Gpr126 functions in schwann cells to control differentiation and myelination via G-protein activation

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The myelin sheath surrounding axons ensures that nerve impulses travel quickly and efficiently, allowing for the proper function of the vertebrate nervous system. We previously showed that the adhesion G-protein-coupled receptor (aGPCR) Gpr126 is essential for peripheral nervous system myelination, although the molecular mechanisms by which Gpr126 functions were incompletely understood. aGPCRs are a significantly understudied protein class, and it was unknown whether Gpr126 couples to G-proteins. Here, we analyze DhhCre; Gpr126<sup>fl/fl</sup> conditional mutants, and show that Gpr126 functions in Schwann cells (SCs) for radial sorting of axons and myelination. Furthermore, we demonstrate that elevation of cAMP levels or protein kinase A activation suppresses myelin defects in Gpr126 mouse mutants and that cAMP levels are reduced in conditional Gpr126 mutant peripheral nerve. Finally, we show that Gpr126 directly increases cAMP by coupling to heterotrimeric G-proteins. Together, these data support a model in which Gpr126 functions in SCs for proper development and myelination and provide evidence that these functions are mediated via G-protein-signaling pathways.

Introduction

Myelin is essential for the proper function of the vertebrate nervous system. During peripheral nervous system (PNS) development, immature Schwann cells (SCs) are associated with many axons; in a process called radial sorting, immature SCs insert cytoplasmic projections into axon bundles to separate individual axons. Some immature SCs develop into nonmyelinating Remak SCs that ensheath multiple small caliber axons. Other immature SCs become promyelinating SCs that are associated in a 1:1 relationship with a single axonal segment. Myelinating SCs iteratively wrap their membrane around their associated axonal segment to generate the myelin sheath (Jessen and Mirsky, 2005).

We previously showed that the adhesion G-protein-coupled receptor (aGPCR) Gpr126 is essential for PNS myelination. aGPCRs represent a unique protein class defined structurally by a seven-transmembrane helix region (7TM) and an extremely large extracellular N terminus, separated from the 7TM by a GPCR autoproteolysis-inducing (GAIN) domain and a GPCR proteolytic site (GPS) domain. These domains split the receptor during the maturation process into N-terminal and C-terminal fragments that are thought to remain noncovalently attached at the cell surface (Arac¸ et al., 2012). The extracellular regions of many aGPCRs contain domains that are involved in cell–cell or cell–matrix adhesion in other proteins (Yona et al., 2008). The 7TM is classically defined by its ability to bind heterotrimeric G-proteins within a cell, through which signal transduction cascades are activated upon agonist binding. For example, the G-protein subunit Goα activates adenyl cyclases to elevate cAMP levels, whereas Goi inhibits adenyl cyclases to decrease cAMP levels (Gilman, 1984). Via release of Gβγ dimers, G<sub>i</sub>-proteins mediate diverse additional functions, including modulation of phosphatidylinositol 3-kinase (PI3K; Lin and Smrcka, 2011). The majority of aGPCRs remain undercharacterized, and for most family members, the biological functions, activating ligands, and downstream effectors are unknown.

Our previous work defined the first known function of Gpr126 as an essential regulator of PNS myelination. In germline zebrafish and mouse Gpr126 mutants, SCs fail to myelinate axons (Monk et al., 2009, 2011). Analysis of genetic chimeras in zebrafish suggested that Gpr126 is required in SCs for myelin protein expression (Monk et al., 2009). However, mouse Gpr126<sup>−/−</sup> mutant peripheral nerves have many defects not observed in zebrafish mutants, including radial sorting delays, limb contracture defects, and axon degeneration (Monk et al., 2011), raising questions regarding the cellular autonomy of Gpr126 in...
mammals. Furthermore, germline deletion of Gpr126 is lethal in mouse, precluding analysis beyond early postnatal stages. Here, we analyze Gpr126 conditional mutant mice, and our data suggest that Gpr126 functions in SCs for timely radial sorting and myelination. We also show that elevating cAMP levels and activating protein kinase A (PKA) restores myelination in Gpr126−/− myelinating cultures, that cAMP is downregulated in Gpr126 conditional mutant nerves, and that Gpr126 couples to the Gs-protein and Gq-protein families. These data demonstrate that Gpr126 is required autonomously in SCs for proper development and strongly support a model in which Gpr126, via interactions with G-proteins, modulates cAMP levels in SCs to control differentiation and myelination.

