The adhesion GPCR Gpr56 regulates oligodendrocyte development via interactions with G\(\alpha_{12/13}\) and RhoA

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In the vertebrate central nervous system, myelinating oligodendrocytes are postmitotic and derive from proliferative oligodendrocyte precursor cells (OPCs). The molecular mechanisms that govern oligodendrocyte development are incompletely understood, but recent studies implicate the adhesion class of G protein-coupled receptors (aGPCRs) as important regulators of myelination. Here, we use zebrafish and mouse models to dissect the function of the aGPCR Gpr56 in oligodendrocyte development. We show that \(gpr56\) is expressed during early stages of oligodendrocyte development. In addition, we observe a significant reduction of mature oligodendrocyte number and myelinated axons in \(gpr56\) zebrafish mutants. This reduction results from decreased OPC proliferation, rather than increased cell death or altered neural precursor differentiation potential. Finally, we show that these functions are mediated by G\(\alpha_{12/13}\) proteins and Rho activation. Together, our data establish Gpr56 as a regulator of oligodendrocyte development.
Myelin is a multilamellar, lipid-rich membrane that insulates axons in the vertebrate nervous system to facilitate rapid conduction of action potentials. In addition, myelinating glia promote neuronal health by providing vital trophic support to the axons they ensheath. Myelin is generated by specialized glial cells—Schwann cells (SCs) in the peripheral nervous system (PNS) and oligodendrocytes in the central nervous system (CNS). Oligodendrocytes form the myelin sheath by extending and iteratively wrapping their plasma membranes around axons, while glial cell cytoplasm is extruded to ultimately form compact myelin. Impaired myelination causes debilitating symptoms in many severe neurological disorders such as multiple sclerosis, and loss of mature oligodendrocytes and myelin can lead to neuronal death and eventual paralysis. Therefore, continued research to uncover new pathways that promote oligodendrocyte development and myelination is essential.

Although myelin is required for human life, the molecular mechanisms that underlie glial cell development and myelination are incompletely understood. Previous studies indicate that extracellular matrix proteins and their receptors, in addition to intracellular signal-transduction cascades, are essential for proper development of myelinating glial cells. Adhesion G protein-coupled receptors (aGPCRs) represent good candidate regulators of glial cell development and myelination because they are purported to regulate both cell–cell and cell–matrix interactions as well as signal-transduction by heterotrimeric G proteins, and recent studies implicate aGPCRs as important regulators of myelination.

aGPCRs are the second largest class of GPCRs, but remain relatively understudied due to their structural complexity. In addition to the canonical seven-transmembrane (7TM) domain common to all GPCRs that usually facilitates intracellular signalling, aGPCRs are characterized by the presence of a very large N-terminal domain rich in functional motifs associated with cell–cell and cell–matrix adhesion, which is separated from the 7TM by a GPCR proteolytic site (GPS) domain. The GPS motif is encompassed by a larger GPCR autoproteolysis-inducing (GAIN) domain. Together, these domains are required for autoproteolytic cleavage of the N terminus from the C terminus, a process that may be required for proper intracellular aGPCR trafficking and signalling. The dual roles of aGPCRs in promoting cell–cell/cell–matrix interactions and facilitating intracellular signalling, in addition to the importance of Gpr126 for SC myelination, led us to hypothesize that additional aGPCRs regulate glial cell development and myelination in the CNS.

Previously published data sets indicate that Gpr56, an aGPCR related to Gpr126, is highly expressed during mouse CNS development in oligodendrocyte precursor cells (OPCs) and in early larval stages (2 through 5 days post-fertilization (dpf)) when OPCs are first specified and differentiated. These findings are consistent with previously published data in mammals showing that Gpr56 is also expressed in astrocytes, and to a lesser extent, in neurons. At 2 dpf, when OPCs have been specified and are actively migrating and dividing, there was a marked increase in gpr56 expression in the CNS, which is consistent with expression in neural precursors. In contrast, gpr56 expression was not detected by WISH in the CNS at larval stages > 3 dpf (Supplementary Fig. 1a,b), during which time oligodendrocytes undergo terminal differentiation. We also never detected signal in sense control WISH experiments (Supplementary Fig. 1c). These findings demonstrate that gpr56 expression during CNS development is conserved from zebrafish to mammals and suggest that levels are highest at earlier stages.

**Results**

**gpr56 is expressed during oligodendrocyte development.** Myelin is an evolutionary innovation of the jawed vertebrate lineage, and zebrafish represent one of the simplest genetically tractable organisms in which to study myelinating glial cell development. We therefore took advantage of the zebrafish model system to interrogate the necessity of Gpr56 function in oligodendrocyte development and myelination. First, we analysed the spatiotemporal expression of gpr56 by RT–PCR in developing zebrafish. We detected gpr56 expression during embryogenesis and in early larval stages (2 through 5 days post-fertilization (dpf)) when OPCs are first specified and differentiated(Fig. 1a). In addition, whole-mount in situ hybridization (WISH) revealed low levels of gpr56 expression throughout the CNS at 1 dpf, before OPC specification (Fig. 1b). These findings are consistent with previously published data in mammals showing that Gpr56 is also expressed in astrocytes, and to a lesser extent, in neurons.

**Targeted disruption of gpr56 in zebrafish.** Given the expression pattern of gpr56 in the developing CNS, we sought to test the hypothesis that Gpr56 regulates oligodendrocyte development. To this end, we generated Transcription Activator-Like Effector Nucleases (TALENs) designed to cleave zebrafish genomic sequences between exons 8 and 9 (Fig. 1c), which encode the GPS motif. We recovered two mutant alleles of gpr56: stl13 representing a 6 bp deletion that is predicted to remove a completely conserved tryptophan residue, and stl14 representing a 26 bp deletion (Fig. 1f,g). The GPS is a cysteine-rich domain containing four cysteine residues and two tryptophan residues required for autoproteolytic cleavage. Previous reports show that inhibiting autoproteolytic cleavage of GPR56 prevents receptor trafficking and therefore impairs signalling. In addition, two missense mutations that disrupt the highly conserved cysteine and tryptophan residues in humans cause BFD. Together, these findings suggest that gpr56 loss-of-function mutants may generate a non-functional Gpr56 due to failed trafficking of the receptor to the plasma membrane. Therefore, we chose to primarily characterize and utilize stl13 as a tool for understanding gpr56 function.

