon degeneration, how placental growth factor (Plgf) regulates axon fragmentation by SCs and how SC JUN promotes axon outgrowth after injury (Arthur-Farraj et al., 2011; Babetto et al., 2020; Salzer and Bunge, 1980; Vaquié et al., 2019). The use of a coculture system to study axon-SC interactions during axon degeneration and regeneration offers some advantages over *in vivo* approaches as both neurons and SCs can be genetically manipulated separately and live imaged with ease. While there are available methodological descriptions for dissociated cocultures using rat SCs with either rat or mouse DRG neurons, in addition to fully compartmentalised rat cocultures, and mouse explant cocultures, there are no current published protocols for fully dissociated and compartmentalised mouse myelinating cocultures (Taveggia and Bolino, 2018; Vaquié et al., 2018). Indeed there has only ever been one laboratory detailing convincing myelin formation in dissociated mouse myelinating SC-DRG neuron cocultures, however this was never published as a step by step detailed protocol (Stevens and Fields, 2000; Stevens et al., 1998). In the last twenty years, there have been no published studies demonstrating myelination in fully dissociated mouse SC-mouse DRG cocultures. This has largely prevented the use of cells, particularly SCs, from transgenic mice in cocultures and thus restricted the ability to study SC-axon interactions in a system that can be readily manipulated and live imaged and results directly applied back to *in vivo* findings in the same species.

The consensus within the field is that inducing myelination in dissociated mouse SCs is challenging. Certainly, induction of myelin differentiation with cyclic adenosine monophosphate (cAMP) analogues or elevating agents, such as forskolin, is more difficult in mouse SC monocultures compared to rat SC cultures. This is because mouse SCs require additional exogenous β-neuregulin-1 (βNRG1), plating on poly-L-lysine (PLL) instead of poly-D-lysine (PDL), and low concentration horse serum as opposed to foetal calf serum (Arthur-Farraj et al., 2011; Päiväläinen et al., 2008; Stevens et al., 1998). Protocols exist where endogenous mouse SCs are used to myelinate dissociated or non-dissociated DRG explant

cultures. (Harty et al., 2019; Numata-Uematasu et al., 2023; Shen et al., 2014; Stettner et al., 2013; Sundaram et al., 2021). Furthermore, another protocol seeded exogenous SCs onto non-dissociated DRG explant cultures (Päiväläinen et al., 2008). Other laboratories seed cultured rat SCs onto dissociated mouse DRG axons (Taveggia and Bolino, 2018). Use of dissociated or non-dissociated DRG explants cultures precludes many experimental uses, such as using SCs from different transgenic animals and separate transfection of SCs and neurons with viruses for live imaging or genetic manipulation, and easy use of microfluidic chambers to allow injury studies and separate drug treatments to neurons or SCs. The reason for this is that antimitotics cannot be used in dissociated or non-dissociated DRG explant cultures as this depletes SCs, and the culture quickly becomes contaminated with other non-neuronal cell types, such as satellite cells and fibroblasts migrating out of the DRG. Furthermore, use of exogenous SCs in a non-dissociated DRG explant culture, after a period of antimitotic exposure, as developed by Päiväläinen et al., 2008, still risks potential contamination from endogenous SCs and satellite glia migrating out of the DRG explant over time. This occurs because antimitotic treatment is unlikely to fully penetrate the whole DRG without prior dissociation. Additionally, a compartmentalised culture system cannot be readily used with non-dissociated DRG explant cultures (Päiväläinen et al., 2008).

Recent studies using SC-DRG cocultures to investigate axon-SC interactions after injury have found differing results. A study using dissociated rat myelinating SC-DRG neuron cocultures in microfluidic chambers found that the presence of SCs accelerated the disintegration of axons after traumatic axotomy at late timepoints (Vaquié et al., 2019). A second study seeded rat SC on mouse DRG axons, in microfluidic chambers in short term culture, but did not induce them to myelinate, and they found that the presence of SCs delayed axon degeneration (Babetto et al., 2020). Certainly data from *in vivo* studies, first in zebrafish and later in mouse have shown that SCs do participate in the breakup of the axon (Catenaccio et al., 2017; Rosenberg et al., 2014; Vaquié et al., 2019; Villegas et al., 2012). It is possible that the different conclusions of the two coculture studies could be confounded by a species difference, given Babetto et al., 2020 combined rat and mouse cells whereas Vaquié et al., 2019 studied solely rat cells. Another explanation is that myelination status of the SCs may influence the outcome of the experiment as Vaquié et al., 2019 induced myelination prior to injury whereas Babetto et al., 2020 did not. Given these outstanding questions we investigated whether SCs accelerate or delay axon degeneration, and whether the outcome depends upon myelination status, in a fully mouse SC-DRG coculture system.

We describe a detailed protocol for setting up dissociated mouse myelinating SC-DRG neuron cocultures in microfluidic chambers. We demonstrate how these compartmentalised

cocultures can be used for performing axotomies, drug treatments, lentiviral infection of DRG neurons and SCs separately, and live imaging. SCs can be induced to robustly express myelin markers periaxin (PRX), myelin protein zero (MPZ) and myelin basic protein (MBP), and form electron dense myelin and nodal and paranodal structures. Additionally, SCs that align with axons and are not induced to myelinate appear to ensheath multiple axons. After axotomy, cocultured SCs replicate key parts of the *in vivo* injury response, upregulating the major injury transcription factor, c-JUN (JUN), demyelinating and forming myelin ovoids (Arthur-Farraj and Coleman, 2021; Arthur-Farraj et al., 2012). We then show that after traumatic axotomy, SCs first have an axo-protective role, as severed DRG axons in the presence of SCs degenerate more slowly, compared to severed DRG axons cultured on their own. We find that both myelinating SCs and aligned SCs, which have not been induced to myelinate, are capable of delaying axon degeneration, suggesting that myelination status is not important in regulating this phenomenon. At later timepoints after axotomy, live imaging of cultures reveals that SCs help break up and ingest axonal fragments, clearing the debris.

Results

Establishing dissociated mouse myelinating SC-DRG neuron cocultures in microfluidic chambers.

To establish dissociated mouse myelinating SC-DRG neuron cocultures, we dissected DRGs from E14 mice, enzyme dissociated these (see methods), and seeded the DRG neuronal cell suspension into the top compartment of microfluidic chambers on PLL and Matrigel® coated Aclar® coverslips (Fig. 1A). DRG neurons are then purified using the anti-mitotic cytosine Arabinoside (Ara-C) and are allowed to extend axons across a 150 μm microgroove barrier into an axonal compartment for up to seven days (Fig. 1B). Due to their size, DRG neurons cannot cross this microgroove barrier, and are exclusively present in the top compartment of the microfluidic chamber. By maintaining a hydrostatic pressure gradient between the two compartments, we were able to apply this anti-mitotic solely to the DRG compartment. Postnatal day 2-4 (P2-P4) neonatal sciatic nerves were then dissected, enzyme dissociated, and Ara-C purified in DMEM/5% horse serum (HS) for 72 hours to obtain cultured mouse SCs (Arthur-Farraj et al., 2011). Ara-C was withdrawn from microfluidic chambers for 48 hours before cultured mouse SCs were seeded into the axonal compartment of the chambers, where they were allowed to align with axons and proliferate for up to one week (Fig. 1C). SCs can then be induced to myelinate over the course of approximately three weeks through supplementation of their cell culture media with forskolin (10 μM), β neuregulin-1 (10 ng ml^{-1}, βNRG1), Matrigel® (1/100) and L-ascorbic acid (50 μg ml^{-1}, Fig. 1C). Importantly, we found that L-ascorbic acid was insufficient to induce substantial myelination in our cultures, unlike in rat SC-DRG cocultures, and in the one previously published dissociated mouse SC-DRG protocol (Stevens et al., 1998). In fact, plating cocultures on laminin, adding ascorbic acid (50 μg ml^{-1}), βNRG1 (10 ng ml^{-1}) and forskolin (10 μM) induced very few myelin sheaths (Fig. S1). Only when cultures were plated on Matrigel® and further Matrigel® was added to the myelination medium for each medium change, were we able to visualise robust reproducible myelination in our cocultures (Fig. S1). Forskolin and βNRG1 were included in the myelination medium as we have previously shown that these agents can induce myelin proteins in cultured mouse SCs (Arthur-Farraj et al., 2011). For a detailed step by step description of how to set up these cultures please see our methods section.