Materials and Methods

Mice. All animal experiments were performed in compliance with Washington University’s institutional animal protocols. Gpr126−/− constitutive knock-out mouse generation and genotyping have been previously described (Monk et al., 2011). Conditional Gpr126 mutant mice (Gpr126−/− mice) were generated by Lexicon Pharmaceuticals, and Gpr126+/+ mice were obtained from Taconic (catalog number TF0269) on a mixed background (129Sv/EvBrd). We used the following primers to detect the presence of the floxed allele: 5′-CATTGAGCCTCCCTGG-TATGG-3′; 5′-CCCTGACGTTACCCACATG-3′. Gpr126−/− mice were crossed to βActinCre mice on a pure C57BL/6 background (Lewandoski et al., 1997), and βActinCre;Gpr126−/− mice were crossed to Gpr126−/− mice to generate βActinCre;Gpr126−/− mice and their sibling controls. βActinCre mice were genotyped as described previously (Abdulkadir et al., 2002). Gpr126−/− mice were also crossed to DhhCre mice on a pure C57BL/6 background (Jaegle et al., 2003), and DhhCre;Gpr126−/− mice were crossed to Gpr126−/− mice to generate DhhCre;Gpr126−/− mice and their sibling controls. DhhCre mice were genotyped as described previously (Jaegle et al., 2003). To assess recombination of the floxed allele, we used the ROSA26 LacZ reporter strain (Soriano, 1999). For all mouse experiments, mice of either sex were analyzed, and mutants were always compared with littermate sibling controls.

RT-PCR. Standard RT-PCR was performed as described previously (Monk et al., 2011) on cDNA derived from βActinCre;Gpr126−/− and sibling control postnatal day (P) 1 lung tissue or on cDNA derived from DhhCre;Gpr126−/− and sibling control P14 lung or sciatic nerve tissue. For sciatic nerve, we pooled four nerves from two Gpr126−/− mice and six nerves from three DhhCre;Gpr126−/− mice (all mice were siblings). In situ hybridization. Mice were intracardially perfused with 4% PFA. Tissues were dissected and postfixed in the same fixative at 4°C overnight. After protecting in 30% sucrose and embedding in optimal cutting temperature compound, frozen sections were prepared at 7 μm on a cryostat (Leica). In situ hybridization was performed as previously described (VanDunk et al., 2011) on sections from DhhCre;Gpr126−/− (n = 2) and sibling control (n = 2 DhhCre;Gpr126−/− siblings) P21 sciatic nerve and dorsal root ganglia (DRGs). Antisense and sense control digoxigenen-labeled riboprobes were transcribed as described previously (Patra et al., 2013).

Immunohistochemistry. For tissue sections, mice were processed as described in situ hybridization. Antibody staining was performed as described previously (Jeong et al., 2012). For myelinating DRG cultures, cells were fixed at room temperature with 4% PFA for 30 min. After washing with PBS, cells were permeabilized with ice-cold methanol and plates were kept at −20°C for 20 min. Cells were washed again with PBS and blocked with PBS containing 2% BSA plus 10% goat serum plus 0.1% Triton X-100 for 30 min at room temperature. Primary antibodies were suspended in the blocking buffer and cells were incubated for 1 h at room temperature. Cells were washed with PBS and incubated with appropriate fluorescently labeled secondary antibodies (1:1000; Invitrogen) in blocking buffer for 1 h at room temperature. After washing with PBS, coverslips were mounted on glass slides using Vectashield with DAPI (Vector Labs). We used the following primary antibodies: rat anti-MBP (1:10; AbD Serotec), rabbit anti-si100 (1:400; Dako), rabbit anti-TUJ1 (1:1000; Covance), chicken anti-β-galactosidase (1:200; Abcam).

Transmission electron microscopy. Mice were intracardially perfused with modified Karnovsky’s fix (2% glutaraldehyde plus 4% PFA in 0.1 M sodium cacodylate, pH 7.4), and sciatic nerves were removed and postfixed in the same fixative at 4°C overnight. Nerves were postfixed and embedded as described previously (Monk et al., 2011). Semithin sections (200 nm) were stained with toluidine blue, viewed on a light microscope (Zeiss AxiosImager M2), and images were recorded with an AxioCam MRm. Thin sections (70 nm) were stained with uranyl acetate and Sato’s lead stain, and then viewed on a Jeol (JEM-1400) electron microscope. Images were recorded with an Advanced Microscopy Techniques V601 digital camera. We examined three siblings (one Gpr126+, one Gpr126−/−, and one DhhCre;Gpr126−/−) and four DhhCre;Gpr126−/− mutants at P1; four siblings (two Gpr126−/− and two DhhCre;Gpr126−/−) and three DhhCre;Gpr126−/− mutants at P10; four siblings (four DhhCre; Gpr126−/−) and three DhhCre;Gpr126−/− mutants at P21; three siblings (two Gpr126−/− and one DhhCre;Gpr126−/−) and three DhhCre;Gpr126−/− mutants at P42. For quantifications, we quantified all visible axons and their relationships with SCs at 3000× magnification from four to five randomly selected areas of two DhhCre;Gpr126−/− mutant and three sibling (DhhCre;Gpr126−/−) controls.

