Neuronal Regulation of Schwann Cell Mitochondrial Ca\textsuperscript{2+} Signaling during Myelination

Graphical Abstract

**Highlights**

- A purinergic agonist stimulates mitochondrial metabolism in Schwann cells (SCs)
- Firing peripheral nervous system axons release ATP to the extracellular space
- ATP released from firing axons activates the P2RY2-IP\textsubscript{3}-MCU pathway in myelinating SCs
- Suppression of the P2RY2-IP\textsubscript{3}-MCU pathway in myelinating SCs causes hypomyelination

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**In Brief**

Ino et al. use microdialysis and in vivo transfection to demonstrate that peripheral nervous system axons release ATP to regulate mitochondrial Ca\textsuperscript{2+} signaling in surrounding Schwann cells (SCs). The authors propose that this axon-to-SC signaling has an important role in myelination.
Neuronal Regulation of Schwann Cell Mitochondrial Ca\(^{2+}\) Signaling during Myelination

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SUMMARY

Schwann cells (SCs) myelinate peripheral neurons to promote the rapid conduction of action potentials, and the process of myelination is known to be regulated by signals from axons to SCs. Given that SC mitochondria are one of the potential regulators of myelination, we investigated whether SC mitochondria are regulated by axonal signaling. Here, we show a purinergic mechanism that sends information from neurons to SC mitochondria during myelination. Our results show that electrical stimulation of rat sciatic nerve increases extracellular ATP levels enough to activate purinergic receptors. Indeed, electrical stimulation of sciatic nerves induces Ca\(^{2+}\) increases in the cytosol and the mitochondrial matrix of surrounding SCs via purinergic receptor activation. Chronic suppression of this pathway during active myelination suppressed the longitudinal and radial development of myelinating SCs and caused hypomyelination. These results demonstrate a neuron-to-SC mitochondria signaling, which is likely to have an important role in proper myelination.

INTRODUCTION

Schwann cells (SCs) form spiral membrane insulators, called myelin sheaths, around axons in the peripheral nervous system (PNS) to enable the rapid transmission of sensory and motor information by saltatory conduction. Myelin is produced by differentiated SCs, called myelinating SCs, and the plasma membrane of myelinating SCs extends radially and longitudinally to wrap many layers of membrane around the axons. This requires massive amounts of lipid and protein synthesis (Garbay et al., 2000), so that elevation of cellular metabolism within SCs during myelination is expected (Harris and Attwell, 2012). Mitochondria, which are pivotal in energy production and metabolism of lipids and amino acids, can therefore be a potential regulator of myelination. In line with this, it has recently been reported that an increase in the production of tricarboxylic acid (TCA) cycle metabolites by SC mitochondria plays a key role in myelination (Pooya et al., 2014). Furthermore, SC-specific disruption of mitochondrial oxidative phosphorylation in mice results in hypomyelination (Fünschilling et al., 2012) and demyelination (Viader et al., 2011, 2013), and human patients with a certain form of peripheral neuropathy have been shown to have SC mitochondrial abnormalities (Schröder, 1993).

For SCs to fully develop, they require interaction with axons. Numerous studies have been dedicated to this research field and have identified the key axon-derived molecules such as neuregulin 1, notch ligands, and neurotrophins (Taveggia et al., 2010). Among those, neuregulin 1, in concert with the other molecules, plays critical roles in most developmental processes including mitosis, differentiation, and myelination. Furthermore, neuronal electrical activity is an important modulator of SC development (Stevens and Fields, 2000). Firing axons release ATP via maxi-anion channels to activate purinergic receptors on SCs (Fields and Ni, 2010; Jahromi et al., 1992; Mayer et al., 1998; Stevens and Fields, 2000). The ATP-mediated axon-to-SC signaling inhibits proliferation of SCs, and controls the timing of SC differentiation before the initiation of myelination (Stevens and Fields, 2000). Although the important roles of both mitochondria and the axon-to-SC signaling in myelination have been documented, the relationship between the axonal activity and mitochondrial functions in SCs requires further clarification. Therefore, we set out to examine whether axons send signals to SC mitochondria during myelination.