**Gpr56 is required for proper oligodendrocyte development.** To determine whether Gpr56 is required for normal oligodendrocyte development and myelination, we performed marker analyses to observe oligodendrocyte development in gpr56stl13/stl13 mutants. In the

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CNS, OPCs differentiate from neural progenitors that reside within subventricular zones of the embryonic brain and spinal cord through coordinated expression of the transcription factors Olig2 and Sox10 (ref. 28). Some OPCs remain as non-myelinating OPC progenitors throughout life, while others are further specified by expression of the transcription factor Nkx2.2, which allows OPCs to enter the terminal differentiation pathway. Expression of Nkx2.2 is characteristic of axon-associated pro-oligodendrocytes (pro-OLs)25, and mature myelinating oligodendrocytes express Myelin basic protein (MbP), which encodes a structural component of the myelin sheath23. Using transgenic sox10(- / −:memgfp zebrafish (a kind gift from M. Voigt, manuscript in preparation), we did not detect any difference in the number of sox10+ OPCs in gpr56<sup>stl13</sup> larvae (N = 19) compared with controls (N = 20) at 28 hours post-fertilization (hpf), the earliest time point at which sox10 expression was detected (Fig. 2a–c, P < 0.007, Student’s t-test). These data suggest that specification of the oligodendrocyte lineage is not impaired in gpr56<sup>stl13</sup> mutants. Consistent with this observation, general dorsal-ventral patterning (visualized by Sonic hedgehog pathway markers) was also unaffected in gpr56<sup>stl13</sup> mutants (Supplementary Fig. 2). Furthermore, we did not observe overt defects in the development of neurons, radial glia, or in microglia number at any stage examined in gpr56<sup>stl13</sup> mutants compared with controls (Supplementary Fig. 3). However, by 2.5 dpf, we detected a significant decrease (P < 5.223x10<sup>-6</sup>, Student’s t-test) in nkx2.2+ pro-OL number in gpr56<sup>stl13</sup> mutants (N = 19) by WISH relative to controls (gpr56<sup>+/+</sup> and gpr56<sup>stl14</sup>: N = 22), and we observed a subsequent reduction in MbP expression in gpr56<sup>stl13</sup> (N = 20/27, gpr56<sup>stl14</sup>: N = 14/19) by 4 dpf (Fig. 2d–i, WT control: N = 13/13). Although gpr56 is maternally expressed (Fig. 1a), we did not observe enhanced defects in oligodendrocyte development in gpr56<sup>stl13</sup> maternal-zygotic mutants (no WT maternal contribution) compared with gpr56<sup>stl13</sup> zygotic mutants (Fig. 2j, N = 5/5). Importantly, injection of synthetic mRNA encoding WT zebrafish (50 pg) or mouse (200 pg) Gpr56 was sufficient to rescue the CNS mbp deficits in gpr56<sup>stl13</sup> mutants, confirming the specificity of the TALEN-induced mutations (Supplementary Fig. 4). Together, these results demonstrate that Gpr56 is required for the proper development of mature, myelinating oligodendrocytes.

**Figure 1** | gpr56 expression and mutant generation in zebrafish. (a) Representative image of gpr56 expression assessed by RT-PCR from fertilization through larval development (three technical replicates). From left to right: Mat (maternal expression), 8 h (8 h post-fertilization), 1 d (days post-fertilization) to 5 d, RT (-) control and H<sub>2</sub>O control. (b,c) Whole-mount in situ hybridization (WISH) of zebrafish larva at (b) 1 and (c) 2 dpf shows robust gpr56 expression within the central nervous system (black arrows in b) during larval development (lateral views shown, anterior to the left, dorsal is up, two technical replicates performed). (d) Cross-section through the spinal cord (white dashed line in c depicts approximate location to the left) of gpr56 WISH embryo at 2 dpf shows gpr56 expression in the spinal cord midline (white arrow) and in bands consistent with neural precursors (black arrows). (e) Diagram of the zebrafish gpr56 gene (top) and protein (bottom) structures. TALENs used to generate gpr56 zebrafish mutants targeted between the 8th and 9th exons (text in red). Gpr56 contains a signal sequence (ss), GPCR Autoproteolysis-Inducing Domain (GAIN), GPCR Proteolytic Site (GPS) and the canonical 7-Transmembrane Domain (7TM). (f) Recovered mutant alleles of gpr56—<i>stl13</i> representing a 6-bp deletion and <i>stl14</i> representing a 26 bp deletion. (g) Amino-acid sequence alignment of the GPS motif from representative species showing perfect conservation of the Trp residue that is deleted in the gpr56<sup>stl13</sup> allele (highlighted yellow). Mutation of the second, highly conserved Trp residue within the GPS causes BFPP (black arrowhead).