After approximately six weeks in culture, we were able to generate cultures with only axons in the axonal compartment, cultures with SCs aligned to axons but not induced to myelinate (aligned SC), and cultures with SCs myelinating axons (myelinating SC; Fig. 1C). Myelinating cocultures and cultures with aligned SCs can be easily distinguished by phase contrast microscopy, as myelin segments could be identified as long phase bright structures

surrounding axons (Fig. 1C). In cultures with aligned SCs, they labelled readily with p75NTR, and EM analysis demonstrated SCs ensheathing multiple axons (Fig. 1D and E). Seven days of pulsed Ara-C treatment to the top compartment removed any non-neuronal cells. To confirm that axon only cultures are not contaminated by any potential surviving endogenous SCs that have migrated from the top compartment, we show no DAPI or SC specific SOX10 nuclear staining in the axonal compartment (Fig. 1F). In cocultures with aligned and myelinating SCs, we labelled SCs with antibodies against the myelin associated protein, periaxin (PRX), which is expressed in the mouse sciatic nerve around birth, at the initiation of myelination (Gillespie et al., 1994). In cultures with aligned SCs, these cells have a much more diffuse, spread-out morphology, and a subset of these cells express PRX, while in myelinating cultures, strongly PRX positive myelin segments are present (Fig. 2A-C). Quantifying the number of PRX positive myelin segments we found that there were 325.33 ± 12.3 sheaths per mm^2, comparable to what has been originally described in rat SC/DRG cocultures and two fold more extensive myelination than recently described compartmentalised rat cocultures models (Eldridge et al., 1987; Vaquié et al., 2019). Furthermore, 25.47 ± 1% of Schwann cells were myelinating in our cultures (n=3; Table 1). To confirm that cocultured myelinated SCs formed compact myelin we performed electron microscopy (EM), which revealed compact myelin formation with multiple myelin wraps and formation of readily visible major dense (MDL) and intraperiod lines (IPL; Fig. 2D). Additionally, we measured the periodicity, i.e., the distance between two adjacent major dense lines, to make sure myelin was compacted. Interperiodic distance was 12.16 ± 0.28 nm, in line with previous reports (n=3; Table 1; (Boutary et al., 2021; Fernando et al., 2016; García-Mateo et al., 2018; Giese et al., 1992; Perrot et al., 2007). Additionally, we immunolabelled cultures with antibodies to compact myelin proteins and found myelin sheaths were positive for MPZ and MBP (Fig. 2E and F). As myelinated fibres are organised into distinct domains, including the node of Ranvier, paranodal and juxtaparanodal regions, and the internode, we wanted to confirm if contactin associated protein 1 (CASPR1, also known as neurexin IV or paranodin) is confined to paranodal regions, where it normally accumulates in mature sheaths (Einheber et al., 1997; Salzer, 2015). We detected CASPR1 protein in the correct distribution, labelling the paranodal region, adjacent to regions of MPZ labelling (indicating compact myelin) in our myelinating SC-DRG neurons cocultures (Fig. 2G).

In summary our dissociated mouse myelinating SC-DRG neuron cocultures develop robust compact myelin, as evidenced by myelin protein immunostaining and EM. Our cocultures also develop nodal/paranodal structures and the myelination efficiency is comparable to dissociated rat SC/DRG neurons cocultures.

Axotomy in SC-DRG neuron cocultures replicates characteristic axonal and SC injury responses.

After injury, SCs transform into repair SCs, which are characterised by a strong upregulation of the transcription factor JUN, and myelin breakdown by SCs through a process termed myelinophagy (Arthur-Farraj et al., 2012; Gomez-Sanchez et al., 2015; Jessen and Arthur-Farraj, 2019; Parkinson et al., 2008). To test whether axons and SCs respond to injury in a similar way in our coculture model as they do *in vivo*, we performed axotomies on cultures, using a scalpel under a light microscope to cut axons at the level of the microfluidic barrier, after carefully removing the chamber. In myelinating SC-DRG cocultures, 12 hours after axotomy, we fixed and immunolabelled cultures for neurofilament light chain (NFL) and found that many axons distal to the site of axotomy had started to degenerate (n=4; Fig. 3A). Additionally, we noted a strong upregulation of JUN protein in SCs 12 hours after axotomy (Fig. 3B and C). We also saw significant JUN upregulation 12 hours after axotomy in cocultures with aligned SCs (Fig. 3D and E). Fluoromyelin labelling 48 hours post axotomy demonstrated myelin-ovoid formation, suggestive of active SC demyelination (Jung et al., 2011); Fig. 3F). We confirmed SC demyelination in our axotomised cocultures using electron microscopy, identifying characteristic demyelinated profiles surrounding degenerated axons, similar to what has been previously shown in rat SC-DRG cocultures (Fernandez-Valle et al., 1995) Fig. 3G).

In addition to traumatic axotomy, our cocultures can also be used for drug treatments. To demonstrate this, we treated only DRG cell bodies with the specific SARM1 agonist, vacor, which induces specific degeneration of axons and neuronal cell bodies but not of SCs, which are completely insensitive to SARM1 agonists (Fazal et al., 2023; Loreto et al., 2021). 50 μM vacor addition to DRG cell bodies induced axon degeneration within 6-8 hours in our cocultures (n=4, Fig. 3H). Finally, we also demonstrate that DRG neurons and SCs can be separately infected with lentiviruses (LVs) to permit live cell imaging. Here we infected mouse DRGs directly after the DRG dissociation step with an mCherry expressing LV (LV-CMV-mCherry) and mouse SCs were infected with a GFP expressing LV (LV-CMV-GFP) after Ara-C purification and prior to seeding in the microfluidic chamber (Fig. 3I). We found both mouse DRGs and SCs transduced best with LVs in suspension with slow centrifugation (see methods). Importantly dissociated mouse SCs required a much higher multiplicity of infection (MOI) than dissociated mouse DRGs (see methods
). Interestingly, we found that embryonic DRG neurons were almost completely resistant to LV transduction if they had already been cultured for 2-3 days (data not shown).

In summary, our mouse myelinating SC-DRG cocultures can be axotomised to study SC-axon interactions as they reliably replicate *in vivo* cell behaviours. Furthermore, these cocultures can be used to study drug induced neurodegeneration and both DRG neurons and SCs can be separately transduced with LVs for live imaging and genetic disruption studies.

At early timepoints after axotomy, Schwann cells are axo-protective, independent of myelination status, and at later timepoints they clear axonal debris.