In this study, we first searched for potential axonal ligands that affect intracellular energy state in SCs, and we found that purinergic signaling enhances SC mitochondrial bioenergetics regulating mitochondrial Ca\(^{2+}\) increase via the mitochondrial Ca\(^{2+}\) uniporter (MCU). Next, we reexamined whether the concentration of purinergic agonists increases in the vicinity of myelinating SCs in vivo. Our results showed that firing axons increase extracellular ATP concentration enough to activate purinergic receptors. In line with this, firing axons also induced Ca\(^{2+}\) increase in SC mitochondria via P2Y2 purinergic receptor (P2RY2), inositol 1,4,5-trisphosphate (IP\(_3\)), and the MCU signaling pathway. Finally, we showed that chronic suppression of the P2RY2-IP\(_3\)-MCU signaling pathway during myelination in vivo attenuates the development of myelinating SCs. Taken together, our study shows a signaling mechanism from axons to SC mitochondria that is involved in the development of myelinating SCs.
RESULTS

Molecules Signaling to SC Mitochondrial Metabolism

Given that myelination is a metabolically demanding process, potential axonal signaling molecules may have an effect on SC mitochondrial metabolism. Because mitochondria are involved in the generation of ATP, an increase in intracellular ATP levels is expected when mitochondrial metabolism is stimulated by external signals. We tested a panel of ligands for receptors, which are linked to distinct signaling pathways in SCs, and examined whether they have an effect on the intracellular ATP content in SCs in primary culture (Figure S1A). Among the tested ligands, ATP$_\gamma$S, a purinergic receptor agonist, significantly increased intracellular ATP levels (Figure S1B). This ATP increase was suppressed by an application of suramin, a purinergic receptor antagonist (Figure S1B). Because SCs express Gq-coupled P2Y receptors, ATP$_\gamma$S is likely to drive the generation of IP$_3$, which then induces intracellular Ca$^{2+}$ release (Stevens and Fields, 2000). Indeed, ATP$_\gamma$S induced a robust Ca$^{2+}$ response in SCs, but other ligands in the panel failed to induce an intracellular Ca$^{2+}$ increase (Figures S1E and S1F). Because the influx of Ca$^{2+}$ into the mitochondrial matrix through mitochondrial Ca$^{2+}$ uniporter (MCU) enhances mitochondrial ATP production (Baughman et al., 2011; De Stefani et al., 2011), we then examined whether the blockade of MCU has any effect of ATP$_\gamma$S on ATP production. Indeed, Ru360, an inhibitor of MCU, suppressed the ATP$_\gamma$S-induced increase in intracellular ATP levels (Figure S1B). We found no change in the levels of housekeeping proteins in mitochondria (COX IV) in ATP$_\gamma$S-stimulated SCs (Figure S1C), excluding the possibility of quantitative change of mitochondria. These results raise a possibility that purinergic receptor sends signals to mitochondria in SCs.

ATP-Induced Mitochondrial Ca$^{2+}$ Signaling in SCs

Next, we examined whether purinergic receptor-mediated mitochondrial stimulation operates in SCs in vivo. To perform functional experiments, we developed an electroporation technique to deliver exogenous genes to SCs in rat sciatic nerves (Figure 1A). When a red fluorescent protein (RFP)-expressing plasmid under the control of CAG promoter was electroporated on postnatal day 3 (P3), the RFP-positive cells appeared by P7 and showed bipolar morphology, a characteristic of SCs (Figure 1B). Most (~96%) RFP-positive cells overlapped with S100, an SC marker, on P7 (Figure 1C), and 91% of RFP-positive cells overlapped with MBP, a myelinating SC marker, on P14 (Figure 1D). We did not find any axons expressing RFP. Thus, highly selective gene expression in myelinating SCs can be achieved by the in vivo electroporation method. As observed in the previously reported viral transduction method (Ozcelik et al., 2010), this method sparsely introduces the exogenous genes; we can usually find 10–20 myelinating SCs in a radial section of the most densely transfected region. When a mixture of each data point and mean ± SEM are shown (n = 8–9 cells from three to four nerves). Ligands were applied during the period indicated by gray rectangles (A and D). Scale bar, 200 μm (B) or 50 μm (C–F). *p < 0.0008, ***p < 0.0001, Student’s t test (H).
of GFP- and RFP-expression plasmids was electroporated, we found highly overlapping (~97%) expression of these proteins in SCs (Figure 1E). Therefore, simultaneous expression of multiple genes is possible.