Proliferation assay and nucleic quantification. To quantify proliferation, sciatic nerve cryosections were obtained and processed as described for immunocytochemistry. Rabbit anti-Ki67 (1:200; Abcam) and appropriate secondary antibody (1:1000; Invitrogen) were used to identify proliferating cells. The total number of nuclei (DAPI+) and the total number of proliferating cells (Ki67+) cells per sciatic nerve were counted manually for four control (DhhCre;Gpr126−/−) and four DhhCre;Gpr126−/− mutants at P42. To quantify total nuclei, we counted the total number of nuclei from semithin sections obtained from the same P21 animals described for transmission electron microscopy (TEM). SC nuclei were identified based on morphology (see Fig. 3D) and were confirmed by electron microscopy to be associated with axons and in possession of a basal lamina (see Fig. 5F).

Myelinating DRG cultures. Myelinating DRG cultures were performed according to standard methods (Eldridge et al., 1987) with minor modifications. Briefly, DRGs were removed from embryonic day (E) 12.5 mouse embryos and plated on Matrigel (BD Biosciences)-coated coverslips. Embryos were genotyped as described previously (Monk et al., 2011). DRGs were cultured in neurobasal media containing 2% B27 (Invitrogen) and 50 mg/ml NGF (Harlan Labs). Media was changed every other day for the duration of culture. After 7–8 d in vitro, SCs were confluent along axons and 50 μg/ml ascorbic acid (Sigma-Aldrich) was added to induce the formation of basal lamina. We added 5 μM forskolin (Sigma-Aldrich) or 250 μM 8-CTP-cAMP (BioLog) to experimental wells at the same time as ascorbic acid. These conditions were maintained for 3 weeks, at which point the cultures were fixed and subjected to immunocytochemistry. Three independent experiments were performed in triplicate.

In vivo CAMP measurement. CAMP concentration in the sciatic nerves was measured using CAMP measurement kit (Enzo Life Sciences) according to the manufacturer’s instructions. Briefly, sciatic nerves from DhhCre; Gpr126−/− mice (n = 3) and control animals (n = 5 Gpr126−/− mice) were homogenized in ice-cold trichloroacetic acid. After centrifugation, the supernatant was clarified of cell debris by water-saturated ether, the clarified supernatant was lyophilized overnight, and the dried pellet was resuspended in manufacturer-provided buffer. CAMP levels were measured in this suspension, and CAMP concentrations were quantified using Assay Blaster software (Enzo Life Sciences). Control values were set to 100%, and mutant CAMP concentrations were calculated as a percentage of control.

In vitro functional assays. Full-length human (NM001032395.2) GPR126 protein was directly cloned from human monocye cDNA library (primers: forward, ATGATGTTTCGCTCAGATCGAATG, and reverse, TTAAAACCTTGTGCTGTGGCTG) and inserted into the mammalian expression vector pcDps. The human GPR126 cDNA was N-terminally tagged with the initial signal peptide at amino acid position 38 with a hemagglutinin (HA) epitope and C-terminally tagged with a FLAG epitope by a PCR-based site-directed mutagenesis and fragment replacement strategy.
COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 7% CO₂ in a humidified atmosphere. Cells were split into 12-well plates (1 × 10⁵ cells/well, for inositol phosphate (IP₃) assay) and 48-well plates (3 × 10⁴ cells/well for cAMP assay) and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. To measure IP₃ formation, transfected COS-7 cells were incubated with 2 μCi/ml myo-[³H]inositol (18.6 Ci/mmol; PerkinElmer Life Sciences) for 16 h. Thereafter, cells were washed once with serum-free DMEM containing 10 mM LiCl followed by incubation with serum-free DMEM containing 10 mM LiCl for 1 h at 37°C. Intracellular IP₃ levels were determined by anion-exchange chromatography as described previously (Bertride, 1983). IP₃ accumulation data were analyzed using GraphPad Prism version 5.0 for Windows (GraphPad Software). For cAMP measurements, 48 h after transfection, cells were incubated with 3-isobutyl-methyl-xanthine (1 μM)-containing medium for 1 h. Incubation was stopped by washing with ice-cold PBS. Cells were lysed in Li buffer (PerkinElmer Life Sciences) and frozen at −20°C until measurement. To measure cAMP concentration, the AlphaScreen cAMP assay kit (PerkinElmer Life Sciences) was used according to the manufacturer’s protocol. The accumulated cAMP was measured in 384-well white OptiPlate microplates (PerkinElmer Life Sciences) with the Fusion AlphaScreen multilabel reader (PerkinElmer Life Sciences). To estimate cell surface expression of receptors carrying an N-terminal HA tag, we used an indirect cellular ELISA as described previously (Schoenberg et al., 1998).