Recent evidence has shown both axo-protective and axon debris clearance roles for SCs in cocultures. Babetto et al., 2020 used axotomy in rat SCs seeded on mouse DRG axons without inducing myelination, whereas Vaquié et al., 2019 studied laser axotomy in myelinating rat SC-DRG cocultures (Babetto et al., 2020; Vaquié et al., 2019). One outstanding question resulting from both studies is whether myelination status influences the axon degeneration rate *in vitro*? Therefore, we set out to use our cocultures to replicate the findings of both studies in a coculture model purely from mouse cells and also investigate whether the myelination status of SCs influences the rate of axon degeneration. We performed axotomies on axon only cultures, cultures with aligned SCs and cultures with myelinating SCs. Cocultures were fixed at three, six, nine, and 12-hours post axotomy and immunolabelled with NFL to assay axonal integrity (Fig 4A). Importantly, we found that fixed myelinated cultures needed to be permeabilised with acetone to allow full penetration of NFL antibodies to label axons in their entirety through myelinated segments (data not shown). While there was negligible degeneration in all cultures at three hours post axotomy, there was noticeably more degeneration at six hours post axotomy in axon only cultures (29.00 $\pm$ 1.64%, n=5), compared to both aligned SC, and myelinating SC cocultures (8.19 $\pm$ 2.57%, n=3 $p<0.0001$, and 8.14 $\pm$ 0.65%, n=4 respectively, $p<0.0001$). Both aligned and myelinating SC cocultures also showed significantly lower amounts of degeneration at both nine and 12 hours post axotomy in comparison to axon only cultures (axon only: 9h, 47.39 $\pm$ 1.34%, n=3, and 12h, 76.68 $\pm$ 6.60%, n=3; aligned SC: 9h, 30.05 $\pm$ 3.05%, n=4, $p=0.0004$ and 12h, 44.61 $\pm$ 4.72%, n=3, $p<0.0001$; myelinating SC: 9h, 33.12 $\pm$ 0.61%, n=3, $p=0.0059$ and 12h, 48.38 $\pm$ 7.75%, n=3, $p<0.0001$) (Fig. 4B). Between aligned and myelinating SC cocultures, there were no significant differences in amounts of axon degeneration (3h: $p=0.65$, 6h: $p=0.98$, 9h: $p=0.44$, 12h: $p=0.70$).

Since Vaquié et al., 2019 showed SCs appear to accelerate axon degeneration in rat cocultures at later timepoints after axotomy, we were interested to see whether SCs in our cocultures help break up, ingest, and clear axonal fragments. We used cocultures where axons were transduced with LV-CMV-mCherry and SCs with LV-CMV-GFP and we live imaged cultures for up to 48 hours post axotomy. In these cocultures, we visualised axons

break into large fragments surrounded by GFP positive SC processes, similar to the constricting actin spheres described by Vaquié et al., 2019. We then visualised SCs phagocytose and digest mCherry-labelled axonal fragments (Fig. 4C and D; movie S1). When we quantified this phenomenon, we found that 97.84 ± 1.462% (n=2) of SCs in our cocultures contained mCherry-labelled axonal fragments.

Thus, in a dissociated mouse coculture system, the presence of SCs delays the onset of axon degeneration at sites distal from the axotomy. Furthermore, this delay in degeneration does not appear to be reliant on the myelination status of the SCs. At later timepoints, once axons start to degenerate, SCs fragment, ingest and clear axonal debris.

DISCUSSION

Here we describe a protocol to set up dissociated mouse myelinating SC-DRG neuron cocultures in microfluidic chambers. These can be utilised to study myelination and injury responses and can be adapted for the application of drugs to different cellular compartments and for live imaging. Furthermore, as this is a dissociated and compartmentalised purely mouse cell culture system, one can utilise the vast array of transgenic and knockout lines available to study neuron-SC interactions in more detail, without concern of contaminating endogenous SCs and other non-neuronal cells that remains a drawback of current mouse dissociated or non-dissociated DRG explant models. Our cultures display several hallmarks of mature myelin sheaths, with electron dense myelin, compact myelin proteins and a CASPR immunolabelling patterning suggesting assembly of paranodal and nodal structures. Furthermore, we show comparable levels of myelination to dissociated rat SC/DRG coculture models. Axotomy of our cocultures faithfully replicates several of the key cellular events seen after nerve injury, including axon degeneration, upregulation of the key injury transcription factor JUN in both aligned and myelinating SCs and SC demyelination with myelin ovoid formation.

Achieving myelin differentiation of mouse SCs, whether in monocultures or in coculture, has historically been very difficult. There are very few published reports of robust myelination in dissociated mouse SC-DRG neuron cocultures or of myelin differentiation in mouse monocultures (Arthur-Farraj et al., 2011; Stevens et al., 1998). Furthermore, there are no detailed published protocols for either. The majority of the field use rat SCs for *in vitro* myelination studies as it is much easier to achieve myelin differentiation and there are a number of published protocols (Eldridge et al., 1987; Monje, 2020; Morgan et al., 1991; Taveggia and Bolino, 2018; Vaquié et al., 2018). Our protocol differs somewhat from the one used by Stevens et al., 1998 to induce myelination in dissociated mouse SC-DRG cocultures, as they used ascorbic acid and 10% horse serum and presumably plated their cultures on laminin, though they do not explicitly detail this (Stevens et al., 1998). In our preliminary experiments we were unable to visualise much myelination with use of laminin, ascorbic acid or indeed if βNRG1 and high concentration forskolin was added to the medium for up to four weeks. However, if we plated cocultures on Matrigel® and continuously added it to the myelination medium then we saw comparable levels of myelination in our mouse cocultures to that of rat cocultures (Eldridge et al., 1987). This approach of using Matrigel® to enhance myelination has previously been successfully employed in cultures of human iPSC sensory neurons with rat SCs and in non-dissociated mouse DRG explant cultures (Clark et al., 2017; Päiväläinen et al., 2008). Importantly, we used growth factor depleted Matrigel® as standard Matrigel® preparations contain substantial amounts of Transforming growth factor β (TGFβ)

which is a known inhibitor of myelination (Einheber et al., 1995). Additionally, the majority of rat and mouse coculture protocols plate cells on glass whereas we found cultures were healthier and myelinated better when cultured on plastic Alcar® coverslips. Using our protocol, it is possible to set up dissociated compartmentalised mouse myelinating SC-DRG neuron cocultures and take advantage of the ability to use cells from various transgenic mouse lines to study axon-SC interactions during myelination, injury, and regeneration. Furthermore, our protocol is complementary to the recently described 3D mouse myelinating SC-motor neuron coculture system using collagen hydrogels (Hyung et al., 2021; Park et al., 2021). It will be interesting in the future to up titrate the concentration of Matrigel®, which is similar to collagen hydrogels, in our cultures to see whether further increasing extracellular matrix viscosity and stiffness improves our myelination efficiency even further. While it is possible to study cell migration in microfluidic cell culture devices, transwell models offer significant advantages to study this cellular phenomenon (Negro et al., 2022). To date, there have been no published studies of successful myelination in human SC-neuron coculture systems. Despite this, rat SCs have been shown to readily myelinate human-induced pluripotent stem cell (iPSC)-derived sensory neurons and an iPSC-derived peripheral nerve organoid system which does contain myelinating SCs has recently been described (Clark et al., 2017; Van Lent et al., 2022).

We used our cocultures to confirm the recently published findings using rat SCs that the presence of SCs can delay the onset of axon degeneration and that SCs are able to clear axonal debris after degeneration is initiated. We have identified two distinct phases of SC-axonal interaction post axotomy in coculture. At timepoints up to 12 hours post axotomy we observe that SCs delay the initiation of axon degeneration, however once the axon degenerates, live imaging up to 48 hours post axotomy demonstrates that SCs help break axons into large fragments and then ingest and clear axonal debris. These findings confirm the observations of both Babetto et al., 2020 and Vaquié et al., 2019 who used rat SCs in similar microfluidic culture systems (Babetto et al., 2020; Vaquié et al., 2019). We have shown that the axo-protective observation seen by Babetto et al., 2020 does not rely on myelination status, which was an outstanding question from that study (Babetto et al., 2020). Furthermore, in an advance from previous studies, we have visualised the axo-protective and axon clearance phenomena in the same culture and shown that they are temporally separated, with axon fragmentation and debris clearance by SCs occurring at much later timepoints after axotomy. Several different culture and experimental conditions preclude direct comparison of our study with both those of Vaquié et al., 2019 and Babetto et al., 2020. These include the use of rat SCs in both prior studies and that Babetto et al., 2020 mixed rat SC with mouse DRG axons; length of time in culture, time points quantified after injury and distance from injury

and site of analysis. Babetto et al., 2020 performed axotomy on relatively short term cocultures (6 days in vitro) whereas Vaiquié et al., 2019 cultured for at least 4 weeks and in our case 6 weeks prior to axotomy. Vaiquié et al., 2019 removed nerve growth factor (NGF) prior to laser axotomy and quantified proximally (though also imaged distally) whereas both our study and Babetto et al., 2020 kept NGF in the medium, performed axotomy with a scalpel, and quantified more distally and in our case extremely distal, where only individual neurites and no axon bundles were visible. Vaquié et al., 2019 had SCs on both sides of the barrier in the microfluidic chambers whereas we seeded SCs only in the axonal/bottom compartment. Additionally, our cocultures had both forskolin and βNRG1 added to help induce myelination, whereas these factors are not required in rat myelinating cocultures. Finally, it is important to permeabilise myelinated cultures with acetone after fixation, as we did, to visualise the entire axon through heavily myelinated segments otherwise axon integrity cannot be reliably assessed in a quantitative manner (Babetto et al., 2020; Vaquié et al., 2019).