Using this method, we examined whether activation of the purinergic receptor triggers mitochondrial Ca\(^{2+}\) increase in intact SCs. We imaged the cytosolic Ca\(^{2+}\) signaling using G-GECO1.1, a green fluorescent cytosolic Ca\(^{2+}\) indicator (Zhao et al., 2011). In addition, R-GECO1mt, a red fluorescent Ca\(^{2+}\) indicator targeted to the mitochondrial matrix, was used for mitochondrial Ca\(^{2+}\) imaging (Suzuki et al., 2014). We simultaneously imaged the Ca\(^{2+}\) signaling in the cytosol and mitochondrial matrix of SCs by coexpressing these genetically coded Ca\(^{2+}\) indicators (Figure 1F). Because SCs actively undergo myelination during the neonatal period, Ca\(^{2+}\) imaging experiments were conducted using sciatic nerves dissected from neonatal rats (P6–P9) following delivery of the Ca\(^{2+}\) indicator genes to SCs by electroporation on P3. Upon stimulation with ATP, Ca\(^{2+}\) increases were observed in both the cytosol and mitochondrial matrix of myelinating SCs expressing scrambled small hairpin RNA (shRNA) for MCU (scMCU; Figures 1G, 1H, and S2A). On the other hand, the average mitochondrial Ca\(^{2+}\) response was significantly suppressed in SCs expressing shRNA for MCU (shMCU; Figure S2A), while no significant difference in the cytosolic Ca\(^{2+}\) signaling in the cytosol and mitochondrial matrix of SCs expressing scrambled small hairpin RNA (shRNA) for MCU was observed (Figures 1G and 1H). These results indicate that purinergic receptor stimulation-induced cytosolic Ca\(^{2+}\) increases cause MCU-mediated Ca\(^{2+}\) entry into mitochondria in myelinating SCs.

**Firing Axons Increase Extracellular ATP Levels Enough to Activate Purinergic Receptors**

A previous in vitro study showed that SC purinergic receptors are activated by an activity-dependent axonal ATP release in co-cultures of neurons and SCs (Fields and Ni, 2010; Stevens and Fields, 2000). It is also reported that ATP or its metabolite are released from intact sciatic nerves in an activity-dependent manner (Fields and Ni, 2010; Kuperman et al., 1964; Liu and Bennett, 2003). Therefore, firing axons are a potential regulator of SC mitochondrial metabolism. In order to estimate the ATP concentrations in the close proximity of firing axons, we assessed the ATP concentration during nerve stimulation. Sciatic nerves were dissected from P6–P9 rats after the delivery of Ca\(^{2+}\) indicator genes (G-GECO1.1 and R-GECO1mt) by electroporation to SCs on P3. Electrical stimulation to acutely isolated nerves resulted in reversibly Ca\(^{2+}\) increases in the cytosol and mitochondrial matrix of SCs (Figure 2D). Cytosolic and mitochondrial Ca\(^{2+}\) responses were observed throughout the SCs including perineurial region, internodes, and paranodal loops (Figure 2E). MCU silencing using shMCU inhibited the mitochondrial Ca\(^{2+}\) response without affecting the cytosolic Ca\(^{2+}\) response (Figures 2F and 2G).

