

Glutamate Signaling via the AMPAR Subunit GluR4 Regulates Oligodendrocyte Progenitor Cell Migration in the Developing Spinal Cord

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Oligodendrocyte progenitor cells (OPCs) are specified from discrete precursor populations during gliogenesis and migrate extensively from their origins, ultimately distributing throughout the brain and spinal cord during early development. Subsequently, a subset of OPCs differentiates into mature oligodendrocytes, which myelinate axons. This process is necessary for efficient neuronal signaling and organism survival. Previous studies have identified several factors that influence OPC development, including excitatory glutamatergic synapses that form between neurons and OPCs during myelination. However, little is known about how glutamate signaling affects OPC migration before myelination. In this study, we use *in vivo*, time-lapse imaging in zebrafish in conjunction with genetic and pharmacological perturbation to investigate OPC migration and myelination when the GluR4A ionotropic glutamate receptor subunit is disrupted. In our studies, we observed that *gria4a* mutant embryos and larvae displayed abnormal OPC migration and altered dorsoventral distribution in the spinal cord. Genetic mosaic analysis confirmed that these effects were cell-autonomous, and we identified that voltage-gated calcium channels were downstream of glutamate receptor signaling in OPCs and could rescue the migration and myelination defects we observed when glutamate signaling was perturbed. These results offer new insights into the complex system of neuron-OPC interactions and reveal a cell-autonomous role for glutamatergic signaling in OPCs during neural development.

Key words: glutamate signaling; myelin; oligodendrocyte; oligodendrocyte progenitor cell; zebrafish

Significance Statement

The migration of oligodendrocyte progenitor cells (OPCs) is an essential process during development that leads to uniform oligodendrocyte distribution and sufficient myelination for central nervous system function. Here, we demonstrate that the AMPA receptor (AMPA) subunit GluR4A is an important driver of OPC migration and myelination *in vivo* and that activated voltage-gated calcium channels are downstream of glutamate receptor signaling in mediating this migration.

Introduction

During development, oligodendrocyte progenitor cells (OPCs) migrate from their ventral spinal cord origin and distribute throughout the CNS, and a subset differentiates into mature oligodendrocytes that myelinate axonal segments (Barres and Raff, 1999; Kucenas et al., 2008; Emery, 2010; de Faria et al., 2019;

Mitew et al., 2014; Nave and Werner, 2014; Marisca et al., 2020). During this process, OPCs interact extensively with each other and their environment, and are influenced by a variety of signals, including growth factors, chemokines, neurotransmitters, and extracellular matrix components (Kirby et al., 2006; Frost et al., 2009; Hughes et al., 2013; Sanchez-Rodriguez et al., 2018; Suzuki et al., 2019). Previously, we identified adenosine signaling via neuronal A2A receptors as an upstream regulator of OPC migration (Fontenas et al., 2019). In this study, we sought to extend those findings and identify an axonally-derived cue that modulates OPC migration during development.

Previous studies demonstrate that neuronal signaling influences OPC development via various mechanisms, including OPC-expressed ionotropic AMPA receptors (AMPA; Bergles et al., 2000; Lin and Bergles, 2004). The activation of OPC AMPARs can affect cellular processes by facilitating membrane depolarization and subsequent activation of voltage-gated calcium channels, resulting in Ca^{2+} influx (Gudz et al., 2006; Paez et al., 2010; Giesen et al., 2020). *In vivo*, neuronal activity affects oligodendrocyte axon

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Table 1. Zebrafish lines

Name	Source	Description
AB*		Wild type
<i>Tg(olig2:dsred)^{vu19}</i>	Shin et al. (2003)	Red fluorescent line that labels <i>olig2</i> ⁺ motor neurons and OLCs
<i>Tg(olig2:egfp)^{vu12}</i>	Shin et al. (2003)	Green fluorescent line that labels <i>olig2</i> ⁺ motor neurons and OLCs
<i>Tg(mbp:egfp-CAAX)</i>	Almeida et al. (2011)	Green fluorescent membrane line that labels <i>mbp</i> ⁺ myelinating cells
<i>Tg(sox10:megfp)^{l3}</i>	Kirby et al. (2006)	Green fluorescent membrane line that labels <i>sox10</i> ⁺ glia and neural crest cells
<i>Gt(foxd3:mCherry)^{ct110R}</i>	Hochgreb-Hägele and Bronner (2013)	Red fluorescent gene-trap line that labels <i>foxd3</i> ⁺ neural crest cells and peripheral glia
<i>Tg(Xla.Tubb:dsred)</i>	Peri and Nüsslein-Volhard (2008)	Red fluorescent line that labels <i>tubulin</i> ⁺ neurons
<i>gria4a^{uv43}</i>	This article	13-bp insertion resulting in frameshift and early stop codon (see Fig. 1D)
<i>Tg(sox10:SF-iGluSnFR)^{uv456}</i>	This article	i-GluSnFR line that labels <i>sox10</i> ⁺ glia with a cell membrane glutamate sensor

selection for myelination and stabilization of myelin sheath formation (Hines et al., 2015; Koudelka et al., 2016), and neuronal signaling is also implicated in OPC proliferation (Barres and Raff, 1999; Gibson et al., 2014) and survival (Kougioumtzidou et al., 2017). Several studies have reported that AMPAR-mediated Ca^{2+} influx activates OPC migration *in vitro* (Gudz et al., 2006; Paez et al., 2010; Harlow et al., 2015), but this effect has not been described *in vivo* (Harlow et al., 2015; Kougioumtzidou et al., 2017) and currently, there is no consensus on how AMPARs affect OPC migration *in vivo*.

In addition to AMPAR-mediated activation of voltage-gated calcium channels, extracellular Ca^{2+} influx can occur via calcium-permeable AMPARs. There are eight AMPAR subunit genes encoded in the zebrafish genome, compared with four in mammals, because of a whole-genome duplication event in the evolutionary history of teleost fish (Meyer and Schartl, 1999; Sobolevsky et al., 2003). Of these eight zebrafish AMPAR subunit genes, *gria2a* and *gria2b* are calcium impermeable (Kung et al., 2001; Sobolevsky et al., 2003). The remaining six AMPAR subunit genes, including *gria4a*, which encodes GluR4A, are calcium-permeable (Kung et al., 2001). The calcium permeability of an AMPAR is determined by the permeability of each of its subunits (Burnashev, 1998), and calcium-permeable AMPARs are a major source of Ca^{2+} influx in OPCs (Ge et al., 2006). This mechanism of Ca^{2+} influx via calcium-permeable AMPARs has been studied extensively in the context of excitotoxicity (Feldmeyer et al., 1999; Higuchi et al., 2000; Lee et al., 2001; Isaac et al., 2007; Evonuk et al., 2020) and extracellular Ca^{2+} influx can cause a wide array of cellular effects in OPCs, including changes in proliferation and differentiation (Chen et al., 2018), cell death and survival (Danesi et al., 2019), myelination (Krasnow et al., 2018), and cell morphology and migration (Gudz et al., 2006; Paez et al., 2010; Harlow et al., 2015). In primary rat cultures, OPCs and immature oligodendrocytes highly express GluR4, while GluR4 expression and Ca^{2+} influx was decreased in mature, myelin basic protein (MBP)-expressing oligodendrocytes, indicating that AMPAR subunit composition and downstream Ca^{2+} influx may control the onset of myelination (Itoh et al., 2002). However, the relationship between AMPAR subunit composition and downstream Ca^{2+} influx with regard to OPC migration *in vivo*, is not fully understood (Harlow et al., 2015).

Here, using *in vivo*, time-lapse imaging coupled with a new *gria4a* CRISPR mutant, we demonstrate that the migration and myelination patterns of spinal cord OPCs and oligodendrocytes are disrupted in the presence of perturbed GluR4A. Our results highlight, *in vivo*, a role for AMPAR activation downstream of neuronal activity and identify the GluR4A subunit as a regulator of OPC migration before myelination during vertebrate development.