Multiple independent findings from *in vivo* studies have demonstrated that SCs help break up the axon during or slightly after programmed axonal degeneration is initiated during nerve trauma (Catenaccio et al., 2017; Rosenberg et al., 2014; Vaquié et al., 2019; Villegas et al., 2012). We have also confirmed this phenomenon in a 2-photon axotomy model in zebrafish larvae (P. Arthur-Farraj unpublished observation). More recently Babetto et al., 2020 showed that SCs upregulate glycolysis within the first two days after traumatic nerve injury and this has an axo-protective effect *in vivo* (Babetto et al., 2020). SCs have been shown to promote axonal and neuronal survival in other situations, including during axon regeneration in both the acute and chronic setting, old age and in neuropathy (Arthur-Farraj et al., 2012; Fontana et al., 2012; Hantke et al., 2014; Painter et al., 2014; Wagstaff et al., 2021). It is thus likely that SCs have both axo-protective and axon fragmentation roles, *in vivo*, after traumatic nerve injury. Future studies will be needed to detail the precise *in vivo* timing of these different cellular phases of SCs on axon integrity after nerve trauma.

One limitation of our coculture model and indeed all coculture and cell culture models that are used to investigate cellular and molecular mechanisms in nerve injury is that the cells are obtained from embryonic or neonatal animals. This is an important caveat when applying results from cell culture to adult *in vivo* nerve injury. However, while we would argue that cell culture approaches should always be used in combination with *in vivo* study it is important to remember that nerve injury is not restricted to adults and brachial plexus injury secondary to birth trauma is unfortunately a significant clinical problem (Pondaag et al., 2007). Furthermore, neonatal SCs replicate many of key cellular and molecular mechanisms seen in adult SCs after injury, including JUN upregulation, myelinophagy, promotion of axon growth and

expression of key repair program transcripts (Arthur-Farraj et al., 2012; Arthur-Farraj et al., 2017; Gomez-Sanchez et al., 2015; Parkinson et al., 2008). A future development would be to try to adapt this protocol to make a coculture model with adult mouse or even human cells.

In summary, we have described a detailed method of setting up dissociated mouse myelinating SC-DRG neuron cocultures in microfluidic chambers. These cultures can be transfected, readily live imaged, used for studying myelination and cellular responses to injury and regeneration, and used for drug studies. Most importantly SCs and DRG neurons from various transgenic mice can be used to perform *in vitro* analysis to complement findings from *in vivo* transgenic mouse studies.

Myelin segments/mm^2	325.33 ± 12.3	N=3
% Myelinating Schwann cells	25.47 ± 1 %	N=3
Myelin Interperiodic distance	12.16 ± 0.28 nm	N=3

Table 1 Quantification of myelination in dissociated mouse SC/DRG cocultures

Table demonstrating percentage of myelinating Schwann cells and number of myelin segments per mm^2 in dissociated mouse cocultures. These cocultures have comparable levels of myelination to dissociated rat cocultures. Measurement of myelin interperiodic distance shows that myelin is normally compacted. n number refers to independently prepared cultures from separate litters of mice.

Methods

Animals

All research complied with the Animals (Scientific Procedures) Act 1986 and the University of Cambridge Animal Welfare and Ethical Review Body (AWERB). Wild-type C57BL/6J mice were obtained from Charles River Laboratories and were held under standard specific pathogen free conditions.

Immunocytochemistry

Cells were fixed for 10 minutes in 4% paraformaldehyde (PFA; Electron Microscopy Sciences) diluted in phosphate buffered saline (PBS) at RT. Axon only cultures related to Figure 1 were permeabilised in PBS + 0.5% Triton (Merck) + 5% horse serum (HS, Thermo Fisher - 16050130) + 5% donkey serum (DS, Merck - D9663) at room temperature (RT) for 1 hour. For the purposes of quantifying the rate of axon degeneration (Figure 4) both axon only cultures and cocultures with SCs were permeabilised in 50% Acetone for 2 minutes, 100% Acetone for 2 minutes, 50% Acetone for 2 minutes (all at RT), and then blocked in PBS + 0.5% Triton + 5% HS + 5% DS at RT for 1 hour. Myelinating cocultures stained for MBP, MPZ or CASPR were permeabilised in 50% Acetone for 2 minutes, 100% Acetone for 2 minutes, 50% Acetone for 2 minutes (all at RT), 100% Methanol at -20°C for 10 minutes, and then blocked in PBS + 5% HS + 5% DS at RT for 1 hour. Cultures were immunolabelled by incubating overnight at 4°C with primary antibodies. Primary antibodies were visualised using Alexa 488-, 568, and 647-conjugated secondary antibodies. DAPI (4',6-diamidino-2-phenylindole, Thermo Fisher Scientific - D1306) was used 1:10000. Cells were mounted using Citifluor Glycerol Pbs Solution AF1 (Agar Scientific Ltd) and sealed using nail varnish. For confocal imaging a Zeiss LSM700 or LSM900 with airyscan 2 were used. Images were then processed using Fiji (Schindelin et al., 2012)

Antibodies

Primary antibodies: CASPR (1:500, Antibodies Inc., 75-001, RRID: AB_2083496), JUN (1:500, Cell Signalling Technology, 9165, RRID:AB_2130165), MBP (EMD Millipore, 1:1000, AB9348, RRID: AB_2140366), MPZ (1:500, Aves Labs, PZO, RRID: AB_2313561), NFL (1:500, Abcam, ab72997, RRID: AB_1267598), PRX (1:500, kind gift from Peter Brophy), SOX10 (1:100, R&D Systems, AF2864, RRID:AB_442208).

Secondary antibodies: Donkey anti-goat IgG (H+L) Alexa Fluor 488 (1:500, Thermo Fisher Scientific, A11057, RRID:AB_2534102), Goat anti-mouse IgG (H+L) Alexa Fluor 488 (1:500, Thermo Fisher Scientific, A11001, RRID:AB_2534069), Goat anti-mouse IgG (H+L) Alexa Fluor 647 (1:500, Thermo Fisher Scientific, A-21235, RRID:AB_2535804), Goat anti-rabbit IgG (H+L) Alexa Fluor 568 (1:500, Thermo Fisher Scientific, A11011, RRID:AB_143157), Donkey anti-chicken IgY (H+L) Alexa Fluor 488 (1:500, Jackson ImmunoResearch, 703-545-155, RRID:AB_2340375), Donkey anti-chicken IgY (H+L) Alexa Fluor 647 (1:500, Thermo Fisher Scientific, A78952, RRID:AB_2921074).

Quantification of Myelination in Cocultures

To quantify the number of myelin segments per area, we counted the number of myelin segments for five areas per culture for three cultures and normalised this per mm^2. To quantify the percentage of Schwann cells in myelinating cocultures that are actively myelinating, we quantified the number of myelin segments and the number of DAPI-positive nuclei for five areas per culture for three cultures. To measure interperiodic distance, we measured at least 10 periods per myelinated fibre for at least three fibres per sample for three separate samples.