**Statistical analysis.** For TEM quantification, we performed a two-tailed Student's t test. For in vitro cAMP assays, we performed a Mann–Whitney U test. For IP₃ assays, we performed one-way ANOVA followed by a Bonferroni post hoc test. For in vitro cAMP assays, proliferation, and nuclei quantification, we performed one-way ANOVA followed by the Dunn's multiple post hoc test. For values < 0.05 were considered to be significant; specifically, *p < 0.05; **p < 0.01; ***p < 0.001.

**Results**

**Dhh<sup>Cre</sup>;Gpr126<sup>fl/fl</sup> mutant mice are viable**

In an attempt to circumvent the lethality of constitutive Gpr126 mutant mice, we used conditional mutant mice (Gpr126<sup>b/b</sup>). In the mutant allele, exons 2 and 3 are replaced by a selection cassette flanked by loxP sites (Fig. 1A, B). To verify the utility of this line, we globally ablated Gpr126 by mating Gpr126<sup>b/b</sup> mice with mice that express Cre recombinase under control of the β-actin (β-Act) promoter (Lewandoski et al., 1997). We observed the same limb contracture defects (Fig. 1C) and early lethality in βAct<sup>Cre</sup>; Gpr126<sup>b/b</sup> mutants as previously reported in constitutive Gpr126<sup>b/b</sup> mutants (Monk et al., 2011). Additionally, we did not detect Gpr126 mRNA in lung tissue from βAct<sup>Cre</sup>; Gpr126<sup>b/b</sup> mutants (Fig. 1D), though it is normally highly expressed in this tissue (Moriguchi et al., 2004; Haitina et al., 2008).

To begin to interrogate the function of Gpr126 in SCs, we crossed Gpr126<sup>b/b</sup> mice with mice that express Cre recombinase under control of the Desert Hedgehog (Dhh) promoter, which drives recombination in SC precursors at ~E12.5 (Jaegle et al., 2003). Unlike constitutive Gpr126<sup>b/b</sup> mutants, which showed significant embryonic lethality (Monk et al., 2011), Dhh<sup>Cre</sup>; Gpr126<sup>b/b</sup> animals were born in near-Mendelian ratios (53:315, 16.8%) and survived for >1 year. Constitutive Gpr126<sup>b/b</sup> mutants were generally immotile (Monk et al., 2011); in contrast, Dhh<sup>Cre</sup>; Gpr126<sup>b/b</sup> mutants were ambulatory, although they exhibited trembling by ~P10. Dhh<sup>Cre</sup>; Gpr126<sup>b/b</sup> mutants were also sometimes smaller than littermate controls, but this phenotype was variable and not fully penetrant. We did not detect Gpr126 mRNA in sciatic nerve of Dhh<sup>Cre</sup>; Gpr126<sup>b/b</sup> mutants (Fig. 1E), although Gpr126 was robustly expressed in the lungs of Dhh<sup>Cre</sup>;
Gpr126fl/fl mutants (data not shown). Because most mRNAs in the sciatic nerve are transcribed by SCs, this result suggests that Gpr126 was efficiently recombined in DhhCre;Gpr126fl/fl mutant SCs. To more thoroughly assess cell type-specific recombination efficiency of Gpr126 in DhhCre;Gpr126fl/fl mutants, we performed in situ hybridization on tissue sections from DRGs and sciatic nerve from DhhCre;Gpr126fl/fl and control DhhCre;Gpr126fl/+ animals. We observed Gpr126 expression in a pattern consistent with DRG neurons in both control and DhhCre;Gpr126fl/fl mutants (Fig. 1F, G). We observed robust Gpr126 expression in control nerve, in a pattern consistent with SCs (Fig. 1H). In contrast, DhhCre;Gpr126fl/+ mutant sciatic nerve did not express Gpr126 (Fig. 1I) and staining was indistinguishable from sense controls (data not shown).