Materials and Methods

Zebrafish

The use of all animals in this study was approved by the University of Virginia Institutional Animal Care and Use Committee. The zebrafish lines used were: AB* (wild type), *Tg(olig2:dsred)^{vu19}*, *Tg(olig2:egfp)^{vu12}* (Shin et al., 2003), *Tg(mbp:egfp-CAAX)* (Almeida et al., 2011), *Tg(sox10:megfp)^{l3}* (Smith et al., 2014), *Gt(foxd3:mCherry)^{ct110R}* (Hochgreb-Hägele and Bronner, 2013), *Tg(Xla.Tubb:dsred)* (Peri and Nüsslein-Volhard, 2008), *Tg(sox10:SF-iGluSnFR)^{uv456}*, and *gria4a^{uv43}* (Table 1). To visualize individual oligodendrocyte lineage cells (OLCs), one-cell *gria4a*^{+/+}, *gria4a*^{+/uv43} and *gria4a*^{uv43/uv43} embryos were transiently injected with 2 nl of 20 ng/μl *mbp:egfp-CAAX* (Almeida et al., 2011). Embryos were incubated at 28.5°C in egg water, and phenylthiourea (PTU; 0.004%) was used to reduce pigment formation for imaging (Karlsson et al., 2001). Embryos of either sex were used in all experiments (Kimmel et al., 1995), and ages are listed in hours postfertilization (hpf) or days postfertilization (dpf).

Generation of *gria4a^{uv43/uv43}* mutants and the *Tg(sox10:SF-iGluSnFR)* line

Guide RNA targeting *gria4a* (TGAGAGGTTTCATGCACGGCG) was designed using CHOPCHOP (Labun et al., 2018) and the protocol described by Gagnon et al. (2014). The 3' constant and 5' *gria4a*-specific oligonucleotides were combined as described by Nakayama et al. (2014), and the sgRNA transcribed using the Ambion Megascript T7 kit. An injection of 2 nl of the sgRNA (200–400 ng/μl) with Cas9 protein (500 ng/μl) was performed into one-cell stage AB* embryos. Once the injected fish reached adulthood, they were bred to AB* wild-type adults and DNA isolated from a pool of 8 embryos was amplified with PCR using the primers TTAGTGTGTCAGCATTGGGAC and TAGTTTGATCACCATCGGTTTG, then sequenced to identify potential founders. *Tg(olig2:dsred)* and *Tg(mbp:egfp-CAAX)* were crossed with the injected adult fish (F0) with a germline mutation in *gria4a* to produce stable heterozygote mutants. These fish (F1) were raised to adulthood, and fin clipping and sequencing was used to determine each mutation. We identified a 13-bp insertion resulting in a premature stop codon, and fish bearing this mutation were crossed to produce all subsequent generations. This 13-bp insertion produces a restriction site for MspI not found in the wild-type *gria4a* gene, and restriction digest of the amplified fragment followed by agarose gel electrophoresis on a 1.5% agarose gel was used for genotyping. This digest produces two bands at 149 and 75 bp for the mutant gene and does not affect the 224-bp wild-type fragment. All three bands are visible following digestion of heterozygous DNA. Whenever possible, genotyping was performed after analyzing results to allow for blind analysis of all studies.

Tg(sox10:SF-iGluSnFR) fish were generated via the Tol2kit Gateway-based cloning system (Kwan et al., 2007). Vectors used for making the Gateway expression constructs were p5E-sox10(–4.9) (Carney et al., 2006), pME-SF-iGluSnFR.A184V (Marvin et al., 2018), p3E-poly A (Kwan et al., 2007), and the destination vector pDesTol2CG2 (Kwan et al., 2007). All three expression vectors were inserted into the pDesTol2CG2 destination vector via LR reactions (Kwan et al., 2007). Resulting LR plasmids were amplified and sequenced to confirm appropriate insertion of each expression construct. After sequence confirmation, the plasmid was injected into one-cell stage AB* embryos at a

concentration of 25 ng/ μ l with an equal concentration of *Tol2* transposase mRNA. Fish expressing the pDesTol2CG2 heart reporter and SF-iGluSnFR fluorescence were raised to adulthood and were crossed to AB* adults to screen for founders (Kawakami, 2004).

Cell transplantation

Donor embryos were injected with ThermoFisher Dextran Cascade Blue dye (3000 MW) immediately following fertilization at the one-cell stage. All embryos were manually dechorionated at 4 hpf in 2% agarose-lined dishes and flamed pipettes were used to transfer dechorionated embryos. Transplantation was performed using the Eppendorf CellTram vario transplantation rig, with $\sim$ 30 cells transferred from each donor embryo and inserted into the expected spinal cord region of the 4- to 5-hpf host embryo according to fate mapping (Kimmel et al., 1995). Transplantation was performed in 1 $\times$ Danieau with 1 $\times$ penicillin-streptomycin, and hosts were incubated at 28.5°C in 0.3 $\times$ Danieau with 1 $\times$ penicillin-streptomycin following the procedure. The hosts were later imaged as described below and both donor and host embryos were kept and genotyped as described above.

Chemical treatments

Drug treatments [NBQX, ($\pm$)-Bay K 8644] were performed by adding a working solution of each drug, dissolved in 1% dimethylsulfoxide (DMSO), to 96-well plates containing one embryo per well. ($\pm$)-Bay K 8644 was applied at a treatment concentration of 5 μ M as previously described (Koleilat et al., 2019). A treatment of 1% DMSO was used as a control for each drug.

Confocal imaging

All confocal images were taken with a 40 $\times$ water objective (NA = 1.1) mounted on a motorized Zeiss AxioObserver Z1 microscope equipped with a Quorum WaveFX-XI (Quorum Technologies Inc.) or Andor CSU-W (Andor Oxford Instruments) spinning disk confocal system. Embryos used for imaging were anesthetized with 0.01% 3-aminobenzoic acid ester (Tricaine) and mounted in 0.8% low-melting-point agarose in 35 mm glass-bottomed Petri dishes, then immersed in egg water with PTU (0.004%). For drug treatment experiments, the immersion during imaging contained the same dosage administered in the 96-well plate, described above. In time-lapse experiments, images were taken every 10 min unless otherwise noted. Images were processed with MetaMorph and ImageJ softwares, and adjustments were limited to levels and contrast. Cell migration tracking was performed on time-lapses with images every 20 min, for consistency, using the ImageJ Manual Tracking plugin, as described below. Myelin quantification was performed through the z-stack of each image using the measurement feature of ImageJ.

Cell tracking

The ImageJ plugin Manual Tracking was used to determine speed and distance traveled of migrating cells. For each cell, a track was created by clicking the center of the cell body at each time point in a time-lapse. Distance migrated was calculated by summing the “distance” output, and migration speed was calculated by averaging the “velocity” output, both scaled to μ m. Further documentation can be found on <https://imagej.nih.gov/ij/plugins/track/track.html>.

Myelin measurement

The “measure” feature of ImageJ was used to quantify the length of myelin internodes. To do this, the line tool was used to mark myelin sheaths appearing in either individual z-planes or z-stacks of images of *Tg(mbp:egfp-CAAX)* embryos or embryos transiently injected with the *mbp:egfp-CAAX* construct (see figure legends). The results were compiled and scaled to μ m.

Photo-uncaging glutamate

We bathed embryos and larvae in 1 μ M MNI-glutamate (Tocris catalogue number 1490; 25 mM stock solution in DMSO) 1 h before imaging. An equal amount of DMSO (0.4%) was used as a vehicle control. During treatment, larvae were kept in the dark as much as possible and we mounted larvae for live imaging as described above. We performed focal

uncaging of MNI-glutamate with a 404-nm laser focused to a circular region of interest (50- μ m² surface, $\sim$ 20–40 μ m from *sox10*⁺ OPC membranes) in the center of the acquired z-stack, followed by time-lapse imaging for 1 min, imaging every 32 ms, or for 2 h, imaging every minute.