Coculture Axotomy

All cultures (axon only, aligned SCs and myelinating SCs) were cultured for 6 weeks prior to axotomy experiments. To minimise the possibility that medium constituents were responsible for differences in axon degeneration rates, axonal compartments of axon only cultures were cultured in medium containing 10 ng ml^{-1} βNRG1 and 10 μM forskolin (axon only medium, extended methods section D6) once SCs were seeded on other cultures, and then switched into myelination medium (additional Matrigel® and 50 μg ml^{-1} L-Ascorbic Acid), 24 hours before axotomy. Bottom compartments of aligned SC cultures, 24 hours before axotomy, were switched into DRG/SC medium containing 10 ng ml^{-1} βNRG-1, 10 μM forskolin and 50 μg ml^{-1} L-Ascorbic Acid, which is insufficient to induce myelination in mouse cultures. Bottom compartments of myelinating cocultures were medium changed into fresh myelination medium (Extended methods section D7) 24 hours prior to axotomy. Traumatic axotomies were carried out by carefully removing the microfluidic chamber (SND150 and RND150, Xona Microfluidics®) from the Aclar® coverslip using sterile forceps and severing axons with a surgical blade under a light microscope. Axotomies were carried out at the level of the microgroove barrier. To confirm all axons were severed, a second higher cut was performed and axons between the cut sites removed using the surgical blade. Vacor axotomies were carried out by media changing the top compartment to media supplemented with 50 μM vacor (Greyhound Chromatography - N-13738).

Quantification of Degeneration

Five images at a distance of between 1.2-1.4mm from the microgroove barrier (the most distal part of the culture that could be imaged) were quantified per culture, taken in comparable locations in each culture. A line was drawn across each image, and each axon crossing this line was either scored as degenerated or intact. Images were blinded prior to quantification. A minimum of three cultures was assessed per timepoint for each condition.

Electron Microscopy

After removal of the microfluidic chamber, the orientation of the coverslips was marked, and cells were fixed overnight at 4°C in 2% glutaraldehyde/2% formaldehyde in 0.05 M sodium cacodylate buffer pH 7.4 containing 2 mM calcium chloride. After washing 5x with 0.05 M sodium cacodylate buffer pH 7.4, samples were osmicated (1% osmium tetroxide, 1.5% potassium ferricyanide, 0.05 M sodium cacodylate buffer pH 7.4) for 3 days at 4°C. Samples were washed 5x in deionised water (DIW) and treated with 0.1% thiocarbohydrazide/DIW for 20 minutes at RT in the dark. After 5 further washes in DIW, samples were osmicated a second time for 1 hour at RT (2% osmium tetroxide/DIW). After washing 5x in DIW, samples were block stained with uranyl acetate (2% uranyl acetate in 0.05 M maleate buffer, pH 5.5) for 3 days at 4°C. Samples were washed 5x in DIW and then dehydrated in a graded series of ethanol (50%/70%/95%/100%/100% dry) and 100% dry acetonitrile, 3x in each for at least 5 minutes. Samples were infiltrated with a 50/50 mixture of 100% dry acetonitrile/Quetol resin (12 g Quetol 651, 15.7 g NSA, 5.7 g MNA, all from TAAB) overnight, followed by 5 days in 100% Quetol resin with 0.5 g BDMA (TAAB), exchanging the resin each day. Aclar® coverslips were placed on top of round polyethylene cups, with cells facing the resin. Samples were cured at 60°C for 3 days, and coverslips removed. The required section plane was marked on the block, and smaller sample blocks were cut from the resin using a hacksaw and mounted on resin stubs. Thin sections (~ 70 nm) were prepared using an ultramicrotome (Leica Ultracut E) and collected on bare Cu TEM grids or Cu/carbon film grids. Samples were imaged in a Tecnai G2 TEM (FEI/Thermo Fisher Scientific) run at 200 keV using a 20 μm objective aperture to improve contrast. Images were acquired using an ORCA HR high resolution CCD camera (Advanced Microscopy Techniques Corp, Danvers USA).

Live imaging of cocultures

Microfluidic chambers were placed on a Zeiss LSM 900 confocal microscope equipped with a temperature-controlled chamber at 37°C 5% CO_2. Multiple areas of interest were selected for

each microfluidic chamber and imaged every 10 minutes for up to 48 hours. To quantify number of SCs with fragments, each cell was defined as a region of interest and checked for the presence of mCherry positive fragments at all timepoints. Two separate independently prepared cultures and cells in 10 areas per culture were analysed.

Statistical Analysis

Results are shown as mean ± SEM. Statistical significance was estimated by two tailed Student's t test or 2-way ANOVA with post-hoc Tukey test to correct for multiple comparisons. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism software (version 9.5.0).

<u>Step by step dissociated mouse myelinating SC/DRG compartmentalised coculture protocol.</u>

Section A: Dissociated embryonic mouse DRG neuron culture

A1 Aclar® plastic coverslip preparation (Electron microscopy sciences 50425-10)

- Cut into 40x22 mm pieces.
- Autoclave in glass dish.

A2 Day 1

A2.1 Preparing the dishes

- Make a 750 µl drop of 0.5 mg ml^{-1} PLL (Merck - P1274) in a dish and place one Aclar® coverslip on this drop, using sterilised forceps (kept in 70-100% ethanol for at least 24 hours prior to usage).
- Leave to coat overnight at room temperature (RT).

A2.2 DRG dissection

- Dissect as many DRGs as possible from E14 mice:
    - Place the embryo in a dish containing ice-cold L15 (Thermo Fisher - 11415049) in a sterile dissection hood.
    - Lay the embryo on its side and remove head and ventral part of the embryo (carefully remove skin and internal organs, liver and gut particularly, using no. 5 forceps).
    - Remove any remaining tissue in front of the vertebral column, using forceps.
    - Place vertebral column ventral side up and use micro-dissecting scissors or forceps to cut/crush through vertebral column.

    - Open up the vertebral column by gently teasing apart the right and left side to expose the spinal cord and DRGs.
    - From the cranial end use a no. 5 forceps to gently remove the spinal cord from the open vertebral column and move it to a 35 mm dish containing Hibernate media (see 3.2). All the DRGs should remain attached to the spinal cord.
    - In the 35mm dish simply remove DRGs one by one using no. 5 forceps. Transfer all DRGs using a P1000 into fresh Hibernate media. Only transfer 50 or so at a time and use just the tip of the P1000 pipette tip as the DRGs will get stuck on the plastic if they are sucked too far up the P1000 tip.
- Store overnight at 4°C.

A3 Day 2

A3.1 Preparing the PLL-coated Aclar® coverslips

- Remove the PLL and always keep the side of the Aclar® coverslips that is coated with PLL facing up through the washing process below. Using sterile forceps to manipulate the coverslips.
- Wash coverslip with sterile ultrapure H_2O (resistivity 18.2 MΩ·cm at 25°C).
- Airdry coverslips and move to a new sterile 60 mm dish.
- Place Xona Microfluidics®chambers (either SND150 or RND150), which have a 150 µm microgroove barrier, on top of coverslip using sterile forceps.
- Check chamber attachment under microscope until there are no visible air bubbles between the chamber and the PLL coated Aclar® coverslip.
- Place 3x 60 mm dishes containing a PLL coated Aclar® coverslip in an upside down large 200 mm sterile petri dish.

A3.2 Matrigel® Coating

- Thaw an aliquot of growth factor depleted Matrigel® (Corning - 356231) on ice and dilute 1:200 in cold DMEM high glucose (4500 g dl^{-1} glucose; Thermo Fisher - 41966029).
- For both top compartments of the microfluidic chamber (See diagram in Fig. 1), pipette 150 µl into the right well making sure it flows through the top channel into the left well: make sure to pipette forcefully in one fluid motion right at the channel entrance to minimise any air bubbles in the channel.