To further ensure that Gpr126 was efficiently recombined in SCs but not in neurons, we examined LacZ expression in DRGs, spinal cord, and sciatic nerve of DhhCre;Gpr126fl/+;Rosa26m mice. We detected robust LacZ expression in SCs of the sciatic nerve at P15, but not in DRG neurons or in the CNS (Fig. 2), which is consistent with previous reports (Jaegle et al., 2003; Wu et al., 2008). Importantly, Gpr126fl/+ mice are healthy, fertile, and show no obvious defects in SC development or myelination (Fig. 3A, C; Fig. 4A; data not shown), indicating that the neomycin selection cassette and LoxP sites introduced at the Gpr126 locus (Fig. 1B) did not affect Gpr126 function. Further, we observed no difference in expression of genes flanking Gpr126 (Pex3, Aig1, and Hivep2) in cDNA derived from P4 sciatic nerve of Gpr126fl/+ or DhhCre;Gpr126fl/+ animals compared with Gpr126fl/+ by quantitative PCR (qPCR; data not shown). Similarly, Gpr126 expression was not altered in cDNA derived from P4 lung of Gpr126fl/+ or DhhCre;Gpr126fl/+ animals compared with Gpr126fl/+ by qPCR (data not shown). Therefore, Gpr126fl/+ mice represent a new tool with which to conditionally ablate Gpr126 with cell-type specificity, and DhhCre;Gpr126fl/+ mutants represent a useful model to examine the function of Gpr126 in SCs.

**DhhCre;Gpr126fl/+ mutant mice show defects in radial sorting and myelination**

Gross examination revealed that DhhCre;Gpr126fl/+ mutant sciatic nerves were less opaque than control nerves at P21 (Fig. 3A, B), indicative of reduced myelination. To test this, we examined sciatic nerve ultrastructure at multiple developmental stages between P1 and P42 by transmission electron microscopy (TEM). Analysis of semithin sections stained with toluidine blue revealed a lack of myelin in DhhCre;Gpr126fl/+ mice (Fig. 4A, B, C). Interestingly, we did not observe peripheral axon degeneration in DhhCre;Gpr126fl/+ mutant sciatic nerve, although this phenotype was evident in constitutive Gpr126−/− mutants (Mogha et al., 2011). Future analyses will determine whether Gpr126 has a neu-
ronal function in survival/axonal maintenance or whether neurons require a Gpr126-mediated signal from SCs before E12.5. In sum, this analysis suggests that Gpr126 is required in SCs for timely radial sorting.

At P21 and P42, large-caliber axons in control nerves were well myelinated while small-caliber axons were organized into Remak bundles (Fig. 4D,G). In DhhCre; Gpr126fl/fl mutant sciatic nerves at these stages, many axons were sorted into a 1:1 relationship by SCs (Fig. 4E,H,I), although no myelin was observed and radial sorting defects persisted (Fig. 4E,F,H–M). Furthermore, we observed aberrant abaxonal cytoplasmic protrusions in mutant SCs (Fig. 4F,1, similar to defects previously reported in Rac1 mutants (Benniger et al., 2007; Nodari et al., 2007; Guo et al., 2012). These results suggest that Gpr126 is required in SCs for myelination, which is consistent with chimeric analysis in zebrafish mutants (Monk et al., 2009). These results also suggest that Gpr126, like Rac1, is required in SCs for membrane stabilization.

**DhhCre;Gpr126fl/fl** mutant SCs are hyperproliferative

Examination of semithin sections stained by toluidine blue suggested that more SC nuclei were present in DhhCre;Gpr126fl/fl mutant nerves compared with control (Fig. 3). We assessed proliferation in P4 sciatic nerve by Ki67 immunostaining. Proliferation was significantly increased in DhhCre;Gpr126fl/fl mutant nerves compared with control nerves (Fig. 5A–C), and this increase in SC number persisted at P21 (Fig. 5D). These data indicate that loss of Gpr126 causes SCs to overproliferate and show that defects in radial sorting and myelination are not due to a reduction in SC number.

cAMP elevation suppresses myelin defects in Gpr126 mutants

For the majority of aGPCRs, downstream signaling effectors are unknown or not well defined. Moreover, although previous studies have supported a link between aGPCRs and G-protein signaling (Liebercher et al., 2013), direct G-protein coupling has only been recently demonstrated for two family members, GPR133 and GPR114 (Bohnkamp and Schöneberg, 2011; Gupte et al., 2012). We previously reported that forskolin treatment to elevate cAMP suppresses myelin defects in gpr126 mutant zebrafish (Monk et al., 2009), suggesting that Gpr126 might couple to G-protein and elevate cAMP. To begin to dissect this signaling pathway, we first tested the hypothesis that cAMP elevation would suppress myelin defects in Gpr126 mouse mutants. To this end, we performed myelinating assays in vitro using DRGs derived from constitutive Gpr126+/+, Gpr126−/−, or Gpr126−/− animals with or without stimulation by compounds that elevate cAMP. Myelin internodes, as assessed by myelin basic protein [MBP(+)]) segments, were observed in Gpr126+/+ and Gpr126−/− (control) cultures after stimulation with ascorbic acid to promote SC basal lamina formation and myelination (Eldridge et al., 1987; Fig. 6A,G). No differences were observed between Gpr126+/+ and Gpr126−/− cultures (data not shown). MBP(+) internodes were never observed in Gpr126−/− cultures.
GPR126 elevates cAMP and couples to heterotrimeric G-proteins