Mutations in gpr56 cause CNS hypomyelination. To further delineate how impaired Gpr56 function affects oligodendrocyte myelination, we performed transmission electron microscopy (TEM) to measure the extent and quality of myelin present in the ventral spinal cord of gpr56 mutants (gpr56<sup>stl13</sup>: N = 5, gpr56<sup>stl14</sup>: N = 4, WT: N = 6) during development (Fig. 3a–k). We found a significant reduction (gpr56<sup>stl13</sup>: P < 0.019, gpr56<sup>stl14</sup>: P < 0.039, Student’s t-test) in the percent of myelinated axons in mutant larva compared with WT controls at 5 dpf (Fig. 3l), but total axon number was unchanged (gpr56<sup>stl13</sup>: P = 0.5, gpr56<sup>stl14</sup>: P = 0.78, Student’s t-test). We also did not observe a change in the number of myelin wraps surrounding those axons that were myelinated in gpr56<sup>stl13</sup> mutants compared with controls (Fig. 3n,o; non-Mauthner: gpr56<sup>stl13</sup>: P = 0.29, gpr56<sup>stl14</sup>: P = 0.6; Mauthner: gpr56<sup>stl13</sup>: P = 0.25, gpr56<sup>stl14</sup>: P = 0.76, Student’s t-test). In contrast to the hypomyelination phenotype shown in the ventral spinal cord of gpr56 mutants at 5 dpf (Fig. 3f–k), we did not observe any effect on oligodendrocyte myelination in the dorsal spinal cord at this developmental stage (Supplementary Fig. 5). By 21 dpf, however, we observed a statistically significant decrease in the percent of myelinated axons in the ventral (P = 0.007, Student’s t-test) and dorsal (P = 0.002) spinal cord of gpr56<sup>stl13</sup> (N = 4) mutants relative to WT siblings (N = 2), with no change in axon number (Supplementary Fig. 6, dorsal spinal cord: P = 0.89, ventral spinal cord: P = 0.96, Student’s t-test). We suspect that we were unable to detect a difference in the dorsal spinal cord at larval stages because very few axons are myelinated in this region at 5 dpf. Interestingly, we also observed many oligodendrocytes with distended endoplasmic
Figure 2 | Oligodendrocyte development is impaired in gpr56 zebrafish mutants. (a,b) Representative fluorescent images of (a) control (gpr56+/-) and (b) gpr56+/-/- larvae at 28 hpf expressing Tg(sox10(-7.2):memGFP) to mark OPCs. (c) Quantification of OPC number (sox10+; white arrows) in control (N = 20) and gpr56+/-/- (N = 19) larvae. (d-f) Representative WISH images of the spinal cord of (e) gpr56+/-/-+ (N = 19) and (d) control embryos (gpr56+/-+ and gpr56+/-+), N = 22) showing nkx2.2a expression to mark pro-Ols. (f) Quantification of pro-OL number (nkx2.2a+) (P<5.223 x 10^-6). (g-j) mbp expression (CNS marked by white arrow, hindbrain) in (h) gpr56+/-/- (N = 20/27), (i) gpr56+/-/-+ (N = 14/19) and (j) MZgpr56+/-/-+ (5/5) larvae compared with (g) WT controls at 4 dpf (N = 13/13). (a-f) All images and quantification thereof were taken from segments 5 to 6 for consistency. For (a,b,d,e) lateral views are shown, anterior to the left, dorsal is up; for (g-j) dorsal views are shown, anterior to the left. (a,b) Scale bar, 50 μm. (d,e) Scale bar, 100 μm. (g-j) Scale bar, 200 μm. Student’s t-test used to test for statistical significance and error bars shown as ± s.d. A minimum of two technical replicates were performed for each marker. NS, not significant.

Gpr56 is not required for neural stem cell differentiation. One possible explanation for the observed decrease in myelinating oligodendrocyte numbers in gpr56+/-/-+ mutants could be reduced differentiation of the oligodendrocyte lineage from neural stem cells. The fact that we did not detect defects in early OPC specification (Fig. 2a–c) in gpr56+/-/-+ zebrafish mutants argued against this, but, to definitively test this model, we harvested neurospheres from the subventricular zone of Gpr56–/– knockout mice29 at postnatal day 3 and assessed their ability to differentiate into Tuj1+ neurons, GFAP+ astrocytes and O4+ oligodendrocytes. Importantly, we found robust levels of the Gpr56 protein in neurospheres harvested from WT and Gpr56+/-+– animals, which was absent from mutant neurospheres (Supplementary Fig. 8). We observed no differences in the ability of Gpr56–/– neural stem cells to differentiate into any cell-type tested (Fig. 4), consistent with our analysis of gpr56+/-/-+ zebrafish mutants (Fig. 2a–c; Supplementary Fig. 3). Of note, neurospheres harvested from the spinal cord of Gpr56–/– animals showed a slightly increased potential to differentiate into neurons (Supplementary Fig. 9), highlighting the variation that exists between neural progenitor populations from different tissues30. Importantly, neurospheres harvested from both tissues showed no difference in their ability to differentiate into oligodendrocytes compared with controls, supporting the hypothesis that Gpr56 is not required for specification and differentiation of the oligodendrocyte lineage.