**Firing Axons Control Mitochondrial Ca\(^{2+}\) Responses via the P2RY2-IP3 Pathway in Myelinating SCs**

To confirm that activity-dependent axonal ATP release activates purinergic receptors in SCs to induce cytosolic and mitochondrial Ca\(^{2+}\) responses, we performed Ca\(^{2+}\) imaging during nerve stimulation. Sciatic nerves were dissected from P6–P9 rats after the delivery of Ca\(^{2+}\) indicator genes (G-GECO1.1 and R-GECO1mt) by electroporation to SCs on P3. Electrical stimulation to acutely isolated nerves resulted in reversibly Ca\(^{2+}\) increases in the cytosol and mitochondrial matrix of SCs (Figure 2D). Cytosolic and mitochondrial Ca\(^{2+}\) responses were observed throughout the SCs including perineurial region, internodes, and paranodal loops (Figure 2E). MCU silencing using shMCU inhibited the mitochondrial Ca\(^{2+}\) response without affecting the cytosolic Ca\(^{2+}\) response (Figures 2F and 2G).

Myelinating SCs express P2RY2 purinergic receptors (Burnstock, 2007; Mayer et al., 1998). Stimulation of P2RY2 produces IP\(_3\) and increases in the cytosolic Ca\(^{2+}\) concentration via IP\(_3\) receptor-mediated Ca\(^{2+}\) release from the ER. Thus, we next examined the involvement of this pathway in electrical stimulation-evoked mitochondrial Ca\(^{2+}\) responses. To inhibit this pathway, shRNA for P2RY2 (shP2RY2), which efficiently silences P2RY2 expression (Figure S2C), and IP\(_3\) 5-phosphatase (5ppase\textsuperscript{WT}), which efficiently suppresses IP\(_3\) signaling (Kanemaru et al., 2007), were prepared. Either shP2RY2 or 5ppase\textsuperscript{WT} suppressed ATP-induced cytosolic Ca\(^{2+}\) responses (Figures S2D and S2E) as compared with the controls (scP2RY2 for shP2RY2, 5ppase\textsuperscript{Nhact} for 5ppase\textsuperscript{WT}). As expected, electrical-stimulation-evoked cytosolic and mitochondrial Ca\(^{2+}\) responses were significantly suppressed by expression of either shP2RY2 or 5ppase\textsuperscript{WT} (Figures 2H–2K).

We further examined axonal involvement in the SC mitochondrial Ca\(^{2+}\) response by blocking individual signaling steps. Application of lidocaine to the sciatic nerve suppressed stimulation-evoked cytosolic and mitochondrial Ca\(^{2+}\) responses (Figures
Figure 2. Firing Sciatic Nerves Induce P2RY2-IP₃-MCU-Dependent Mitochondrial Ca²⁺ Response in Myelinating SCs

(A) A schematic of extracellular ATP measurement by microdialysis. Dialysates from neonatal rat sciatic nerves at P6–P9 were collected through a microdialysis probe for 60 min before, during, and after nerve stimulation and analyzed by HPLC. Before nerve stimulation, vehicle or lidocaine was applied to sciatic nerves.

(B) HPLC traces of dialysates collected before (black), during (red), or after stimulation (gray). The bottom traces show ATP, ADP, AMP, and ADO standards.

(C) ATP (top) and total adenine nucleotide/nucleoside concentrations (bottom) calculated from the data in (B) (mean ± SEM; n = 5–7 nerves).