The ability of cAMP elevation to suppress mutant phenotypes in Gpr126−/− cultures (Fig. 6D,F,G). These data show that cAMP elevation suppresses Gpr126−/− myelin defects in mammals. Moreover, since 8-CPT-cAMP is a site-selective activator of PKA (Dostmann et al., 1990), these results also support a model in which Gpr126 elevates cAMP in SCs to activate PKA before myelination, which is consistent with a recent report in zebrafish (Glenn and Talbot, 2013).

transfected cDNA-encoding full-length human GPR126 into COS-7 cells and measured cAMP accumulation using a second messenger assay. GPR126-transfected COS-7 cells showed a concentration-dependent increase of cAMP levels (Fig. 7A). As a control, we transfected human P2Y12, a known Gq-coupling receptor (Yang et al., 2002). As expected, P2Y12-transfected COS-7 cells did not show a concentration-dependent increase in cAMP, and cAMP accumulation was significantly lower than in GPR126-transfected cells (Fig. 7A). Proper cell-surface expression of GPR126 was detected by ELISA (Fig. 7C). These results show that full-length GPR126 can localize to the plasma membrane and elevate cAMP via the Gs/adenylyl cyclase pathway in a concentration-dependent manner.

To further define the G-protein-coupling abilities of GPR126 to Gq-proteins and Gs-proteins, we performed IP3 accumulation assays. Transfection of GPR126 into COS-7 cells alone did not lead to an increase in IP3 levels, indicating that this receptor does not couple to Gq (Fig. 7C). Chimeric G-proteins, which have the C-terminal 4 aa of a Gq-protein exchanged for the corresponding amino acids of Goq or Goi, can redirect the intracellular signaling cascades of these respective GPCRs toward a classic Goq pathway, resulting in the production of IP3 as a downstream effector of PLC (Conklin et al., 1993). Using this well-established system, we cotransfected GPR126 and chimeric G-proteins Goq4 (to assess Goq coupling) or Goi4 (to assess Goi coupling). In both experiments, we observed a robust increase in IP3 levels (Fig. 7C). Therefore, we conclude that GPR126 couples to both Gq-proteins and Gs-proteins, but not Gq-proteins. Finally, we measured cAMP concentration in DhhCre;Gpr126fl/fl mutant sciatic nerve at P4 and found that cAMP levels in mutant nerves were significantly reduced compared with control (Fig. 7D). Together, these data show that GPR126 elevates cAMP and, like many Gq-coupled GPCRs (Guérrmann et al., 1997, Liebscher et al., 2013), couples to Gq-proteins as well as Gs-proteins.

Discussion

Gpr126 couples to G-proteins and elevates cAMP

A role for cAMP in SC development and myelination has long been hypothesized. Elegant studies spanning decades of work have firmly established that axonal signals are required for SC differentiation and myelination in vivo and in vitro (Wood and Bunge, 1975; Weinberg and Spencer, 1978; Salzer and Bunge, 1980; Salzer et al., 1980a). In vitro, embryonic SCs grown in the presence of neurons display a similar developmental progression as SCs in vivo—they migrate along neurites, proliferate, sort axons, and myelinate axons (Wood, 1976). These behaviors rely on the presence of axons because in their absence, SCs fail to differentiate. The requirement for neurons can be overcome in vitro by the addition of axonal membrane fragments (Salzer et al., 1980, 1980b) or by elevating levels of cAMP in the SCs. Upon exogenous axon membrane application or cAMP elevation, SCs upregulate the expression of...
myelin-related molecules and downregulate the expression of immature SC markers (Sobue et al., 1984; Mokuno et al., 1988; Monuki et al., 1989; Mirsky et al., 1990; Morgan et al., 1991; Scherer et al., 1994). Thus, an in vivo role for cAMP in SC myelination was hypothesized; however, the identity of a receptor that might alter its levels was only recently discovered.