In situ hybridization

Embryos and larvae used were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and stored in 100% methanol. *In situ* RNA hybridization was used to localize the expression of *gria4a* in the zebrafish spinal cord. The primers used to produce the probes were as follows: forward primer, ACATCTCTCGCAGAGGCAAT; reverse primer, GGGCTTGGAGAAATCAATCA. Probes were prepared with DIG labeling, as previously described (Fontenas et al., 2019). After *in situ* hybridization, embryos and larvae were embedded in 1.5% agarose and saturated with 30% sucrose in distilled water, then frozen in 2-methylbutane cooled by immersion in liquid nitrogen. We produced 25 μ m cross-sections of the embryos/larvae with a cryostat microtome, then immersed in 75% glycerol and imaged the slides with the 40 $\times$ water objective (NA = 1.1) on a Zeiss AxioObserver microscope. Images were imported to Adobe photoshop, and adjustments were limited to levels, contrast, and cropping.

Immunohistochemistry

Embryos and larvae were fixed in 4% PFA for 1 h, then embedded in 1.5% agarose and saturated with 30% sucrose in distilled water. Then, they were frozen in 2-methylbutane cooled by immersion in liquid nitrogen. We produced 25 μ m cross-sections of the embryos/larvae with a cryostat microtome. Antibodies used were as following: rabbit anti-Sox10 (1:1000; Binari et al., 2013) and chicken anti-GFP (Aves Lab; 1:500). Fluorescent detection of antibody labeling was performed using Alexa Fluor 555 donkey anti-rabbit (1:500) and Alexa Fluor 488 donkey anti-chicken (1:500). Slides were imaged using a 40 $\times$ oil objective (NA = 1.1) on a Zeiss AxioObserver ZI microscope. Images were imported to ImageJ, and adjustments were limited to levels, contrast, and cropping. For immunohistochemistry following *in situ* hybridization, whole mount *in situ* hybridization was performed as described above and then embryos or larvae were embedded in agarose, cryosectioned, and then immunohistochemistry was performed as described.

Cell dissociation for flow cytometry

For cell dissociation, 72 hpf larvae with transgenes *Tg(olig2:dsred);Tg(sox10:megfp)* for OPCs, *Tg(sox10:megfp);Gt(foxd3:mCherry)* for Schwann cells, and *Tg(nbt:dsred)* for neurons, were chilled in egg water on ice, then the heads were removed using a scalpel. The cells were dissociated according to a previously published method (Zhu et al., 2019). Briefly, the trunks were immersed in calcium-free Ringer's solution with 2.5 mM EDTA and rocked for 15 min at 4°C, then washed three times with chilled Dulbecco's PBS (D-PBS) and transferred with 100 μ l D-PBS into microcentrifuge tubes. The trunk pieces were broken up using a pellet pestle followed by the addition of 30 μ l Liberase TM. The samples were incubated for 15 min at 28.5°C, 1 ml Trypsin with 5% EDTA was added, then the samples were incubated again for 15 min at 28.5°C. The samples were pipetted into 5-ml D-PBS with 1% BSA, then filtered by passing through a 40 μ m cell strainer, and a syringe plunger was used to gently mash the samples into a Petri dish. The sample was passed through a new 40 μ m cell strainer and transferred to a microcentrifuge tube, then washed twice with D-PBS with 1% BSA. Three samples of cells (1750 $\pm$ 750) of each type were used for RNA-sequencing. Total RNA was extracted using the RNeasy Micro kit (QIAGEN), then cDNA was prepared using Smart-Seq v4 Ultra Low Input RNA kit for sequencing (Takara). Library preparation was performed using the NEB Next Ultra II DNA Library Prep kit for Illumina (NEB).

RNA-seq analysis

RNA-seq analysis was performed following previously published methods (Zhu et al., 2019). Briefly, abundance of transcripts from RNA-seq datasets were imported into the DESeq2 pipeline using the R package tximport (Love et al., 2014; Sonesson et al., 2015). R package AnnotationDbi was used to acquire ENTREZ IDs and Gene Symbols

(Pagès et al., 2020). Normalized FPKM counts were generated using the `fpkm()` function in the DESeq2 package (Love et al., 2014). Differentially expressed genes were analyzed using the DESeq2 package ($\text{abs}(\log_2\text{FoldChange}) > 1$ and $\text{padj} < 0.1$; Love et al., 2014). Lists of differentially expressed genes were then used to perform functional analysis using the clusterProfiler package (Yu et al., 2012). Genes with $\text{padj} = \text{NA}$ were removed to exclude genes with low counts. Raw sequencing data are available on GEO: [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE174486]. Transcript abundance files are available on Mendeley Data: [https://data.mendeley.com/datasets/cpfwssf2ns/2].

RT-PCR

mRNA was extracted and cDNA synthesized as described previously (Peterson and Freeman, 2009) with the use of RNAeasy Mini kit (QIAGEN) and High-capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer instructions. Equal amounts of mRNA were used for cDNA synthesis, and RT-PCR was performed using GoTaq green mastermix (Promega). Primers used were: *gria4a*: forward, CAAGACTCGGGAAGTAAGGAC and reverse, CCGAGAAGGTGAGCTTCAGTT; *gria4b*: forward, ACAA GGCCTGTTGGACAAATTGAAA and reverse, CACGGTAAGT CGGATGACACC; *gria2b*: forward, CTGAACGAGCAGGGGCTTC and reverse, AGCATCTGTGAACGTCATCCC; and *efl α* : forward, GAGACTGGTGTCTCAAGCC and reverse, CCAACGTTGTCA CCAGGAGT. Expression was quantified using ImageJ and compared with relative expression in wild-type larvae.

Statistical analyses

Statistics for all quantitative experiments were conducted using GraphPad Prism. To compare between controls and treatment groups, a two-sided unpaired *t* test was used. To compare across all genotypes or to compare multiple treatment groups with control, a one-way ANOVA with Tukey's multiple comparison test was used. For quantification of the difference in fluorescence intensity on cell membranes relative to the background, a one-sample *t* test with a hypothetical value of 1 was used. Categorical data were analyzed using GraphPad Prism's one-tailed Fisher's exact test. The significance level was set at $\alpha = 0.05$ ($p < 0.05$ were considered significant).

Results

The AMPAR subunit gene *gria4a* is expressed by spinal cord OPCs

While the AMPAR subunit genes *gria1a*, *gria1b*, *gria2b*, and *gria4a* are expressed in the developing zebrafish spinal cord, it was unclear whether any were expressed by OPCs (Hoppmann et al., 2008). To determine which, if any, of these genes are selectively expressed in spinal cord OPCs, we performed RNA sequencing of OLCs (*olig2*⁺/*sox10*⁺ cells collected from the trunks of *Tg(olig2:dsred);Tg(sox10:megfp)* larvae at 72 hpf), Schwann cells (*foxd3*⁺/*sox10*⁺ cells collected from the trunks of *Gt(foxd3:mCherry);Tg(sox10:megfp)* larvae at 72 hpf), and neurons (*nbt*⁺ cells collected from the trunks of *Tg(nbt:dsred)* larvae at 72 hpf) and compared the expression of each AMPAR subunit gene across these cell types. RNA sequencing revealed that at 72 hpf, both *gria2b* and *gria4a* were highly expressed in OLCs, but *gria4a* was the subunit with unique expression in OLCs and was not highly expressed by either Schwann cells or neurons (Fig. 1A). To validate these results, we performed *in situ* hybridization with a mRNA probe specific to *gria4a* at 48 and 72 hpf and found expression in cells closely associated with the spinal cord white matter (Fig. 1B). To confirm that *gria4a* was expressed by OLCs, we performed *in situ* hybridization for *gria4a* and immunohistochemistry with an antibody specific to Sox10, which labels OLCs, on 80 hpf *Tg(olig2:egfp)* larvae, and observed Sox10⁺/*olig2*⁺/*gria4a*⁺ OLCs in the dorsal spinal