(legend continued on next page)
S2F–S2H), confirming the involvement of axonal electrical activity in the Ca^{2+} response. It has been reported that firing peripheral axons release ATP through maxi-anion channels, which have a 1.3-nm-radius open pore through which ATP (which has a radius of 0.6 nm) can permeate (Fields and Ni, 2010). We thus applied three different inhibitors of maxi-anion channels (GdCl_{3}, glibenclamide, and 5-nitro-2-(3-phenylpropylamino)-benzoic acid [NPPB]). The electrical-stimulation-evoked cytosolic and mitochondrial Ca^{2+} responses in SCs were significantly suppressed by these inhibitors (Figures S2F–S2H). We also assessed other potential pathways for ATP release (Burnstock, 2007) and found that inhibitors of vesicular exocytosis (tetanus toxin and bafilomycin A) and connexin-mediated release (carbenoxolone and 1-octanol) had no significant effect (Figures S2F–S2H). Thus, these results are consistent with the notion that firing axons release ATP in a non-vesicular, maxi-anion-channel-mediated manner. Taken together, the findings suggest that firing axons control P2RY2-IP_{3}-MCU signaling in SCs through axonal ATP release.

**Inhibition of P2RY2-IP_{3}-MCU Signaling in SCs Results in Hypomyelination**

We finally examined the physiological significance of P2RY2-IP_{3}-MCU signaling pathway in myelinating SCs in vivo. Because in vivo electroporation method allows long-term expression of the introduced genes (Figure S3A), we were able to analyze the development of transfected SCs at various ages using this method (Figures S3B and S3C). We introduced shP2RY2, 5ppase\textsuperscript{WT}, or shMCU into sciatic nerve SCs on P3, which is when myelination begins in these animals, and we also expressed RFP under the control of the internal ribosome entry site (IRES) sequence to analyze the morphology of the transfected SCs. Four weeks after electroporation, SCs expressing shP2RY2, 5ppase\textsuperscript{WT}, or shMCU were significantly shorter and thinner than control cells (Figures 3A, 3B, and S3D–S3F). Exogenous expression of shRNA-resistant genes (resP2RY2, Figure S2C; resMCU, Figure S2A) rescued the shRNA-induced decrease in the longitudinal and radial extension of SCs, excluding off-target effects of shRNAs (Figures 3A, 3B, and S3D–S3F). These results indicate that functional P2RY2-IP_{3}-MCU signaling pathway is essential for proper development of myelinating SCs. In line with the notion that the P2RY2-IP_{3}-MCU signaling pathway is involved in mitochondrial metabolism, we observed increased phosphorylated AMP-activated protein kinase (p-AMPK) levels (Figures S3G and S3H), which increase in response to decreased energy levels (Cárdenas et al., 2010; Rizzuto et al., 2012).

Electron microscopy (EM) was then used to analyze the structure of myelinating SCs and axons at a higher spatial resolution. To do this, cells were cotransfected with LacZ and labeled with precipitates of the reaction product of β-galactosidase. When P2RY2, IP_{3}, or MCU signaling was silenced in SCs from P3 to P31, a significant increase in the g-ratio (axon diameter/myelinated fiber diameter) was observed (Figures 3C, 3D, and S4A). There was no difference in the average axon diameter between nerve fibers surrounded by SCs with or without expression marker. The control SCs did not show any significant change either in the g-ratio or in the axonal diameter (Figures 3C, 3D, and S4A), indicating that suppression of P2RY2-IP_{3}-MCU results in hypomyelination.

It can be argued that the observed developmental delay in SCs may be caused by abnormal cell proliferation or cell death ( Parkinson et al., 2004) rather than the effect on myelination process itself. However, shP2RY2, 5ppase\textsuperscript{WT}, or shMCU-expressing SCs did not overlap with 5-bromo-2-deoxyuridine (BrdU), a marker for mitotic cells (Figure S3I), or cleaved caspase-3 (Cas3), a marker for cell death (Figure S3J). We also examined whether the ATP signal-dependent mitochondrial metabolism has a role after the end of the myelination period. When P2RY2-IP_{3}-MCU signaling was suppressed from P3 to P14, the time-period during which SCs actively undergo myelination, both longitudinal and radial extensions of SCs were reduced (Figures 4A, 4B, and S3E) and hypomyelination was observed (Figures 4C, 4D, and S4B). In contrast, when P2RY2-IP_{3}-MCU signaling was suppressed from P14 to P31, i.e., after the period of myelination, no significant change in the length and diameter of SCs was observed (Figures 4E, 4F, and S3F), and the g-ratio remained the same (Figures 4G, 4H, and S4C). These results suggest that P2RY2-IP_{3}-MCU signaling is likely to have a key role in the process of myelination rather than in the process of myelin maintenance.