Our previous work in zebrafish offered the first piece of evidence that cAMP elevation is essential for SC myelination in vivo. We showed that treatment of gpr126 mutant zebrafish larvae with forskolin, an adenylyl cyclase activator that elevates cAMP, could suppress myelin defects in vivo (Monk et al., 2009). This suggested, albeit indirectly, that Gpr126 elevates cAMP. Here, we extend these studies to mammals and show that addition of forskolin or 8-CPT-cAMP, a cell-permeable cAMP analog that activates PKA, can suppress Gpr126 mutant phenotypes in myelinating DRG cultures (Fig. 6D,F,G). These results are consistent with our previous studies in zebrafish and support the notion that Gpr126 elevates cAMP. To more definitively test this hypothesis, we show that GPR126 directly elevates cAMP in COS-7 cells (Fig. 7A), and that GPR126 couples to Gαi-proteins and Gαs-proteins (Fig. 7C). Together, our data support a model in which Gpr126, via interactions with Gαi, functions in SCs to elevate cAMP and drive myelination. GPR126 also couples to Gαs-proteins, and although it is not uncommon for GPCRs to couple to more than one G-protein class (Gudermann et al., 1997; Liebscher et al., 2013), this was not predicted from our rescue experiments. In mouse SCs in vitro, low levels of cAMP promote neuregulin-dependent proliferation, while high levels of cAMP promote differentiation (Arthur-Farraj et al., 2011). Consistent with this model, we observe overproliferation of SCs in cAMP-depleted DhhCre; Gpr126−/− mutant sciatic nerve (Fig. 5). Via interactions with both Gαi-proteins and Gαs-proteins and via integration of signals from other essential pathways, Gpr126 could precisely regulate the concentration of cAMP required for a given stage of SC development.

**Cellular autonomy of Gpr126**

In zebrafish, we previously generated genetic chimeras in an attempt to define the autonomy of Gpr126 in myelination. In these transplantation experiments, wild-type (WT) SCs in mutant

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**Figure 6.** cAMP elevation rescues amyelination in Gpr126−/− mutants. A–F, In vitro DRG myelinating cultures from Gpr126+/+ (control; A, C, E) and Gpr126−/− embryos (B, D, F) stained with MBP (red), s100 (green), and DAPI (blue). A, Gpr126+/+ SCs myelinate axons after 2 weeks in culture following ascorbic acid addition (AA). This is potentiated by forskolin (FSK; C) or by 8-CPT-cAMP (E) addition. B, Gpr126−/− SCs do not myelinate axons following AA addition. MBP (+) segments are observed after addition of FSK (D) or 8-CPT-cAMP (F). Scale Bar: (in A) A–F, 100 μm. G, Quantification shows the number of MBP (+) segments observed in 1 mm² in Gpr126+/+ (control) cocultures (gray bars) and in Gpr126−/− cocultures (black bars). Data are given as means ± SD of three independent experiments, each performed in triplicate. ***p < 0.001.
Figure 7. GPR126 directly elevates cAMP, couples to Gas and Gai, and cAMP is downregulated in DhhCre;Gpr126mutant nerves. A, COS-7 cells transfected with increasing amounts (100 – 600 ng/well) of plasmids encoding either human GPR126 (black circles) or human P2Y12 (white circles). After 2 d, intracellular cAMP levels were measured. CAMP concentration is shown as fold change over empty vector (pcDps) control. GPR126, but not P2Y12, caused a dose-dependent increase of cAMP. Data are given as means ± SD of four independent experiments, each performed in triplicate. B, COS-7 cells were transfected with 300 ng of plasmid encoding human GPR126 (black bar), human P2Y12 (gray bar), or empty vector (pcDps). After 2d, cell surface expression levels were determined. OD values are given as the percentage of human P2Y12, which served as a positive control (percentage of positive control). Data are given as means ± SD of three independent experiments, each performed in triplicate. C, To specify G-protein-coupling abilities of GPR126, we performed IP3 accumulation assays, which detect Gαq- or Gαi-mediated activation of PLC and subsequent increase in IP3. Through the use of chimeric Gαqαi proteins or Gαiαq proteins, Gαq and Gαi-coupling GPCRs can be recognized and directed via the Gαq-mediated pathway. For each assay, 1500 ng of either GPR126 plasmid was cotransfected with 100 ng of either chimeric G protein or empty vector. IP3 levels increased only with cotransfection of GPR126 with Gαqαi or Gαiαq showing that GPR126 couples both to Gαq-proteins and Gαi-proteins, but not to Gαs-proteins. Data are given as means ± SD of three independent experiments, each performed in triplicate. D, cAMP is significantly downregulated in P4 DhhCre;Gpr126mutant nerve (black bar, n = 3) compared with Gpr126mutant control (gray bar, n = 3). Data are given as means ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.

hosts were capable of expressing MBP, even though they were associated with gpr126 mutant axons. This suggested that Gpr126 functions in SCs for myelination. However, MBP expression was also rescued when WT SCs and neurons were present in mutant hosts, and importantly, we did not perform TEM analyses to determine whether WT SCs and neurons together rescued myelination more efficiently than SCs alone (Monk et al., 2009). Furthermore, Gpr126 constitutive mutant mice showed many defects that were not observed in gpr126 mutant zebras (including perineurial-like cell invasion throughout the endoneurium, radial sorting delays, severe axon loss, limb contracture defects, and lethality (Monk et al., 2011). These observations raised the question of whether Gpr126 has essential functions in multiple cell types during PNS development.