Gpr56 regulates OPC proliferation. Because OPC specification is unaffected in gpr56+/-/-+ zebrafish mutants, and loss of Gpr56 does not affect the ability of neural precursors to differentiate into oligodendrocytes, reduced pro-OL numbers in gpr56+/-/-+ mutants could result from increased death and/or decreased proliferation of OPCs. To distinguish between these possibilities, we performed time-lapse imaging of gpr56+/-/-+ mutants and controls using transgenic sox10(-7.2):mRFP24 zebrafish to visualize OPC behaviour and development in vivo (Supplementary Movies 1 and 2). Embryos were imaged from 30 to 46 hpf, allowing us to monitor the transition of many OPCs into pro-OLs. At 30 hpf, we found a statistically significant increase in the number of axons associated with pro-Ols in gpr56+/-/-+ mutants (N = 7) compared with controls (N = 7, P<0.04, Student’s t-test). Over time, however, the number of pro-OL associated axons in control embryos increased at a higher rate in gpr56stl13/stl13 mutants at 21 dpf, which was rarely seen in controls, supporting the hypothesis that the protein encoded by gpr56stl13/stl13 does not traffic properly from the endoplasmic reticulum to the plasma membrane (Supplementary Fig. 6). To determine if myelination is simply delayed in gpr56stl13/stl13 mutants, we analysed myelin ultrastructure in the spinal cord of gpr56stl13/stl13 animals at 6 months of age (Supplementary Fig. 7). At 6 months, we likewise found a statistically significant decrease in the percent of myelinated axons in gpr56stl13/stl13 mutants (N = 4) compared with WT controls (N = 3) in both the ventral (Supplementary Fig. 7g, axon diameter of 0.2–0.5 μm (P <0.021), 0.5–1 μm (P <0.005) and 1–2 μm (P <0.029), Student’s t-test) and dorsal spinal cord (Supplementary Fig. 7h, axon diameter of 0.2–0.5 μm (P <0.003) and 0.5–1 μm (P <0.008), Student’s t-test), without any change in myelin thickness for axons that were myelinated (Supplementary Fig. 7h,o, linear regression analysis). Axon number was also unchanged in mutants compared with controls (Supplementary Fig. 7f,m, dorsal spinal cord: P<0.11, ventral spinal cord: P<0.34, Student’s t-test). Collectively, our analysis demonstrates that altered Gpr56 function causes developmental CNS hypomyelination that persists in the adult spinal cord.
rate than in \textit{gpr56}\textsuperscript{stl13/stl13} mutants. This increase resulted in a statistically significant reduction in pro-OL associated axons in \textit{gpr56}\textsuperscript{stl13/stl13} mutants compared with controls by 46 hpf (Fig. 5a–c, \(P<0.004\), Student’s \(t\)-test), consistent with our marker analysis (Fig. 2d–f). These findings suggest that in \textit{gpr56}\textsuperscript{stl13/stl13} mutants, OPCs are born and prematurely form associations with axons, but that they do not proliferate sufficiently to account for the total number of axons that need
to be myelinated. In accordance with this hypothesis, we also observed additional myelinated axons in the ventral spinal cord of gpr56stl13/stl13 mutants (N = 3) compared with controls (WT: N = 3, gpr56+/+ mutants; N = 3) by TEM at 3 dpf (Fig. 5d–g, P < 0.048, Student’s t-test), indicative of precocious myelination. In addition, we observed a statistically significant reduction in the number of proliferating OPCs (sox10+; ph3+ in gpr56stl13/stl13 mutants compared with controls at 32 hpf, when most OPCs have not yet entered the differentiation programme24,25. (Fig. 5h–j, P < 0.0004, Student’s t-test). Importantly, we did not observe any signs of cell death via time-lapse imaging, and there was no increase in the number of cell corpses assessed by acridine orange staining at 32 hpf, 2 dpf or 3 dpf in gpr56stl13/stl13 mutants compared with controls (Fig. 5k–m). Thus, changes in pro-OL number can only be attributed to changes in OPC proliferation and not cell death. Together, these data support a model in which Gpr56 promotes OPC proliferation at the expense of differentiation such that, when Gpr56 function is impaired, insufficient OPC proliferation results in fewer pro-OLs and mature oligodendrocytes, which leads to hypomyelination.

**gpr56 OE increases OPC number but inhibits myelination.** Impaired Gpr56 function results in reduced numbers of myelinating oligodendrocytes and myelin due to decreased OPC proliferation (Figs 2, 3 and 5, Supplementary Figs 6 and 7). Transient overexpression (OE) of WT gpr56 synthetic mRNA rescues CNS mbp expression (Supplementary Fig. 4) by suppressing the OPC proliferation defect observed in gpr56stl13/stl13 mutants (Supplementary Fig. 10). Interestingly, we also observed enhanced mbp expression in a large fraction of gpr56-injected embryos (Supplementary Fig. 4d,e). We therefore hypothesized that transient OE of gpr56 in WT embryos would cause hypermyelination by increasing oligodendrocyte number. Analysis of nkx2.2a expression revealed a statistically significant increase in the number of nkx2.2a+ pro-OLs in the anterior (P < 8.7x10^-7, Student’s t-test) and posterior (P < 0.01, Student’s t-test) spinal cord (Fig. 6a–i) of embryos injected with 50 pg of WT gpr56 synthetic mRNA (anterior: N = 25, posterior: N = 29) relative to control-injected embryos (anterior: N = 26, posterior: N = 31). In addition, we noted an increase in CNS mbp expression in gpr56OE embryos relative to phenol-red-injected controls by WISH (Fig. 6g–j, N = 19/34) at 2.5 dpf. Consistent with these findings, we observed an increase in the numbers of oligodendrocyte lineage nuclei in the spinal cord of gpr56OE embryos relative to phenol-red-injected controls by TEM at 2.5 and 5 dpf (Fig. 6l–q, P < 0.037 at 2.5 dpf (OE: N = 4, control-injected: N = 4); P < 0.15 at 5 dpf (OE: N = 5, control-injected: N = 4), Student’s t-test). We distinguished oligodendrocyte and OPC nuclei from neuronal nuclei by their distinctive heterochromatin

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**Figure 3** gpr56 mutant spinal cord axons are hypomyelinated. (a) Schematic representation of a 5 dpf zebrafish. Larvae were cut between segments 5 and 6 (red dashed line) and prepared for TEM. Axis shows orientation of the embryo (D, dorsal; V, ventral; A, anterior; P, posterior). (b) Diagram of a 5 dpf zebrafish cross-section, dorsal is up (D), ventral is down (V). In this image, the spinal cord is in orange and includes neuronal cell bodies (blue) and myelinated axons (green). Ventral region used for quantification is boxed in green, dorsal region used for quantification boxed in magenta. Muscle in purple. **(c–k)** Represent TEM images from the ventral spinal cord of WT (c–e, N = 6), gpr56stl13/stl13 mutant larvae (f–h, N = 5), and gpr56stl14/stl14 mutant larvae (i–k, N = 4) at 5 dpf. Higher magnifications of n.olfi are shown in d–g, h–j, respectively. **(c,d,g,i)** Myelinated axons are shaded green, unmyelinated large caliber axons (≥ 500 nm) are shaded orange. **(e,h,k)** Images from panels d,j without pseudocolour. **(l)** Quantification of the percent of myelinated axons in the ventral spinal cord of gpr56stl13/stl13 (P < 0.019) and gpr56stl14/stl14 (P < 0.039) compared with WT controls. **(m)** Quantification of the total number of axons in the ventral spinal cord of gpr56stl13/stl13 (P < 0.5) and gpr56stl14/stl14 (P < 0.78) compared with WT controls. **(n)** We also did not observe a significant difference in the number of myelin wraps per myelinated axon in mutants compared with control on the large caliber Mauthner axon (m) or on smaller caliber myelinated axons. **(c,f,i)** Scale bar, 1 μm. **(d,g,h,j,k)** Scale bar, 500 nm. **(l–o)** Quantification performed on a stereotyped 14 μm² region (b) in the ventral spinal cord. Student’s t-test used to test for statistical significance and error bars shown as ± s.d. NS, not significant. Data represent two technical replicates.
P<0.0003; 3MO-injected WT (N = 10) versus 3MO-injected heterozygote (N = 36): P<1.33x10^{-5}; 3MO-injected WT (N = 10) versus 3MO-injected mutant (N = 15): P<2.59x10^{-6}, Student’s t-test). As inhibition of Gα12/13 function preferentially affects oligodendrocyte development in gpr56<sup>±13/+</sup> heterozygotes and mutants compared with WT controls, our findings suggest a genetic interaction and thus support the hypothesis that Gpr56 promotes oligodendrocyte development via interactions with Gα12/13.