- Remove almost all of the volume of 1:200 Matrigel® in DMEM from both wells, making sure to leave medium in the top channel and pipette into the left well to encourage flow.
- Repeat the steps above for the bottom compartment.
- Leave chambers in their 60mm dish coating with Matrigel® for at least 1 hour at 37°C. They can be placed back inside their upside down large sterile petri dish for safety.
- Add an open (remove and discard lid) 35 mm sterile dish full of sterile water to the large sterile petri dish to maintain humidity and stop the chambers from drying out.

A3.3 Dissociation

- You will need cells from approximately 10 DRG/ganglia per chamber for stable long-term cultures.
- Warm up 2.5ml of 0.025% Trypsin (Merck - T9201) dissolved in Ca^{2+} and Mg^{2+} free PBS (Merck) in a 15ml falcon in a waterbath at 37°C.
- Make up DRG media (section D3) and set aside enough DRG media for dissociation and topping up wells the next day. We generally prepare 2.5ml DRG media per 10 ganglia/chamber.
- Transfer approximately 40 ganglia into the warm 15ml falcon containing 2.5ml of 0.025% Trypsin.
- Leave for 30 minutes at 37°C.
- While DRGs are trypsinising, warm up 600 μl of collagenase solution in a 1.5 ml eppendorf at 37°C in a water bath. Collagenase solution 682 U ml^{-1} Collagenase (Worthington Biochemical Corporation - LS004176) in Ca^{2+} and Mg^{2+} free medium (10% Krebs Solution (133 mM NaCl, 177 mM KCL, 1.75 mM NaH2PO4, all Merck), 1% MEM Non-Essential Amino Acids Solution (Thermo Fisher), 0.5% Phenol Red Solution (Merck), 0.2% NaHCO3 (Thermo Fisher), 0.2% Glucose (Thermo Fisher)).
- Transfer ganglia into warmed 600 μl of collagenase in a 1.5 ml eppendorf (transfer only ganglia, not trypsin).
- Mix liquids but not cells.
- Leave for 30 minutes at 37°C.
- Prepare one 1 ml DRG medium in a 15 ml falcon per eppendorf of DRGs.
- Transfer each eppendorf of DRGs to 1 ml DRG medium in a 15 ml falcon.
- Triturate with P1000 pipette very gently - do not over dissociate (this leads to low yields and unhealthy cultures). Move any lumps of tissue left to a separate tube to triturate further, if necessary.

- Centrifuge for 5 minutes at 1200 rpm (room temperature, RT).
- For lentiviral infection: resuspend in 1 ml DRG medium (proceed to section A3.4).
- For plating: resuspend in DRG medium (1 μl per ganglion) and pool tubes (proceed to section A3.5).

A3.4 Lentiviral infection of DRGs

- Lentiviruses stored at -80°C and thawed on ice (can re-use lentivirus aliquot once after thawing but double volume on subsequent experiment as viral copies reduce by roughly 50% every freeze-thaw cycle in our experience).
- Add virus to DRGs resuspended in 1 ml of DRG medium.
- Use a multiplicity of infection (MOI) of 2 -10 for transducing DRGs.
- Leads to approximately 100% transduction.
- Centrifuge for 1 hour at 400 rpm (RT).
- Resuspend pellet in 1 μl per ganglion DRG medium + the same amount of virus added previously.
- Proceed to plating.

A3.5 Plating

- Remove 1:200 Matrigel® in DMEM from top and bottom wells of chambers (leaving just medium in the top and bottom channels to avoid air bubbles) and replace with 100 μl of DRG medium per compartment, pipetting in the same way as is described in section A3.2.
- Immediately afterwards, remove all DRG medium from the top wells (leaving just medium in the channel to avoid air bubbles).
- Load 10 μl cells into the top channel by pipetting gently in one fluid motion right at the channel entrance (either right or left side).
- Check underneath microscope and if cells look sparse, load another 5-10 μl of cells from the other side.
- Allow 4 hours for cells to attach.
- Top up with 100 μl added to top compartment: make sure to pipette from well to well to keep volume the same and reduce flow.
- Check that the media level is higher in the bottom compartment - if not, add one or two drops to establish volume difference.

A4 Day 3

A4.1 Topping up

- For square chambers (SND150, Xona Microfluidics®) top up wells (200 μl in top compartment and 300 μl in bottom compartment).
- For round chambers (RND150, Xona Microfluidics®) top up wells (75 μl in top compartment and 150 μl in bottom compartment).
- check that the media level is higher in the bottom compartment - if not, add one or two drops to establish volume difference.
- Add anti-mitotic agent cytosine arabinoside (Ara-C, Merck - C6645) to top compartment: final concentration 10^{-5} M. Add half of the total volume to the left and right top wells.

A5 Changing Media

- Aim to change medium on Mondays, Wednesdays, and Fridays.
- Make sure to pipette one drop per well into alternating wells to reduce flow.
- Make sure to top up the water in the dish to stop chambers drying out.

A6 Day 10

- After maintaining DRGs in Ara-C for 7 days change media to DRG medium without Ara-C.
- Non-neuronal cells will not return after this stage.
- Make sure to wait for axons to extend to the wells before seeding SCs. If not, then leave cultures for a few more days before seeding SCs. Make sure Ara-C has been removed 3 days prior to SCs being added, and medium has been changed twice, to minimise any possible toxicity (not experienced in our hands).

<u>Section B: Dissociated neonatal mouse Schwann Cell Culture</u>

- Dissect P3-5 sciatic nerves and brachial plexuses from mice and start SC culture within 3-5 days of DRG dissociation.
- After 3 days of Ara-C purification, trypsinise cells and infect with lentiviruses in suspension (see below sections B1-4).
- Expand SCs on 60mm PLL/laminin coated dishes until needed for coculture (section B3).

B1 Preparing dishes

B1.1 PLL Coating

- Prepare a 0.2 mg ml^{-1} solution of PLL and coat 35 or 60 mm sterile dishes overnight.

- Remove PLL (can be re-frozen and used 3 times).
- Wash 3 times with sterile ultrapure H_2O (resistivity 18.2 MΩ·cm at 25 °C).
- leave dishes to air dry, store at RT.

B1.2 Laminin Coating

- Dilute the stock solution of laminin (Merck - L2020) in low glucose DMEM (1000g/dl; Thermo Fisher - 21885025) to a final concentration of 10 μg ml^{-1} (1/100 dilution)
- Add the solution to the dish.
- Leave for at least 1 hour at 37°C.
- Remove laminin immediately prior to plating cells (can be reused 3 times) and do not let dishes dry (add media).

B2 Mouse SC Purification

- Make 20 ml of DMEM low glucose (with 1/100 Pen/Strep (Thermo Fisher - 15140122)) + 5% HS (Thermo Fisher - 16050130) and warm to 37°C.
- Prepare 2x 60 mm tissue culture dishes of L15 and place on ice.
- Dissect out sciatic nerves and brachial plexuses and place in ice-cold L15 (4-6°C).
- De-sheath the nerves and transfer to a separate dish containing L15.
- Place 100 μl trypsin and 100 μl collagenase (per 2 animals) in a 35 mm dish: 2 mg. ml^{-1} Trypsin (Merck – 85450C) and 682 U ml^{-1} Collagenase in Ca^{2+} and Mg^{2+} free medium.
- Transfer the nerves into the trypsin/collagenase and incubate at 37°C for 45 minutes.
- Triturate nerves with a P1000 and then with a P200.
- Stop the digestion by adding an excess 2 ml low glucose DMEM + 5% HS.
- Transfer the cell suspension to a 15 ml centrifuge tube.
- Centrifuge at 1000 rpm for 10 minutes (RT).
- Resuspend the cell pellet in 2 or 4 ml of low glucose DMEM + 5% horse serum.
- Remove laminin/DMEM solution from 35 or 60 mm dishes (store at 4°C for up to one month and can be re-used maximum of three times).
- Transfer cell suspension to the 35 or 60 mm laminin-coated dishes.
- Add Ara-C to a final concentration of 10^{-5} M and culture for 3 days to eliminate fibroblasts.
- After 3 days, replate SCs and expand (see section B3) or lentivirally transduce (see section B4) prior to expanding.