Our analysis of DhhCre;Gpr126mutant mice suggests that Gpr126 is required in SCs for timely radial sorting and for myelination (Figs. 3, 4). We observed a lack of myelination in DhhCre;Gpr126mutant mice at all ages examined up to P42 (Figs. 3, 4). We also observed severe delays in radial sorting in DhhCre;Gpr126mutant mutants at all stages examined (Fig. 4). Interestingly, we did not observe limb contracture defects, perineurial-like cell invasion, axon degeneration, or significant lethality in DhhCre;Gpr126mutant mice, though these phenotypes were observed in constitutive Gpr126 mutants. The lethality of constitutive Gpr126 mutants is likely due to an essential function for Gpr126 in heart development (Waller-Evans et al., 2010; Patra et al., 2013). However, it is unclear whether the other defects observed in constitutive Gpr126 mutant PNS are due to a Gpr126-dependent signal from SCs before E12.5 or whether Gpr126 might function in other cell types in the developing nerve. Previous RT-PCR experiments were inconclusive regarding DRG expression of Gpr126 (Monk et al., 2011), but our in situ hybridization results demonstrate that Gpr126 is expressed in a pattern consistent with DRG neurons (Fig. 1F,G). Importantly, this expression was maintained in DhhCre;Gpr126mutant mice, although the signal was less strong than in control nerve. It is also important to note that DhhCre drives recombination in endoneurial fibroblasts (Joseph et al., 2004), so we cannot formally exclude the possibility that Gpr126 is also required in this cell type. Future work is needed to define the function(s) of Gpr126 in other cell types during peripheral nerve development.
Phenotypic similarities between Gpr126 and Rac1 mutants

In addition to radial sorting delays and amylination, we also observed cytoplasmic protrusion defects in Dhh\(^{Gpr126^+/−}\)Gpr126\(^{+/−}\) mutants at P21 and P42 (Fig. 4 F, I) strikingly similar to defects previously reported in SC-conditioned Rac1 mutants (Benninger et al., 2007; Nodari et al., 2007; Guo et al., 2012). The Rac1-like defects were likely not observed in constitutive Gpr126 mutants because these animals could not be examined past P12 due to lethality. The phenotypic similarities between Gpr126 and Rac1 mutants suggest that these pathways interact. β1 Integrin is known to activate Rac1 in SCs (Nodari et al., 2007), and deletion of Igβ1 or Rac1 in SCs leads to radial sorting delays similar to those observed in Gpr126\(^{−/−}\) and Dhh\(^{Gpr126^+/−}\)Gpr126\(^{+/−}\) mutants (Feltri et al., 2002; Benninger et al., 2007; Nodari et al., 2007; Guo et al., 2012). Gpr126 might indirectly activate Rac1, perhaps by modulating β1 integrin, which would in turn activate Rac1. Other gGPCRs have been shown to bind extracellular matrix molecules via their N termini (Xu et al., 2006; Luo et al., 2011). It is conceivable that the N terminus of Gpr126 binds components of the basal lamina in SCs and that this could modulate β1 integrin/laminin signaling. This is an attractive hypothesis to pursue in future work, as Gpr126 mutant SCs also phenocopy radial sorting defects observed in Laminin mutants (Chen and Strickland, 2003; Yu et al., 2005). Gpr126 may thus indirectly modulate Rac1, and Gpr126 may also directly activate Rac1. The βγ-dimers of G- coupled GPCRs are known to activate Rac1 via PI3K (Vogt et al., 2007), and PKA also promotes Rac1 activation via stimulation of Sif-like and Tiam1-like exchange factor (Goto et al., 2011).

In summary, our data support a model in which Gpr126 functions in SCs for timely radial sorting and myelination, and we show that this gGPCR couples to G\(\beta\)\(\gamma\)-proteins and G\(\alpha\)-proteins. Via these G-protein interactions, Gpr126 can modulate precise levels of cAMP required for different stages of SC development. We hypothesize that both signals from Gpr126, cAMP/PKA activity via G\(\beta\)\(\gamma\)-proteins and βγ-subunits release from G\(\alpha\)-proteins, are essential for SC differentiation and myelination.

References