Rho is the main downstream effector of Gα12/13 (ref. 39), and previous studies in vitro implicate RhoA signalling downstream of Gpr56 (refs 35,36). We therefore hypothesized that Gpr56 couples to Gα12/13 and signals through RhoA to promote oligodendrocyte development. Consistent with this model, transient OE of 10 pg of a constitutively active form of rhoa (rhoA<sup>14</sup>)<sup>38</sup> suppressed gpr56<sup>±13/+</sup> mutant phenotypes, including the OPC proliferation defect (Supplementary Fig. 10) and CNS mbp score (Fig. 7i,j, control WT (N = 22) versus control mutant (N = 27): P<0.0009; OE WT (N = 16) versus OE mutant (N = 17): P<0.343, Student’s t-test). OE of rhoa also caused a marked shift in the distribution of phenotypic classes in gpr56<sup>±13/+</sup> mutants such that the majority (76%) of gpr56<sup>±13/+13</sup> larvae expressed WT levels of mbp (Fig. 7i, N = 13/17). Importantly, all embryos analysed for these studies were morphologically normal, and thus changes in mbp expression could not be attributed to changes in overall heath in treated animals compared with controls (Fig. 7g,h,k and l). Taken together, these experiments demonstrate that Gpr56 interacts with Gα12/13 and signals through RhoA to promote oligodendrocyte development and subsequent myelination.

Discussion

The timing of the transition from OPC proliferation to terminal differentiation is tightly regulated<sup>40–42</sup>. In the CNS, interactions between receptors on the OPC cell surface and axon-bound or axon-secreted molecules ensure that sufficient numbers of OPCs are generated to accommodate the numbers of axons that need to be myelinated. Availability of the axon-derived mitogen PDGF-A, which interacts with PDGFRα on the OPC plasma membrane, is a major determinant of the extent of OPC proliferation. Moreover, numerous mechanisms converge to ensure the timing and execution of terminal differentiation, including downregulation of Pdgfrα by Nkx2.2 in OPCs, cell-cycle exit and vast morphological changes<sup>37,42–45</sup>. Failed differentiation of resident OPC pools in response to demyelination is a major contributor to the degenerative nature of demyelinating diseases such as multiple sclerosis<sup>46</sup>; thus, identification of signalling molecules that regulate OPC behaviour and differentiation could provide new avenues for therapeutics.

In this study, using zebrafish and mouse models, we identified Gpr56 as a new regulator of OPC proliferation and oligodendrocyte myelination. Like PDGFRα, Gpr56 expression is restricted to early stages of oligodendrocyte development and is downregulated before terminal differentiation<sup>18,42</sup>, suggesting that Gpr56 may negatively regulate terminal differentiation and changes in overall heath in treated animals compared with controls (Fig. 7g,h,k and l). Taken together, these experiments demonstrate that Gpr56 interacts with Gα12/13 and signals through RhoA to promote oligodendrocyte development and subsequent myelination.

Figure 4 | Loss of Gpr56 does not affect neural precursor differentiation capacity. Quantification of the percent of (a) GFAP + astrocytes, (b) Nestin + neural progenitors, (c) Tuj1 + neurons and (d) O4 + oligodendrocytes (per field of view) differentiated from neural precursors harvested from WT (N = 3), Gpr56<sup>+/−</sup> (N = 4), and Gpr56<sup>−/−</sup> (N = 5) animals at postnatal day 3 (P3). Representative fluorescent images from each genotype shown to the right of each summary graph. Two-way ANOVA used to test for statistical significance and error bars shown as ± s.d. Data were acquired from two technical replicates.
or positively regulate OPC behaviour/proliferation. Gpr17, another GPCR that is essential for oligodendrocyte development, is also expressed in OPCs and is downregulated in mature oligodendrocytes, and Gpr17 has been shown to directly inhibit terminal differentiation. In the absence of Gpr17, OPCs prematurely differentiate and form myelin, though myelination eventually proceeds to WT levels in Gpr17−/− mice. We found that gpr56stl13/stl13 mutant OPCs likewise formed associations with axons and myelinated prematurely, indicative of precocious differentiation due to relieved inhibition on terminal differentiation. We found that OPCs are specified normally in gpr56stl13/stl13 mutants but are less proliferative,
resulting in reduced numbers of myelinating oligodendrocytes and consequently hypomyelination. In addition, transient OE of gpr56 in WT embryos caused increased numbers of pro-OLs and increased mbp expression in gpr56 OE embryos at 2.5 dpf. However, the apparent increase in mbp expression is likely the result of greater numbers of pro-OLs, as myelination was inhibited in the OE embryos at 5 dpf as assessed by TEM. We also observed many immature oligodendrocyte cell bodies with no axonal contacts in the centre of the spinal cord of the OE embryos at this time point. These findings indicate that the prolonged OE of gpr56 inhibits OPC differentiation and myelination. Together, these findings support the hypothesis that Gpr56 is both a positive regulator of OPC proliferation and an inhibitor of oligodendrocyte terminal differentiation.