B3 Mouse SC Expansion

- Mouse SCs proliferate on PLL/laminin coated dishes in the presence of low serum, βNRG1, and a low concentration cAMP signal (Arthur-Farraj et al., 2011)see section D8).
- Laminin coat 60 mm PLL coated plates (see section B1.2).
- Wash cells 2x with PBS at RT.
- Trypsinise Ara-C purified SCs using 1 ml of 6% 2 mg ml^{-1} Trypsin (Merck - 85450C) in Versene (0.02% EDTA (Thermo Fisher - D/0700/53) + 0.5% Phenol Red in PBS) for up to 5 minutes at 37°C.
- Stop the reaction by adding 2 ml (or more) of low glucose DMEM + 5% HS (pre-warmed to 37°C in a waterbath).
- Transfer the cell suspension to a sterile 15 ml centrifuge tube.
- Centrifuge at 1000 rpm for 10 minutes (RT).
- Pre-plate the cells to eliminate fibroblasts if needed:
    - resuspend pellet in 10 ml of low glucose DMEM + 5% HS (if first passage) or defined medium (DM, table S1) with 0.5% HS (if they have already been passaged once).
    - add to an uncoated sterile 90 mm tissue culture dish (no PLL, no Laminin) for 2-3 hours.
    - fibroblasts will sit down and attach whereas SCs will remain in suspension.
    - collect medium and wash the dish well with a few extra mls of medium.
    - centrifuge at 1000 rpm for 10 mins at RT.
- Resuspend cell pellet in SC expansion medium (see section D8) and plate or lentivirally infect (see section B4) prior to expanding.
- Change medium every 3-4 days e.g., Monday and Thursday.
- Split cells when 80% confluent
- Do not passage cells more than 3 times as mouse SC tend to quiesce after this.

B4 Lentiviral Infection of Mouse SCs

- Pre-plate cells (section B3) and then lentivirally infect, as described below, prior to expanding.
- Resuspend cell pellet in 1 ml of DM + 0.5% HS in a 15ml sterile centrifuge tube.
- Calculate the total number of cells using a haemocytometer or other cell counter.
- LVs are stored at -80°C and thawed on ice.
- Add virus (MOI of 200-500) to cell suspension.

- Centrifuge for 1 hour at 400 rpm at RT. Centrifugation increases the transduction efficiency.
- Resuspend the pellet in the same supernatant so as to allow more LV to infect cells over the next 24 hours.
- Plate SCs on laminin coated PLL 35mm or 60mm dishes (see section B1.2).
- Change medium after 24 hours to fresh SC expansion medium (see section D8).
- Proceed with normal expansion (see section B3).

B5 Quiescing SCs

- The day before they are used in an experiment, SC cell cycles are synchronised by quiescing them.
- Change media to DM + 0.5% HS WITHOUT any forskolin or neuregulin.

<u>Section C: Dissociated mouse DRG neuron/Schwann cell Coculture</u>

C1 Seeding SCs

- Medium change both wells in top compartment (DRG cells side) into DRG/SC medium (section D4).
- there are roughly 400,000-600,000 SCs in an 80-90% confluent 60 mm dish.
- Trypsinise SCs with 1 ml of 6% 2 mg ml^{-1} Trypsin in Versene for max 5 minutes at 37°C.
- Stop the reaction with DMEM low glucose + 5% HS.
- Centrifuge for 10 minutes at 1200 rpm at RT.
- Resuspend in DRG/SC medium to achieve a concentration of 30,000 cells per 10 μl media (3,000,000 ml^{-1}).
- Remove almost all the medium from bottom compartment (Fig.1A), remembering to leave medium in the bottom channel, so as to avoid air bubbles.
- Load 30,000 cells by pipetting at the right or left entrance of the bottom channel (Fig.1A).
- Check underneath microscope and if cells look sparse, load another 5-10 μl of cells from the opposite side of the bottom channel.
- After 4 hours, top up with DRG/SC medium to normal levels (see section A4.1).
- Change medium on Mondays, Wednesdays, and Fridays.

C2 Inducing Myelination

- Allow 7 days for SCs to align and proliferate before inducing myelination (reducing this time will compromise the myelination).

- Keep top compartment in DRG/SC medium.
- Change bottom compartment to axon only media (section D6), keep in DRG/SC media (section D4, for aligned SC), or myelination media (section D7).
- Change medium on Mondays, Wednesdays, and Fridays.

Section D: Media

D1 Supplement stocks

- Nerve growth factor (NGF): 100 μg ml^{-1}, Thermo Fisher Mouse NGF 2.5S Native Protein (13257019).
- Forskolin: 10 mM in ethanol, Merck, Coleus forskohlii - CAS 66575-29-9 – Calbiochem.
- Neuregulin (βNRG1): 10 μg ml^{-1} in in PBS 1% BSA, Recombinant Human NRG1-beta 1/HRG1-beta 1 EGF Domain Protein, RD Systems, 396-HB-050.
- Growth factor depleted Matrigel® (Corning - 356231). Thaw 10ml in fridge (4-6°C) over night. Aliquot into 10 μL and 100 μL aliquots, making sure to keep the Matrigel® at 4-6°C at all times (it polymerises above 10°C). We keep our pipette tips in the freezer beforehand and aliquot on ice. Aliquots can then be stored at -20°C.

D2 Hibernate DRG medium

- for 50 ml of media:
    - 48 ml Hibernate E (Thermo Fisher - A1247601)
    - 1 ml B27 (2%; Thermo Fisher - 17504044)
    - 500 μl Pen/Strep
    - 500 μl L-Glutamine (Thermo Fisher - 25030081)
- on day of use add NGF to a final concentration of 33 ng ml^{-1} (1/3000).

D3 DRG medium

- for 50 ml of media (store at 4°C for up to 4weeks):
    - 48.5 ml DMEM (high glucose)
    - 1 ml B27
    - 500 μl Pen/Strep
- on day of use add NGF 1/3000 (final concentration 33 ng ml^{-1}).

D4 DRG/SC medium

- for 50 ml of media (store at 4°C for up to 4weeks):

- 24.5 ml DMEM (high glucose)
- 24.5 ml DM (Table S1)
- 1 ml B27
- 500 μl Pen/Strep

- on day of use add NGF 1/3000 (final concentration 33 ng ml^{-1}).

D5 Defined medium (DM; Jessen et al., 1994)

- prepare medium according to table S1 and store at 4°C for up to 4 weeks.

D6 Axon only medium

- for 50 ml of media (store at 4°C for up to 4weeks):
  - 24.5 ml DMEM (high glucose)
  - 24.5 ml DM (Table S1)
  - 1 ml B27
  - 500 μl Pen/Strep
- on day of use add
  - NGF 1/3000 (final concentration 33 ng ml^{-1}).
  - Forskolin 1/1000 (final concentration 10 μM).
  - βNRG1 1/1000 (final conc. 10 ng ml^{-1}).

D7 Myelination medium

- for 50 ml of media (store at 4°C for up to 4weeks):
  - 24.5 ml DMEM (high glucose)
  - 24.5 ml DM (Table S1)
  - 1 ml B27
  - 500 μl Pen/Strep

- on day of use add
  - Matrigel®1/100 (you can make a 5 or 10 ml stock with Matrigel® added)
  - NGF 1/3000 (final concentration 33 ng ml^{-1})
  - Forskolin 1/1000 (final concentration 10 μM)
  - βNRG1 1/1000 (final conc. 10 ng ml^{-1})

To wells, also add 50 μg ml^{-1} L-Ascorbic Acid (1/100 from stock; Merck - A4544), make up stock (in H_2O) fresh each time and protect from light.