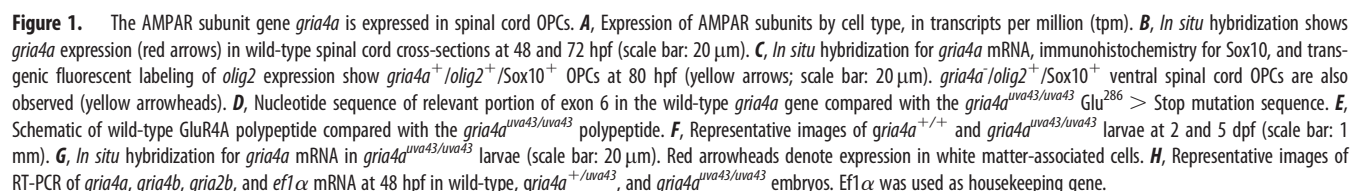
cord (Fig. 1C). OLCs in the ventral spinal cord were typically Sox10⁺/*olig2*⁺/*gria4a*[−] (Fig. 1C). Together, these results indicate that the AMPAR subunit gene *gria4a*, which encodes the AMPAR subunit GluR4A, is expressed by a subset of spinal cord OLCs during development.

Because the AMPAR subunit gene *gria4a* is highly expressed by OLCs and not neurons at 72 hpf (Fig. 1A), we hypothesized that this gene was a good candidate to mediate OPC migration before myelination. To test this hypothesis, we used CRISPR/Cas9 gene editing to create mutants that perturbed the GluR4A subunit. We injected a *gria4a* gRNA that targeted the sixth exon of the *gria4a* gene (Fig. 1D), into one-cell embryos and grew these putative mutant founders to adulthood. Upon crossing them to identify germline founders carrying a mutation in *gria4a*, we identified one allele that caused a 13-bp insertion and subsequent frameshift mutation early in the gene after amino acid 289 (Fig. 1D). This frameshift mutation resulted in a premature stop codon, which terminates the GluR4A polypeptide before translation of the critical glutamate-binding and transmembrane domains (Fig. 1E). These mutant embryos and larvae were morphologically indistinguishable from their clutchmates both at 2 and 5 dpf (Fig. 1F), and adults were homozygous viable.

To investigate whether *gria4a* expression was reduced in *gria4a* mutant embryos and larvae, we performed *in situ* hybridization and this revealed that there was *gria4a* transcript in the spinal cord of *gria4a*^{uva43/uva43} mutants, both at 48 and 72 hpf, although at lower levels than seen in wild-type controls (compare Fig. 1G and B). To confirm this reduction in transcript level, we performed RT-PCR and observed that in 48 hpf *gria4a*^{uva43/uva43} embryos, *gria4a* mRNA expression was decreased by ~20% when compared with wild-type larvae (mutant expression of $81.1 \pm 1.3\%$ of wild-type level; Fig. 1H), possibly caused by non-sense-mediated decay. Because loss of *gria4a* may result in genetic composition by other AMPAR subunits and an altered composition of subunits may change Ca²⁺ influx into the cells, we also analyzed expression of *gria4b* and *gria2b*. We observed that while expression of *gria4b* was low in wild-type larvae, expression increased both in heterozygous ($146.1 \pm 34.7\%$ increase compared with the wild-type level) and homozygous larvae ($142.0 \pm 36.5\%$ increase compared with the wild-type level). Additionally, *gria2b* expression also increased in heterozygous ($123.6 \pm 14.5\%$ increase compared with the wild-type level) and homozygous larvae ($124.8 \pm 17.8\%$ increase compared with the wild-type level; Fig. 1H). Thus, in *gria4a*^{uva43/uva43} and *gria4a*^{+/uva43} larvae there are reduced levels of GluR4A, as well as increased expression of GluR4B and GluR2B, indicating there may be some compensation for the loss of a functional copy of the *gria4a* gene.

GluR4A is required for OPC migration and distribution in the spinal cord

We hypothesized that mutation of GluR4A would likely affect OPC sensing of the neurotransmitter glutamate as we recently identified neuronal activity as important for OPC migration (Fontenas et al., 2019). Previous studies demonstrate that glutamate can be released at sites along CNS axons outside of the synapse (Kukley et al., 2007; Ziskin et al., 2007; Wake et al., 2015; Kula et al., 2019). Therefore, we hypothesized that *gria4a*^{uva43/uva43} mutants would exhibit abnormal OPC migration because they would be unable to sense this axonally-derived glutamate. To investigate this hypothesis, we performed



movies from 56 to 72 hpf, we observed similar numbers of dorsal OPCs across genotypes at 56 hpf, which is just as these cells are migrating from their ventral spinal cord origin (Fig. 2A). However, by 72 hpf, there were significantly fewer dorsal OPCs in *gria4a*^{uva43/uva43} larvae compared with wild-type clutchmates ($p = 0.0041$; Fig. 2B) and a similar trend was observed compared