Though we observed striking defects in oligodendrocyte development and myelination when Gpr56 function is perturbed, we did not observe any defects in the development of neurons, radial glia or in microglia number in gpr56

\[\text{gpr56}^{+/+}\] mutants at any time point analysed. These data suggest that the observed defects in oligodendrocyte development are a cell-autonomous effect, although conditional mouse mutant studies could be leveraged to definitively address the question of cellular autonomy. Together, these data demonstrate that Gpr56 is a positive regulator of OPC proliferation and a negative regulator of OPC differentiation.

Previous in vitro studies place Gza12/13 and RhoA signalling downstream of Gpr56 (refs 35,36). RhoA is the major downstream effector of Gza12/13 and its expression is likewise restricted to early stages of oligodendrocyte development.\(^37,39\). Inactivation of RhoA via Fyn is required for OPC morphological differentiation into pro-OLs, and sustained OE of RhoA inhibits process extension and differentiation of oligodendrocytes\(^37\). Reduction of Gza12/13 function in gpr56\(^{+/+}\) zebrafish mutants and heterozygotes causes enhanced loss of CNS mbp expression. In addition, transient OE of constitutively active RhoA before terminal differentiation suppressed gpr56\(^{+/+}\) defects. In sum, these data support a model in which Gpr56 function in OPCs is mediated via interactions with Gza12/13 and RhoA signalling.

RhoA has not been shown to directly promote OPC proliferation, though our findings do not preclude this possibility. We therefore propose a model in which Gpr56 interacts with Gza12/13 and activates RhoA to prevent OPC differentiation, which facilitates continued OPC proliferation promoted by axon–glia interactions such as those mediated by PDGFRs and PDGF-A.\(^12,44\). When Gpr56 function is perturbed, RhoA activation is therefore reduced, relieving RhoA inhibition of terminal differentiation and causing premature OPC differentiation that manifests in a reduced proliferation phenotype (Fig. 8). In this model, the OPCs that prematurely undergo terminal differentiation should myelinate normally.

Figure 6 | Overexpression of gpr56 causes increased OPC number and inhibits myelination. (a–d) WISH shows nkx2.2a expression in the spinal cord of injected WT larvae at 2.5 dpf. Lateral views are shown, dorsal is up. (e,f) Quantification of the number of nkx2.2a+ pro-OLs (black arrowheads) in WT embryos injected with 50 pg of synthetic gpr56 mRNA (\(N = 30\)) in the anterior (b, segments 5–6, \(P < 8.7 \times 10^{-5}\)) and posterior (d, segments 15–30, \(P < 0.01\)) spinal cord compared with injected controls (a,c, \(N = 31\)). (g–j) Representative images of a (h,j) gpr56–injected WT larva (\(N = 19/31\)) compared with a (g,i) control-injected embryo (\(N = 33/35\)) showing mbp expression by WISH. (g,h) Lateral views shown, anterior on right. (i,j) Dorsal views shown, anterior on right. Black arrows. (k) Cartoon of zebrafish larva in cross-section modified from Fig. 3b. White box represents regions shown in l,m,o,p. Green box represents region shown in r–u. (l,m,o,p) Representative images of spinal cord cross-sections from 2.5 dpf (l,m) and 5 dpf (o,p) WT embryos injected with control (l,o) and 50 pg gpr56 synthetic mRNA (m,p). 2.5 dpf: scale bar, 500 nm. 5 dpf: scale bar, 4 μm. (q) Quantification of the number of oligodendrocyte lineage cell bodies (shaded purple) in gpr56–injected embryos (\(N = 4\)) compared with controls (\(N = 4\)) at 2.5 dpf (\(P < 0.037\)) and 5 dpf (\(P < 0.15\), \(N = 4\) for controls and \(N = 5\) for gpr56–injected embryos). Neuronal cell bodies shaded green. (r–u) Representative TEM images of the spinal cord in cross-section from 5 dpf control (r,s) and gpr56–injected (t,u) embryos. Scale bar, 1 μm. Quantification of total axon number (v), % myelinated axons (shaded blue, w, \(P < 0.05\)) and myelin thickness on the large caliber Mauthner axon (shaded orange, x, \(P < 0.008\)) in gpr56 OE embryos at 5 dpf relative to controls. SC, spinal cord. Student’s t-test used to test for statistical significance and error bars shown as ± s.d. Two technical replicates were performed for each overexpression experiment.
Accordingly, we detected an increase in axons associated with pro-OLs at 30 hpf and increased myelination at 3 dpf, consistent with precocious differentiation. Furthermore, we observed no myelin abnormalities or reduced myelin thickness in those axons that are myelinated in gpr56stl13/13 mutants. In sum, these data support the model that Gpr56 positively regulates OPC proliferation via RhoA activation and inhibition of terminal differentiation.

At present, multiple ligands of Gpr56 have been identified in other cell types. In cancer studies, Gpr56 has been shown to inhibit melanoma cell growth via interactions with tissue transglutaminase (TG2)48. In the developing brain, collagen type III, alpha-1 secreted by meningeal fibroblasts interacts with neuron-bound Gpr56 to facilitate proper neuronal migration36. Impaired differentiation of OPC progenitors is a major cause of failed remyelination in the CNS of patients suffering from demyelinating diseases such as multiple sclerosis46. Therefore, a better understanding of Gpr56 activation and signalling is crucial as targeted inhibition of this receptor could be used to promote...
OPC differentiation and remyelination after injury and disease-induced demyelination. Further studies are required to identify the endogenous ligand(s) and small molecules that modulate Gpr56 signalling in OPCs.