**DISCUSSION**

These findings demonstrate the interaction signaling between axon and SC mitochondria and its physiological significance in myelination. This signaling involves ATP release from axons, P2RY2-IP_{3}-mediated Ca^{2+} mobilization in SCs, and mitochondrial Ca^{2+} influx via MCU (Figure 4I) and plays a key role in the myelin sheath developmental process.

It has been shown that neurotransmitters are released from axons not only at presynaptic sites but also at extrasynaptic sites along the length of axons in an activity-dependent manner. For example, axons in the CNS release glutamate via exocytotic vesicles at extrasynaptic sites (Kukley et al., 2007; Ziskin et al., 2007), whereas axons of dorsal root ganglion (DRG) cells in culture release ATP in a non-vesicular manner upon stimulation (Fields S2F–S2H), confirming the involvement of axonal electrical activity in the Ca^{2+} response. It has been reported that firing peripheral axons release ATP through maxi-anion channels, which have a 1.3-nm-radius open pore through which ATP (which has a radius of 0.6 nm) can permeate (Fields and Ni, 2010). We thus applied three different inhibitors of maxi-anion channels (GdCl_{3}, glibenclamide, and 5-nitro-2-(3-phenylpropylamino)-benzoic acid [NPPB]). The electrical-stimulation-evoked cytosolic and mitochondrial Ca^{2+} responses in SCs were significantly suppressed by these inhibitors (Figures S2F–S2H). We also assessed other potential pathways for ATP release (Burnstock, 2007) and found that inhibitors of vesicular exocytosis (tetanus toxin and bafilomycin A) and connexin-mediated release (carbenoxolone and 1-octanol) had no significant effect (Figures S2F–S2H). Thus, these results are consistent with the notion that firing axons release ATP in a non-vesicular, maxi-anion-channel-mediated manner. Taken together, the findings suggest that firing axons control P2RY2-IP_{3}-MCU signaling in SCs through axonal ATP release.
It was proposed that maxi-anion channels are involved in this activity-dependent axonal ATP release from cultured DRG neurons. Our results are consistent with the notion that ATP is released in a non-vesicular manner, through maxi-anion channels from neonatal rat sciatic nerve axons (Figures S2F–S2H). Because SCs release ATP upon stimulation of P2RY2 in vitro (Liu et al., 2005), it is possible that SCs may partly contribute to the increase in the extracellular ATP concentration during nerve stimulation. It remains to be seen whether the difference in the axonal transmitter release mode, be it vesicular or non-vesicular, depends on the neuron type or on the transmitter type. Because molecular identity of maxi-anion channels remains unknown, we were not able to specify their localization. Considering that the paranodal junction is rigidly sealed with tight junctions (Rosenbluth, 2009), it seems reasonable to assume that ATP is released from the nodes of Ranvier rather than from the intermodal regions. Interestingly, mitochondria, which generate ATP, accumulate at nodes of Ranvier in PNS axons, but such accumulation, if any, is much less pronounced in CNS axons (Berthold et al., 1993; Fabricius et al., 1993). This apparent structural difference may suggest that nodal concentration of mitochondria constitutes one of the components of axonal ATP release machinery in PNS.