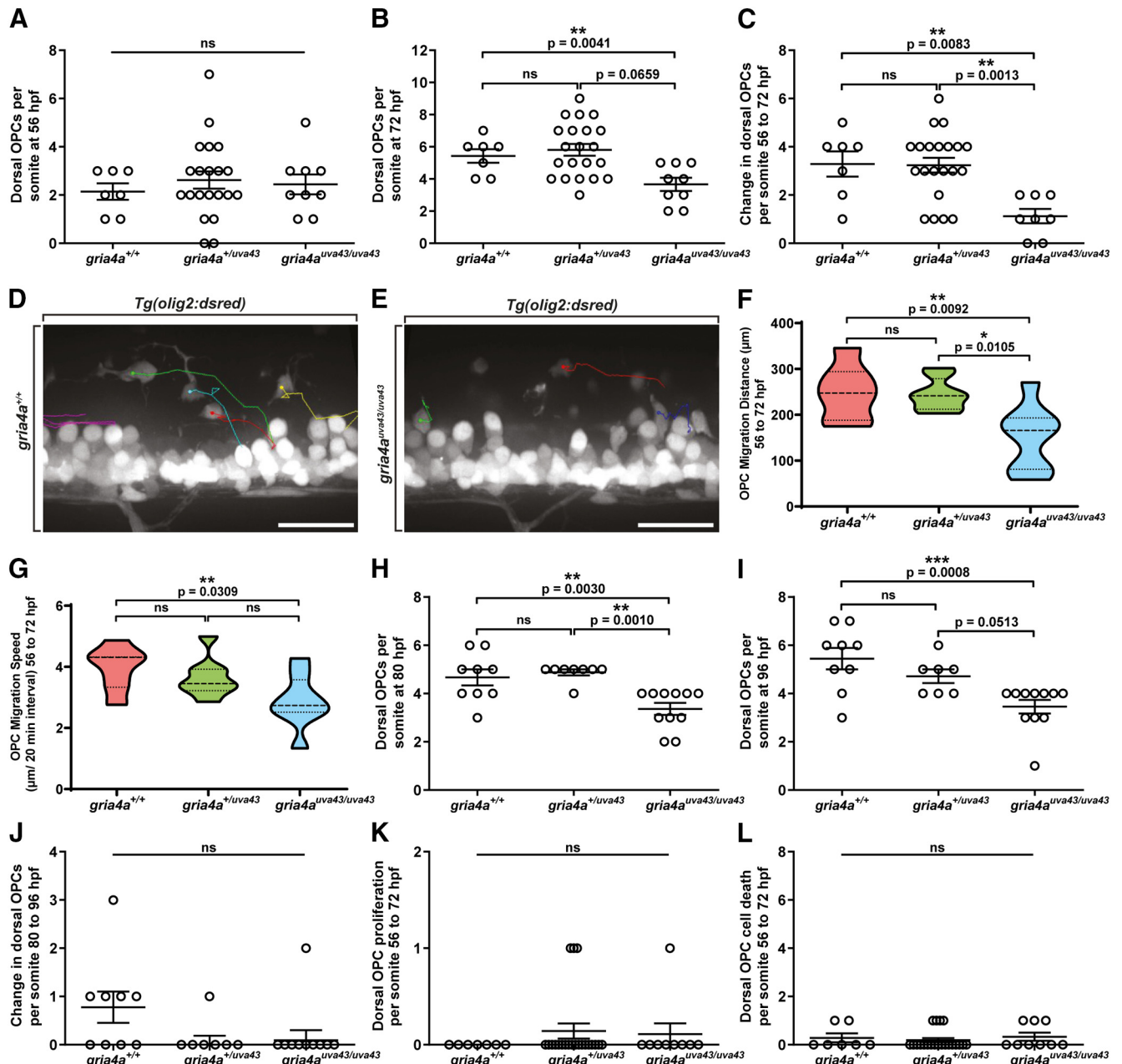


Figure 2. *Gria4a* mutants have reduced dorsal OPC migration and altered OPC migration dynamics. **A, B**, Mean $\pm$ SEM of dorsal OPCs per somite in *Tg(olig2:dsred);Tg(mbp:egfp-CAAX)* *gria4a*^{+/+} (*n* = 6), *gria4a*^{+/uva43} (*n* = 21), and *gria4a*^{uva43/uva43} (*n* = 9) embryos at 56 (*p* = 0.11) and 72 hpf (*gria4a*^{+/+} vs *gria4a*^{uva43/uva43} *p* = 0.0041; *gria4a*^{+/uva43} vs *gria4a*^{uva43/uva43} *p* = 0.0659). **C**, Mean $\pm$ SEM of net change in dorsal OPC number per somite in *gria4a*^{+/+} (*n* = 6), *gria4a*^{+/uva43} (*n* = 21), and *gria4a*^{uva43/uva43} (*n* = 9) embryos between 56 and 72 hpf (*gria4a*^{+/+} vs *gria4a*^{uva43/uva43} *p* = 0.0083; *gria4a*^{+/uva43} vs *gria4a*^{uva43/uva43} *p* = 0.0013). **D, E**, Migration tracks of OPCs in *Tg(olig2:dsred);Tg(mbp:egfp-CAAX)* *gria4a*^{+/+} and *gria4a*^{uva43/uva43} embryos between 56 and 72 hpf. Only fluorescence from *Tg(olig2:dsred)* line shown for clarity. **F, G**, Violin plots of OPC migration distance (*gria4a*^{+/+} vs *gria4a*^{uva43/uva43} *p* = 0.0092; *gria4a*^{+/uva43} vs *gria4a*^{uva43/uva43} *p* = 0.0105) and speed (*gria4a*^{+/+} vs *gria4a*^{uva43/uva43} *p* = 0.0309) between 56 and 72 hpf in *gria4a*^{+/+} (*n* = 7), *gria4a*^{+/uva43} (*n* = 8), and *gria4a*^{uva43/uva43} (*n* = 8) embryos. **H, I**, Mean $\pm$ SEM of dorsal *olig2*⁺ cell number per somite in *gria4a*^{+/+} (*n* = 9), *gria4a*^{+/uva43} (*n* = 8), and *gria4a*^{uva43/uva43} (*n* = 11) larvae at 80 and 96 hpf. At 80 hpf, *gria4a*^{+/+} versus *gria4a*^{uva43/uva43} *p* = 0.0030; *gria4a*^{+/uva43} versus *gria4a*^{uva43/uva43} *p* = 0.0010. At 96 hpf, *gria4a*^{+/+} versus *gria4a*^{uva43/uva43} *p* = 0.0008; *gria4a*^{+/uva43} versus *gria4a*^{uva43/uva43} *p* = 0.0513. **J**, Mean $\pm$ SEM of net change in dorsal *olig2*⁺ cell number per somite in *gria4a*^{+/+}, *gria4a*^{+/uva43}, and *gria4a*^{uva43/uva43} larvae between 80 and 96 hpf; *p* = 0.0794 across all groups. **K, L**, Quantification of dorsal OPC proliferation (**K**) and cell death (**L**) from *in vivo*, time-lapse movies of wild-type (*n* = 7), heterozygous (*n* = 21), and mutant (*n* = 9) larvae between 56 and 72 hpf. ns = no significant differences between groups. Scale bar: 50 μ m.

with heterozygous clutchmates (*p* = 0.0659; Fig. 2B). When we compared the difference between dorsal OPCs at 72 hpf and 56 hpf, OPCs in *gria4a*^{uva43/uva43} larvae exhibited a significantly reduced net dorsal migration when compared with wild-type (*p* = 0.0083) and heterozygous (*p* = 0.0013) larvae (Fig. 2C). These results demonstrate that *gria4a* is necessary for dorsal OPC migration by 72 hpf.

To better understand what causes the reduced dorsal migration, we analyzed our time-lapse videos using the Manual Tracking ImageJ plugin for cell tracking (Fig. 2D–G). Quantification of distance migrated revealed that OPCs in *gria4a*^{uva43/uva43} larvae migrated a shorter distance (*p* = 0.0092; Fig. 2F) and at a slower speed (*p* = 0.0309; Fig. 2G) than their wild-type counterparts between 56 and 72 hpf. Interestingly,