**Methods**

**Zebrafish stocks and rearing conditions.** Zebrafish (*Danio rerio*) were maintained in the Washington University Zebrafish Consortium facility (http://zebrafish.wustl.edu/husbandry.html). All experiments were performed in compliance with Washington University’s institutional animal protocols. Embryos were collected from pair-wise or harem matings and reared at 28.5°C in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4). Embryos were staged in hours post-fertilization (hpf) or days post-fertilization (dpf) using morphological markers. To prevent pigmentation in embryos >1 dpf, egg water was supplemented with phenylthiourea to 0.003%. The following lines of zebrafish were used for these studies: both alleles of *gpr56* (stl13/stl14) were generated in a WT (AB*) background. For marker analysis, we used the following transgenic lines: *Gpr56* (green arrow), which indirectly causes a decrease in OPC proliferation (red dashed lines) as OPCs differentiate at the expense of proliferation.

**Figure 8 | Gpr56 promotes OPC proliferation through inhibiting differentiation.** Model of Gpr56 function in OPCs. In WT OPCs, Gpr56 couples to the endogenous ligand(s) and small molecules that modulate Gpr56 PDGF-A-bound signalling in OPCs. Gpr56 PDGF-A-bound signalling alleviates RhoA inhibition on terminal differentiation (green arrow), which indirectly causes a decrease in OPC proliferation (red dashed lines) as OPCs differentiate at the expense of proliferation.

**Mice.** All mouse (*Mus musculus*) experiments were performed in compliance with Washington University’s institutional animal protocols. The Gpr56-knockout mice were obtained from the mutant Mouse Regional Resource Centers (MMRRC), were maintained on a C57BL/6 background and genotyped as described previously. P3 animals of presumed mixed gender were used for all neurosphere experiments, although pups were not genotyped for gender.

**PCR with reverse transcription.** Standard PCR with reverse transcription (RT–PCR) was performed on cDNAs derived from embryos at the two-cell stage (Maternal), 8 hpf, 24 hpf, 48 hpf, 72 hpf and 5 dpf. Analysis was performed three separate times with similar results. For all stages, cDNA was reverse-transcribed from RNA extracted from pools of 30 embryos with the SuperScript First-Strand Synthesis system for RT–PCR (Invitrogen) using Superscript II reverse transcriptase (RT) and oligo(dT) primers, and genomic DNA was controlled for by omitting RT from reactions using the same RNA. To amplify gpr56, we used primers that amplify a 602-bp fragment from exons 3 to 7: 5'-TCATCCGACAGACTGATT-3' and 5'-GAATTCACGCTATGAGAG-3', gpdh was amplified as a control to produce a 117-bp product: 5'-CTGATTCTCTGCTCTTCTT-3'.

**Cloning.** Full-length gpr56 cDNA was amplified from cDNA derived from 5 dpf embryos and cloned into pcDNA3 using the following primer set: 5'-GATCTACTCTGAGTTGATGAAATGGGATCTGCG-3' (XhoI site underlined) and 5'-AGGGAT CCTAGATCTCTCACAACCTTCC-3' (XbaI site underlined). Both vector and insert were digested with XhoI and XbaI and ligated together using standard T4 ligase (NEB), and the final vector (pcDNA3 + _zfl_gpr56) was sequenced for verification.

**In situ hybridization.** Whole-mount in situ hybridizations were performed using standard protocols. In brief, embryos were fixed at the desired age with 4% parafomaldehyde for 2 h at room temperature or 4°C overnight, and then dehydrated in 100% Methanol overnight. Following dehydration, embryos were washed in 0.2% PBS-Tween (PBStw), permeabilized in proteinase K (20 μg/ml diluted 1:1,000 in 0.2% PBStw), and incubated with the appropriate DIGoxygenin-labelled riboprobe overnight at 65°C in hybridization buffer (50% formamide). Following probe treatment, embryos were washed to remove formamide, blocked in 2% blocking medium supplemented with 10% normal sheep serum and 0.2% Triton, and incubated in the primary antibody (Anti-Dig, Fab fragments (1:2,000), Roche) overnight in a block. Following primary antibody treatment, embryos were washed in Maleic Acid Buffer with 0.2% Triton and developed by alkaline phosphatase treatment. When complete, embryos were post-fixed in 4% parafomaldehyde and stored long-term in 70% glycerol. To synthesize gpr56 riboprobes, a 509 bp region spanning exons 11–13 was PCR amplified from a partially sequenced cDNA clone (Clone ID 7070653, Open Biosystems) using the following primer pair: 5'-ATTTTCTGCTGCTGTGGTCT-3' and 5'-CATGTG GTCCCATACAGACA-3'.
The PCR product was cloned into PCR II using the PCR II- TOPO TA Cloning kit (Invitrogen) and sequenced. This construct was linearized by NotI and transcribed with SP6 for sense and linearized with BamHI and transcribed with T7 for antisense. All other riboprobes: mgb255, nkx2.2a, apoptosis, shh, ptc (ref. 59) and ptc2 (ref. 60) were described previously. Embryos stained with nkx2.2a were mounted in glycerol and cut into 2.2a-positive cells, these were counted manually in all focal planes of the spinal cord from segments 5–7 or 15–30. apoptosis-positive nuclei were counted as previously described77. All quantifications were performed blinded, and genotypes were obtained following quantification.