How does the axonal ATP signal reach mitochondria in SCs? Most cytosolic components and organelles, including mitochondria, are found in the outermost region of myelinating SCs (the abaxonal region), which is separated by myelin sheath from the region that is in direct contact with axons (the adaxonal region). Therefore, released ATP would signal to the axonal membrane of myelinating SCs. There seem to be two possible ways by which the axonal ATP signal might be transmitted to the mitochondria of SCs. One is diffusion of intracellular signaling molecules (Ca^{2+} or IP3) from the adaxonal region to the abaxonal region.
region within the SC; the other is diffusion of ATP within the extracellular space from axons to the abaxonal region of SC in a volume-transmission manner. Gap-junction-mediated radial diffusion in Schmidt-Lanterman incisures, which allow the passage of small molecules, offers one potential intracellular pathway. However, inhibitors of gap junction channels had no significant effect on SC Ca2+ responses (Figures S2G and S2H). Thus, the extracellular pathway seems more likely. Supporting this, the extracellular concentrations of ATP and its metabolites were found to reach levels that are high enough to activate P2Y purinergic receptors during nerve firing (Figure 2C).

It has been well documented that numerous factors are involved in the axon-to-SC signaling in myelination (Taveggia et al., 2010). Although the present results clearly indicated the necessary role of ATP in the axon-to-SC signaling, the magnitude of the effects of P2RY2-IP3-MCU signal inhibition on the myelin development was modest (Figures 3 and 4). This could be partly because the level of expression of shRNA was variable among transfected SCs. It is also possible that the ATP signaling works as a supportive signal, rather than a primary regulator, for myelination, stimulating the SC mitochondrial metabolism to meet the high metabolic demand during myelination.

It has been reported that axonal electrical activity has trophic effects on CNS myelination (Barres and Raff, 1993; Demeren et al., 1996; Wake et al., 2011). Regarding the role of axonal electrical activity in myelination, axonal electrical activity suppresses mitosis and differentiation of SCs before myelination in cocultures of DRG neurons and SCs (Stevens and Fields, 2000). Thus, electrical activities in the developing nervous system may delay terminal differentiation of SC precursors. On the other hand, axonal electrical activity was found to have no effect on the initiation of myelination in zebra fish PNS (Woods et al., 2006). Here, we found that potential axonal activity-dependent signaling works as a positive modulator of SC development after the initiation of myelination. Thus, axonal activity appears to play distinct roles depending on the developmental stage of SCs. Future in vivo studies investigating the effects of myelination upon direct axonal activity manipulation will be needed.

It is worth noting that the afferent axonal firing rate dramatically increases in postnatal developing nerves (Fitzgerald, 1987). Therefore, axonal-activity-dependent ATP signaling may function as an instructive signal to stimulate mitochondrial metabolism in myelinating SCs for the formation of myelin. Because axonal electrical activity stimulates energy consuming subcellular processes such as protein synthesis and trafficking during CNS myelination by oligodendrocytes (Wake et al., 2011), axonal-activity-dependent control of cellular metabolism in myelinating glial cells seems to be a common mechanism in the myelination of both CNS and PNS.

**EXPERIMENTAL PROCEDURES**

All experiments were performed in accordance with the guidelines of the University of Tokyo. Detailed information regarding all experimental procedures is available in the Supplemental Experimental Procedures.

For in vivo electroporation, Sprague-Dawley rats were anesthetized with iso-flurane and the sciatic nerves were exposed. After the injection of plasmid DNA into the sciatic nerve, electric pulses (voltage, 50 V; pulse duration, 5 ms; pulse interval, 100 ms; pulse number, four times) were then delivered to the injection site. Following electroporation, the incision was closed with cyanoacrylate glue.

To analyze extracellular adenine nucleoside/nucleotide levels within the rat sciatic nerves, rats were anesthetized by isoflurane and a microdialysis probe was inserted into the sciatic nerve. To apply electrical stimulation, a platinum bipolar electrode was placed on the sciatic nerve proximal to the microdialysis probe. Bursts of ten pulses (0.5 ms, 10 V, 50 Hz) were applied every 1 s. Quantification of adenine nucleosides/nucleotides in the collected dialysates was performed using an HPLC system.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.039.

**AUTHOR CONTRIBUTIONS**

D.I. conceived the project, performed most of the experiments, and analyzed the data. H.S. helped with the electron microscopic analyses. J.S. constructed four figures, and two tables and can be found with this article online at

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