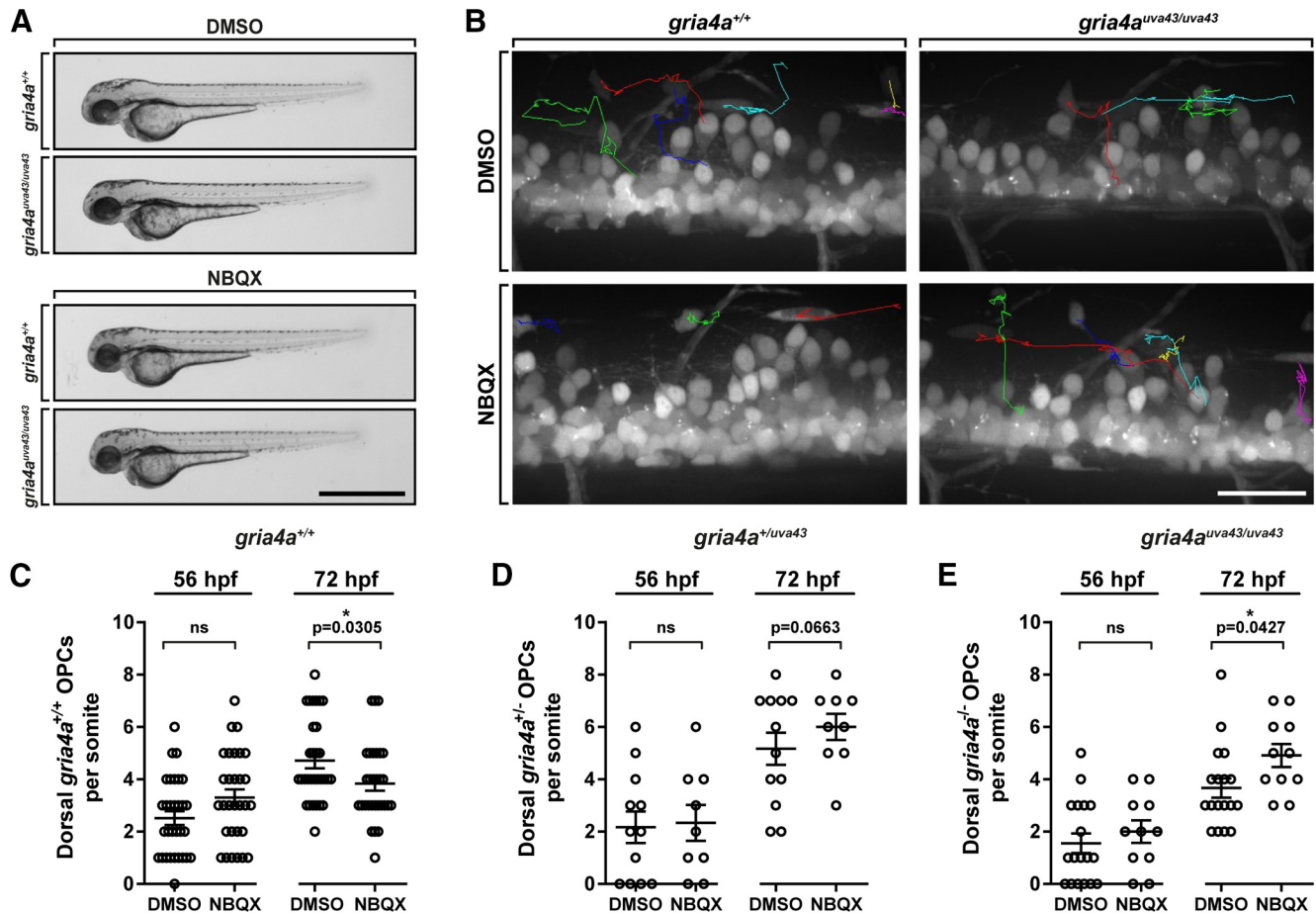


Figure 3. Treatment with ionotropic glutamate receptor inhibitor NBQX reduces dorsal OPC migration between 56 and 72 hpf. **A**, Brightfield images of 56-hpf *gria4a* wild-type and mutant embryos after treatment with DMSO or 40 μ M NBQX. **B**, Representative images and tracing of OPC migration after treatment with 40 μ M NBQX (scale bar: 50 μ m). **C–E**, Mean $\pm$ SEM of dorsal OPCs per somite at 56 to 72 hpf in *Tg(nkx2.2a:megfp);Tg(olig2:dsred)* embryos treated with 40 μ M NBQX in 1% DMSO or 1% DMSO alone from 30 to 72 hpf. **C**, Quantification of dorsal OPCs per somite in DMSO and NBQX-treated *gria4a*^{+/+} embryos and larvae at 56 and 72 hpf (DMSO n = 31, NBQX n = 30, DMSO vs NBQX at 72 hpf p = 0.0305). **D**, Quantification of dorsal OPCs per somite in DMSO and NBQX-treated *gria4a*^{+/uva43} embryos and larvae at 56 and 72 hpf (DMSO n = 12, NBQX n = 11, DMSO vs NBQX at 72 hpf p = 0.0663). **E**, Quantification of dorsal OPCs per somite in DMSO and NBQX-treated *gria4a*^{uva43/uva43} embryos and larvae at 56 and 72 hpf (DMSO n = 18, NBQX n = 11, DMSO vs NBQX at 72 hpf p = 0.0427). ns = no significant differences between groups.

although *gria4a*^{+/uva43} OPCs migrated a significantly longer distance than *gria4a*^{uva43/uva43} OPCs (p = 0.0105; Fig. 2F), their migration speed, although not significantly different from either wild-type or mutant OPCs, was intermediate between the two (Fig. 2G). To determine whether the difference in migration between wild-type and mutant OPCs was the result of a developmental delay, we repeated the experiment at a later stage and imaged the larvae from 80 to 96 hpf. The migration during this period revealed that there were fewer dorsal *olig2*⁺ cells in *gria4a*^{uva43/uva43} larvae compared with *gria4a*^{+/+} and *gria4a*^{+/uva43} larvae at both 80 (p = 0.0030 *gria4a*^{uva43/uva43} vs *gria4a*^{+/+}; p = 0.0010 *gria4a*^{+/uva43} vs *gria4a*^{uva43/uva43}; Fig. 2H) and 96 hpf (p = 0.0008 *gria4a*^{uva43/uva43} vs *gria4a*^{+/+}; p = 0.0513 *gria4a*^{+/uva43} vs *gria4a*^{uva43/uva43}; Fig. 2I). Additionally, there was minimal net dorsal OLC migration with no significant difference across the genotypes from 80 to 96 hpf (Fig. 2J), which demonstrates that the variation in OLC migration observed from 56 to 72 hpf was not the result of a developmental delay. Finally, we wanted to determine whether there were any changes to OPC proliferation or survival in *gria4a* mutant larvae, which has previously been described (Kougioumtzidou et al., 2017). To do this, we quantified the number of cell divisions and cell death events from *in vivo*,

time-lapse imaging data taken from 56 to 72 hpf. These studies revealed that OPC proliferation and survival were unchanged in the *gria4a*^{uva43/uva43} larvae when compared with heterozygous and wild-type siblings (Fig. 2K,L). Together, these findings demonstrate that mutation of GluR4A results in reduced dorsal OPC migration and altered migration dynamics of spinal cord OPCs from 56 to 72 hpf.

To independently confirm that perturbed AMPAR signaling was the cause of the dorsal OPC migration defect we observed in *gria4a*^{uva43/uva43} mutants, we used the drug NBQX, an ionotropic glutamate receptor inhibitor (Deng et al., 2003; Evonuk et al., 2020) to investigate the role of AMPAR signaling in OPC migration. To do this, we treated *Tg(olig2:dsred);Tg(nkx2.2a:megfp)* embryos from 30 to 72 hpf with either 40 μ M NBQX dissolved in 1% DMSO or with 1% DMSO as a control. We then performed *in vivo*, time-lapse imaging from 56 to 72 hpf and compared dorsal OPC migration between the two groups. Similar to *gria4a* mutant embryos and larvae, wild-type embryos treated with NBQX and DMSO larvae looked healthy (Fig. 3A). When we assayed OPC migration between 56 and 72 hpf, we observed a significant reduction in the number of *nkx2.2a*⁺/*olig2*⁺ OPCs that migrated dorsally between 56 and 72 hpf in NBQX-treated larvae compared with DMSO-treated larvae (p = 0.0305; Fig. 3B,

C). Similar to what we observed in *gria4a* mutant larvae, OPC proliferation and cell death were not affected in wild-type larvae treated with NBQX (proliferation: DMSO 0.40 ± 0.51 cells per somite vs NBQX 0.38 ± 0.51 cells per somite; cell death: DMSO 0.20 ± 0.41 cells per somite vs NBQX 0.46 ± 0.66 cells per somite; data not shown).

To reveal any compensatory mechanisms that may be taking place in *gria4a* mutant larvae, we treated both *gria4a*^{+/uva43} and *gria4a*^{uva43/uva43} embryos with NBQX from 30 to 72 hpf, and imaged OPC migration from 56 to 72 hpf. As OPCs already migrate significantly less in mutants, we did not expect to observe any effect of NBQX on dorsal OPC migration in mutant larvae. However, to our surprise, we observed a trend of increased dorsally-migrated OPCs at 72 hpf in NBQX-treated *gria4a* heterozygous larvae ($p = 0.0663$; Fig. 3D), and a significant increase in dorsal OPCs at 72 hpf in *gria4a* mutant larvae when compared with DMSO-treated mutant larvae ($p = 0.0427$; Fig. 3B,E). We hypothesize that this observed difference in the effect of NBQX on migration between wild-type and mutant larvae may be related to the altered composition of the AMPAR subunits in *gria4a* mutant larvae, thereby changing the calcium permeability of the AMPARs, and we will discuss this further in the discussion. Similar to what we observed in wild-type larvae, OPC proliferation and cell death were unchanged in heterozygous and mutant larvae treated with NBQX (*gria4a*^{+/uva43} proliferation: DMSO 0.50 ± 0.90 cells per somite vs NBQX 0.18 ± 0.40 cells per somite; cell death: DMSO 0.17 ± 0.39 cells per somite vs NBQX 0.55 ± 0.69 cells per somite; *gria4a*^{uva43/uva43} proliferation: DMSO 0.18 ± 0.40 cells per somite vs NBQX 0.25 ± 0.25 cells per somite; cell death: DMSO 0.27 ± 0.47 cells per somite vs NBQX 0.17 ± 0.39 cells per somite; data not shown). Based on the effects of NBQX on wild-type and mutant OPC migration (Fig. 3C,E), we conclude that AMPAR signaling plays a key role in promoting dorsal OPC migration, and this finding provides validation for the OPC migration defects observed in *gria4a* mutants.