Transmission electron microscopy. For zebrafish larvae and 21 dpf juveniles, TEM was performed using a PEELCO BioWave Pro with SteadyTemp Digital Plus water-recirculating system to ensure sufficient infiltration of the embryos61. Embryos were microwaved for all steps before EPON treatment. In brief, embryos were fixed in 2% formaldehyde, 0.2% glutaraldehyde, 0.1 M sodium cacodylate and left overnight at 4 °C. After fixation, embryos were postfixed using increasing concentrations of ethanol followed by 100% acetone, and then infiltrated overnight in an acetone-EPON mixture. Embryos were then transferred to 100% EPON and embedded in moulds for baking overnight at 65 °C. We always examined fish of a given stage at approximately the same body segment level to control for variability along the anterior/posterior axis. For preparation of spinal cords from 6–month-old adults, we anesthetized animals in an ice-water bath and then severed the hindbrain before dissecting a portion of the anterior spinal cord, and fixation of adult-tissue occurred as described previously10. In brief, adult tissues were fixed in Karnovsky’s fix overnight, post-fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate, washed and dehydrated in increasing concentrations of ethanol followed by 100% uranyl acetate. The tissues were then submerged in a 2:1 mixture of propylene oxide:EPON for 1 h, followed by a 1:1 mixture overnight. The following day, the tissues were transferred to 100% EPON before embedding and baking at 65 °C. Thin sections (70 nm) were mounted on mesh (5 dpf and 6 month samples) or slot (21 dpf samples) grids and stained with 8% uranyl acetate and 1% lead citrate for 10 min each. Images were acquired using a Nikon Eclipse TE300 microscope equipped with an optical camera (Optronics). Images were acquired using Metamorp software on a Nikon Eclipse TE300 microscope equipped with a 10× objective and digital camera. Images were then processed and analysed using Image J. All quantifications were performed blinded, and genotypes were obtained following quantification.

Neurosphere immunocytochemistry. Immunocytochemistry was performed on trypsined NPCs grown in 50 μg ml–1 poly-D-lysine-coated and 10 μg ml–1 fibronectin-coated 24-well plates containing defined medium ([5:3 mixture of DMEM low glucose (LifeTechnologies); neurobasal medium (LifeTechnologies), 0.5 mM 2-mercaptoethanol, 2 mM 1-glutamine, 5 IU of penicillin and 5 μg ml–1 streptomycin (LifeTechnologies); supplemented with 10% FBS and 2% B27 supplement (LifeTechnologies) and 2% B27 supplement (LifeTechnologies)] without growth factors. After 5 days in culture, cells were fixed and stained with primary antibodies (Nestin: ab6142 (Abcam), GFAP: G9269 (Sigma-Aldrich), TuJ1: M-3433 (Covance) and O4: MAB345 (Millipore)) at 4 °C overnight. After fluorescence detection, Alexa Fluor-tagged secondary antibodies (Molecular Probes) were used to detect polyclonal antibodies (GFP, NTRK4, and GABA) as well as α-tubulin ([1:30,000], T745, Sigma) were used as controls to detect changes in axon formation and quantitation. Active RhoA (RhoA-GTP) was determined by PAK1-BD affinity chromatography ([1:200], M17-294, Millipore) according to the manufacturer’s recommendations. All samples were visualized using a Leica TCS SP5-confocal microscope and quantified using a two-way ANOVA test and all data met the assumptions of normal distribution (i.e. equal variances, independent samples) required for this test.

Neurosphere western blotting. Western blots were performed as previously described82. Images of full western blots can be seen in Supplementary Figs 8 and 12. All quantifications were performed blinded, and genotypes were obtained following quantification.

Time-lapse imaging. Embryos were mounted in 1% low melting agarose with tricaine to prevent movement and 0.003% phenylthiourea to prevent pigmentation. Embryos were oriented laterally and all images were taken of spinal cord regions spanning segments 5–7 using a ×40 dry objective. Time-lapse images were taken from gpr56+/-/mutants (N = 7) and gpr56+/-/+ controls (N = 6) expressing tsg101:nRFP82 from 24 to 46 hpf using a Quorum spinning disc confocal microscope on a heated stage, recorded using Metamorph software, and analysed using Image J. All analyses were performed blinded to genotype.

Zebrafish immunohistochemistry and fluorescence imaging. For imaging of transgenic in fixed embryos, embryos were fixed in fresh, 4% paraformaldehyde with 0.1 M Pipes (pH 7.4), 10% glycerol, and 2 mM EGTA and DEcenadione in 1× PBS to preserve fluorescence84. For phosho-H3 antibody stains (06570MI, Millipore), embryos were fixed in 4% paraformaldehyde in 1× PBS overnight (4 °C), washed in 0.3% PBS/Triton and then blocked for 2 h in 0.3% PBS/Triton plus 10% normal goat serum and 4% BSA. Embryos were then incubated overnight at 4 °C in primary diluted 1:3,000 in blocking solution. For secondary staining, embryos were incubated in Alexa Fluor goat anti-rabbit 488 IgG (Life Technologies) diluted 1:2,000 in 0.3% PBS/Triton plus 2% normal goat serum for 3 h at room temperature. Finally, embryos were washed and mounted in vectashield (Vector Labs) for imaging. All quantifications were performed blinded, and genotypes were obtained following quantification.

Morpholinos and synthetic mRNAs injections. The morpholinos targeting gna12, gna13a and gna13e are previously published88 and were a kind gift from Feng Lin (University of Iowa). Morpholinos were combined and diluted in sterile water supplemented 1:10 with phenol-red dye, and then injected at a final concentration of 1 ng each in a total volume of 1 nl. To control for adverse side-effects from the injection process, we also injected control siblings with an equal volume of phenol-red diluted 1:10 in water. A putatively active Rho (rhoV1A) was kindly provided by Lila Solinica-Krezel and was injected and described as previously88. The full-length mouse Gpr56 cDNA clone was obtained from Open Biosystems (Clone ID: 37690247), linearized with NotI, transcribed using the mMESSAGE mMACHINE SP6 ULTRA kit (Ambion) and injected at a final concentration of 200 pg in 1 nl of 1× PBS with 5% phenol-red dye. This solution was linearized with NotI and also transcribed using the mMESSAGE mMACHINE SP6 ULTRA kit (Ambion) before injection of a total concentration of 50 pg in 3 nl.
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**Author contributions**

S.D.A. and K.R.M. designed research, X.P. provided key reagents, and S.D.A. and C.G. performed experiments. All authors analysed the data, S.D.A. and K.R.M. wrote the manuscript, and all authors edited the manuscript.

**Additional information**

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