D8 Schwann cell expansion medium

- Made on day of use. For 50 ml of media:
    - 50 ml DM (Table S1)
    - 0.5% HS
    - 10 ng ml^{-1} βNRG1 (R&D Systems - 396-HB-050)
    - 2 μM forskolin (Merck - 344270) or 50-100 μM dibutryl-cAMP (dbcAMP; Merck - D0627)

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Competing interests

M.P.C. is a consultant for NuraBio. The remaining authors declare no competing interests.

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Figure legends

Figure 1: Dissociated murine Schwann cell/dorsal root ganglion neuron cocultures in microfluidic chambers. A. Standard Neuron Device with a 150 μm microgroove barrier. Dissociated DRG neurons (magenta), which cannot cross the barrier due to their size, are seeded into the top channel and extend axons into the bottom channel, reaching the bottom wells. SCs (green) are then seeded in the bottom channel where they align with and myelinate axons. **B.** Dissociated DRG neurons (magenta) growing across the microgroove barrier. Scale bar 100 μm. **C.** Phase images of the bottom channel showing axons only, axons with aligned SCs (aligned SC), and axons with myelinating SCs (myelinating SC). Scale bar 100 μm. Higher magnification images showing myelin segments in cultures with myelinating SCs. Scale bar 10 μm. **D.** Aligned Schwann cells can be labelled with P75NTR. Scale bar 20 μm. **E.** Electron micrographs of aligned Schwann cells that show structures resembling Remak bundles. Scale bar 1 nm. **F.** Confocal images of axon only cultures, showing axons (NFL, magenta), but no DAPI (blue) or SOX10 (green) signal in the axonal compartment of the microfluidic chamber. Scale bar 100 μm.

Figure 2: Schwann cells in dissociated myelinating cocultures can be induced to robustly myelinate.

A. Confocal images of dissociated DRG neuron cocultures with aligned SC or myelinating SC. Axons are NFL-labelled (magenta), and SCs PRX-labelled (green). Aligned SCs have a more diffuse morphology (white arrowheads), while myelin segments are present in myelinating SCs (white arrows). Scale bar 100 μm. **B.** Higher magnification images showing NFL-labelled axons (magenta) covered by myelin segments (PRX, green, white arrows). Scale bar 10 μm. **C.** DRG neurons (NFL, magenta) growing across the barrier in chambers with SCs (PRX, green) that have been induced to myelinate. Scale bar 100 μm. **D.** Electron micrograph of electron dense myelin in cocultures with myelinating SCs. Scale bar 100 nm. **E.** MPZ (green) labelled myelinating SCs. Scale bar 20 μm. **F.** MBP (green) labelled myelinating SCs. Scale bar 10 μm. **G.** In mature myelinating cultures, SCs CASPR (white) can be detected in the characteristic staining pattern marking paranodes. Scale bar 5 μm. Paranodal CASPR co-localised on axons (magenta). Scale bar 5 μm. When colabelling with MPZ (green), CASPR is localised to paranodal loops adjacent to a Node of Ranvier. Scale bar 10 μm.

Figure 3: SC-DRG neuron cocultures replicate characteristic axonal and Schwann cell injury responses after axotomy. A. 12 hours post axotomy many of the axons (magenta) in SC-DRG neuron cocultures have degenerated. Scale bar 100 μm. **B.** SC JUN in uninjured cultures and 12 hours post axotomy. With identical imaging conditions, a signal can only be detected 12 hours post axotomy. Scale bar 100 μm. **C.** Relative intensity of JUN signal in uninjured myelinating cultures and 12 hours post axotomy. n=3. Data represented as mean ± SEM. *p<0.05. **D**. SC JUN in uninjured cultures with aligned Schwann cells and 12 hours post axotomy. With identical imaging conditions, a signal can only be detected 12 hours post axotomy. Scale bar 100 μm. **E**. Relative intensity of JUN signal in uninjured cultures with aligned Schwann cells and 12 hours post axotomy. n=3. Data represented as mean ± SEM. *p<0.05. Statistical analysis was performed with two tailed Student's t-test. **F**. Myelinating SCs demyelinate after extended periods of time (48 hours) after axotomy. Myelin ovoids and myelin debris (both identified by white arrowheads) are present in fluoromyelin (green) labelled cultures. Scale bar 10 μm. **G.** Electron micrographs of myelinating SCs 48 hours post axotomy showing characteristic demyelinated profiles surrounding degenerated axons. Scale bar 1 μm. **H.** Vacor induces neurodegeneration when applied to the top compartment of cocultures. NFL (magenta). Scale bar 10 μm. **I.** Neurons and SCs can be lentivirally infected prior to plating in microfluidic chambers. Neurons infected with LV-CMV-mCherry (MOI 2, magenta) and SCs with LV-CMV-GFP (MOI 200, green). Scale bar 20 μm. n number refers to independently prepared cultures from separate litters of mice.

Figure 4: Schwann cells are axo-protective, independent of myelination status at early timepoints, and promote axon fragmentation at later timepoints after axotomy. A. Confocal images of NFL-labelled cocultures prior to axotomy at the microgroove barrier (uninjured), and 3, 6, 9, and 12 hours after axotomy. Axon only cultures show earlier signs of degeneration than cultures with SCs. Scale bar 100 μm. **B.** Quantification of axon degeneration after axotomy. Only axon only cultures show statistically substantial degeneration at 6 hours post axotomy (29 ± 1.44%, n=5). In axon only cultures this degeneration increases to 47.39 ± 1.34% at 9 hours post axotomy (n=3), and finally 76.68 ± 6.60% at 12 hours post axotomy (n=3). Cocultures with SCs show little degeneration at 3 hours post axotomy (aligned SC: 4.87 ± 1.65%, n=3; myelinating SC: 3.048 ± 0.06%, n=3) and 6 hours post axotomy (myelinating SC: 8.14 ± 0.65%, n=4; aligned SC: 9.19 ± 2.57%, n=3). At 9 and 12 hours post axotomy, axons associated with both aligned and myelinating SCs start to degenerate (aligned SC: 9h, 30.05 ± 3.05%, n=4 and 12h, 44.61 ± 4.72%, n=3; myelinating SC: 9h, 33.12 ± 0.61%, n=3 and 12h, 48.38 ± 7.75%, n=3). There were no

significant differences in axon degeneration rates between aligned and myelinating SCs cocultures (3h: p=0.65, 6h: p=0.98, 9h: p=0.44, 12h: p=0.70). Axon only cultures show significant differences to cultures with aligned or myelinating SCs at 6 (aligned SC: p<0.0001, myelinating SC: p<0.0001), 9 (aligned SC: p=0.0004, myelinating SC: p=0.0059), and 12 (aligned SC: p<0.0001, myelinating SC: p<0.0001) hours post axotomy. Results shown as individual data points. Statistical significance determined by 2-way ANOVA. Statistical significance for comparisons between axon only cultures and aligned SC cocultures are displayed on the graph. **p<0.01. ***p<0.001. ****p<0.0001. n number refers to independently prepared cultures from separate litters of mice. Statistical analysis was performed with 2-way ANOVA with post-hoc Tukey test to correct for multiple comparisons **C.** Confocal images of mCherry-labelled axons (magenta) and GFP-labelled SCs (green). At an early timepoint (19 h 10 min) an intact axon is visible (white arrow). 3 hours later (22 h 10 min), the axon is starting to be constricted (white arrowheads). This continues until 23 h 50 min after axotomy, when constrictions are clearly visible, and the axon is swollen between two constrictions. 24 hours after axotomy, the axon then breaks apart. Scale bar 10 μm. **D.** Confocal images of mCherry-labelled axons (magenta) and GFP-labelled SCs (green) with intact axons (white arrow, 28 h post axotomy) just before axon degeneration and with mCherry fragments (white arrowheads) within SCs 30 h post axotomy, once axon degeneration has occurred. Scale bar 10 μm.