To confirm that the reduction in OPC migration was caused by an inability of the *gria4a*^{uva43/uva43} OPCs to detect glutamate, we locally increased the availability of glutamate by photo-uncaging MNI-glutamate in the dorsal spinal cord at 56 hpf (Hughes and Appel, 2020). To verify that the glutamate-uncaging method we used was functional, we used a fluorescent glutamate biosensor (SF-iGluSnFR; Marvin et al., 2018), which is a cell surface biosensor that detects extracellular glutamate, expressed under the *sox10* promoter. To do this, we incubated *Tg(sox10:SF-iGluSnFR)* larvae with 1 μ M MNI-glutamate in 0.4% DMSO for 1 h before imaging. We then mounted the larvae for *in vivo* imaging and focally uncaged the MNI-glutamate using a 404-nm pulsed nitrogen dye laser in a region of interest just dorsal to iGluSnFR⁺ cells (~ 20 – 40 μ m from measured regions on OPC membranes, in line with previous studies used for uncaging MNI-glutamate; Fig. 4A, red circle; Li et al., 2012; Hughes and Appel, 2020). We then imaged every 32 ms for 1 min before and immediately after uncaging of the MNI-glutamate and compared the t-stacks of the two time-lapses. We then measured the change in fluorescence intensity across SF-iGluSnFR⁺ OPC membranes and size-matched control regions immediately next to iGluSnFR⁺ cells (Fig. 4A–C, yellow regions mark cell membranes and cyan regions mark neighboring background). After uncaging, we observed larger changes in iGluSnFR fluorescence intensity over time on *sox10*⁺ cell membranes compared with size-matched regions in the background (Fig. 4B, representative data). Quantification of the

change in fluorescence of the t-stacks revealed that the uncaging of MNI-glutamate resulted in significant changes in SF-iGluSnFR fluorescence on the cell-membranes of *sox10*⁺ cells compared with size-matched regions of background ($p < 0.0001$; Fig. 4C). From these results, we conclude that OPCs can detect glutamate release in the spinal cord.

Once we were confident that OPCs can respond to glutamate *in vivo*, we performed *in vivo*, time-lapse imaging from 56 to 58 hpf in *Tg(olig2:dsred);gria4*^{+/+}, *gria4a*^{+/uva43}, and *gria4a*^{uva43/uva43} larvae after focal uncaging of MNI-glutamate in the dorsal spinal cord (uncaging occurred ~ 20 – 40 μ m from SF-iGluSnFR⁺ OPCs; Fig. 4C). After uncaging, we observed that *gria4a*^{+/+} OPCs increased their migration speed ($p = 0.0048$; Fig. 4D) and average migration distance ($p = 0.0094$; Fig. 4E) in response to the increased local availability of glutamate. In contrast, both *gria4a*^{+/uva43} ($p = 0.0018$) and *gria4a*^{uva43/uva43} OPCs ($p < 0.0001$) migrated significantly slower (Fig. 4D) and for shorter distances (*gria4a*^{+/uva43} $p = 0.0012$; *gria4a*^{uva43/uva43} OPCs $p < 0.0001$) when compared with *gria4a*^{+/+} OPCs in response to the local glutamate burst. In fact, *gria4a*^{uva43/uva43} OPCs treated with DMSO were indistinguishable from *gria4a*^{uva43/uva43} OPCs exposed to uncaged MNI-glutamate, demonstrating that mutation of *gria4a* affects OPC sensing of, and migration toward, glutamate.

Next, we sought to determine whether the altered OPC migration we observed in *gria4a*^{uva43/uva43} larvae was maintained past developmental myelination stages, at 6 dpf. To investigate this, we performed immunohistochemistry on transverse trunk sections of mutant and control larvae to investigate the number and distribution of Sox10⁺ spinal cord OPCs and oligodendrocytes at 3 and 6 dpf. For each spinal cord section, we counted and recorded the XY-coordinates of each Sox10⁺ cell, then compiled these coordinates to generate heat maps for each developmental stage and genotype (Fig. 5A,B). We stratified the positional data into dorsal (upper 2/3 of Y area) and ventral (lower 1/3 of Y area) categories, roughly divided by the upper boundary of the primary motor neuron (PMN) domain. Based on these categories, we quantified dorsal and ventral Sox10⁺ cells in each genotype. At both stages, there were significantly fewer dorsal Sox10⁺ cells in the *gria4a*^{uva43/uva43} spinal cord compared with the *gria4a*^{+/+} spinal cord, and there were more ventral Sox10⁺ cells in the *gria4a*^{uva43/uva43} spinal cord compared with the *gria4a*^{+/+} spinal cord at both 3 ($p = 0.0080$; Fig. 5A,C) and 6 dpf ($p = 0.0159$; Fig. 5B,E), based on the proportion of dorsal cells in each section ($n = 20$ for all groups at 3 dpf, $n = 10$ for all groups at 6 dpf). Interestingly, at 3 dpf, there were slightly more dorsal OPCs in *gria4a*^{+/uva43} larvae compared with in *gria4a*^{+/+} larvae ($p = 0.0478$; Fig. 5A,C). However, at both timepoints, the total number of Sox10⁺ cells (both dorsal and ventral) across genotypes was similar (Fig. 5F), which demonstrates that cell proliferation and survival were not affected by the *gria4a* mutation, and this matches the proliferation and cell death counts we did previously (Fig. 2K,L). These results reveal that the OPC migration defect in *gria4a*^{uva43/uva43} larvae persists beyond early development and that GluR4A is necessary for normal distribution of OPCs throughout the spinal cord.

Mutation of *gria4a* alters OPC migration in a cell-autonomous manner

Our *gria4a* CRISPR mutant line is a global mutant, so we sought to determine whether the observed OPC migration differences were cell-autonomous, or alternatively, the consequence of aberrant GluR4A in other cell types, including neurons. To

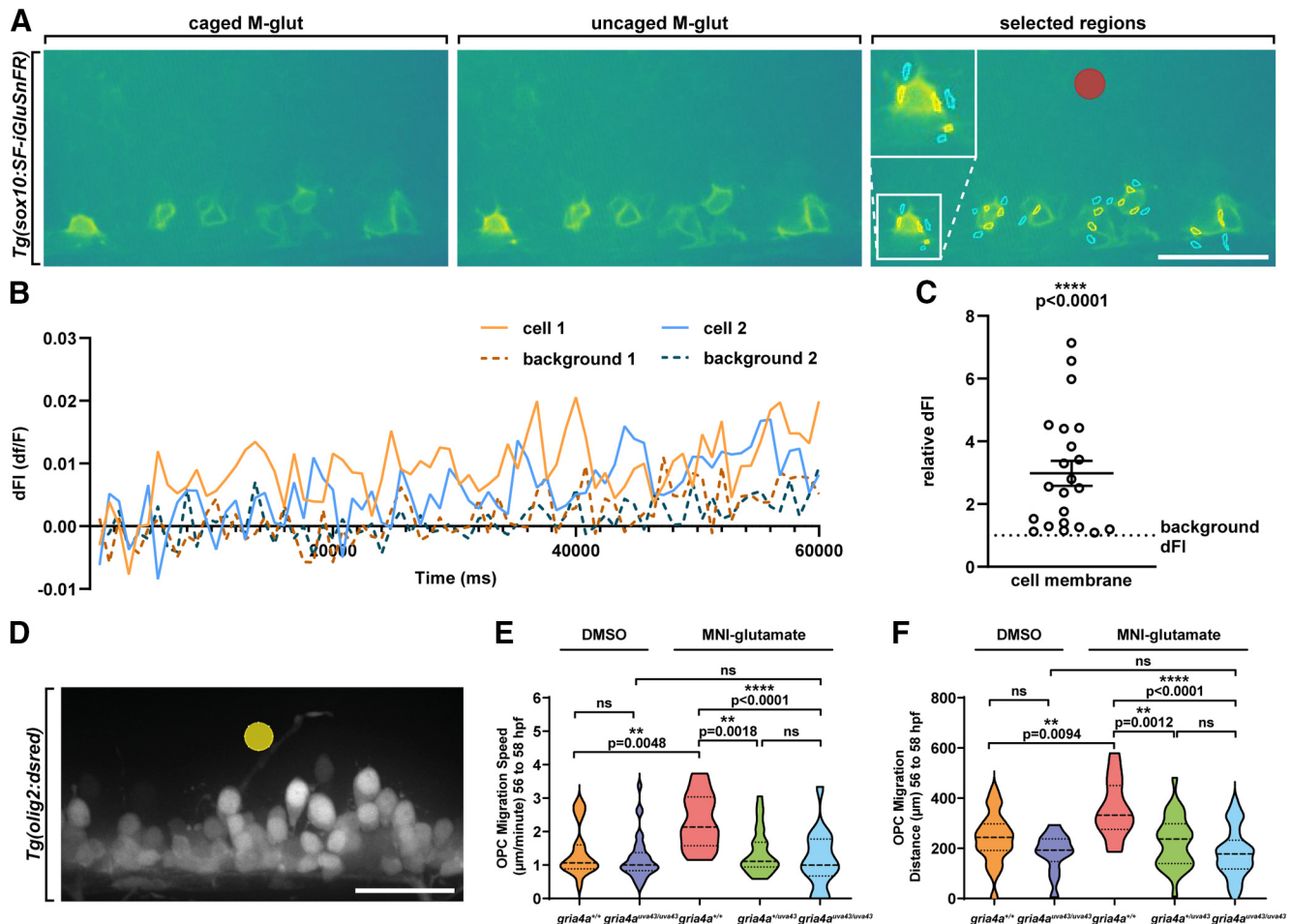


Figure 4. *gria4a* mutant OPCs have a decreased ability to sense glutamate. **A**, Representative images of 56 hpf *Tg(sox10:SF-iGluSnFR)* embryos before and after photo-uncaging of MNI-glutamate (M-glut) by a 404-nm laser. Red circle indicates focal region of interest (ROI) where M-glut was uncaged, ~20–40 μm from *sox10*⁺ OPC membranes. Yellow ROIs indicate where fluorescence intensity was measured on OPC membranes and cyan ROIs indicate size-matched regions where background fluorescence was measured next to the cell ($n = 22$ regions of $n = 18$ cells in $n = 3$ fish). Inset is magnified view of a *sox10*⁺ cell membrane after M-glut uncaging. **B**, Representative quantification of the difference in fluorescence intensity (dFI) before and after MNI-glut uncaging of two different *sox10*⁺ cell membranes and size-matched ROIs in the background, averaged per 25 frames (800 ms) of imaging. **C**, Quantification of relative total dFI after uncaging of M-glut on *sox10*⁺ cell membranes compared with the size-matched regions in the background ($n = 22$, $p < 0.0001$). **D**, Representative image of ROI (yellow, 50 μm^2) in the spinal cord where M-glut was uncaged in *Tg(olig2:dsred)* embryos at 56 hpf for data collected in **D**, **E**, **F**. **E**, **F**, Violin plots of OPC migration speed and distance in *gria4a*^{+/+} (DMSO $n = 9$, M-glut $n = 6$), *gria4a*^{+/uva43} (M-glut $n = 12$), and *gria4a*^{uva43/uva43} (DMSO $n = 6$, M-glut $n = 15$) larvae after uncaging of M-glut (1 μM) over a time course of 2 h. Migration speed across all groups $p < 0.0001$, *gria4a*^{+/+} DMSO versus *gria4a*^{+/+} M-glut $p = 0.0048$, *gria4a*^{+/+} M-glut versus *gria4a*^{+/uva43} M-glut $p = 0.0018$, *gria4a*^{+/+} M-glut versus *gria4a*^{uva43/uva43} M-glut $p < 0.0001$. Migration distance across all groups $p < 0.0001$, *gria4a*^{+/+} DMSO versus *gria4a*^{+/+} M-glut $p = 0.0094$, *gria4a*^{+/+} M-glut versus *gria4a*^{+/uva43} M-glut $p = 0.0012$, *gria4a*^{+/+} M-glut versus *gria4a*^{uva43/uva43} M-glut $p < 0.0001$. ns = no significant differences between groups. Scale bars: 50 μm .

investigate this, we created genetic mosaics (Gansner et al., 2017). To do so, we transplanted blastula cells from dextran-injected *gria4a*^{uva43/uva43}; *Tg(mbp:egfp-CAAX)* embryos into *gria4a*^{+/+} embryos of the same stage, then used *in vivo*, time-lapse imaging to track the migration and dorsal/ventral location of transplanted cells from 56 to 72 hpf (Fig. 6A). At 56 hpf, 66.7% of transplanted *gria4a*^{+/+} *mbp*⁺/dextran⁺ cells were in the dorsal spinal cord, whereas only 37% of transplanted *gria4a*^{uva43/uva43} *mbp*⁺/dextran⁺ cells were located dorsally ($p = 0.0417$; Fig. 7B). At 72 hpf, this difference was even more pronounced, and we observed 83.3% of the transplanted *gria4a*^{+/+} *mbp*⁺/dextran⁺ cells in the dorsal spinal cord, compared with 28% dorsally-located, transplanted *gria4a*^{uva43/uva43} *mbp*⁺/dextran⁺ cells ($p = 0.0003$; Fig. 6C).

To further characterize the cell-autonomy of *gria4a*^{uva43/uva43} in OLCs, we analyzed the migration dynamics of the transplanted cells and found that *gria4a*^{uva43/uva43} cells transplanted into *gria4a*^{+/+} host embryos migrated at a slower speed

($p < 0.0001$; Fig. 6D) and for a shorter total distance ($p < 0.0001$; Fig. 6E) from 56 to 72 hpf, as compared with transplanted *gria4a*^{+/+} cells, similar to what we observed in *gria4a*^{uva43/uva43} larvae. Interestingly, we observed the same trend with *gria4a*^{+/uva43} OLCs with respect to a slower migration speed, although it was not significantly different from wild-type OLCs ($p = 0.0783$; Fig. 6D). However, *gria4a*^{+/uva43} OLCs did migrate significantly less distance than OLCs in *gria4a*^{+/+} larvae ($p = 0.0011$; Fig. 6E), which we hypothesize is evidence of an intermediate phenotype in *gria4a*^{+/uva43} OLCs. Combined, these results indicate that the spinal cord OPC migration and regional distribution phenotypes we observed in *gria4a*^{uva43/uva43} embryos and larvae are cell-autonomous, and mutation of *gria4a* solely in OPCs is sufficient to disrupt their migration.

To determine whether there were any non-cell-autonomous effects of *gria4a* on OLC migration, we transplanted blastula cells from dextran-injected *gria4a*^{+/+}; *Tg(mbp:egfp-CAAX)* embryos

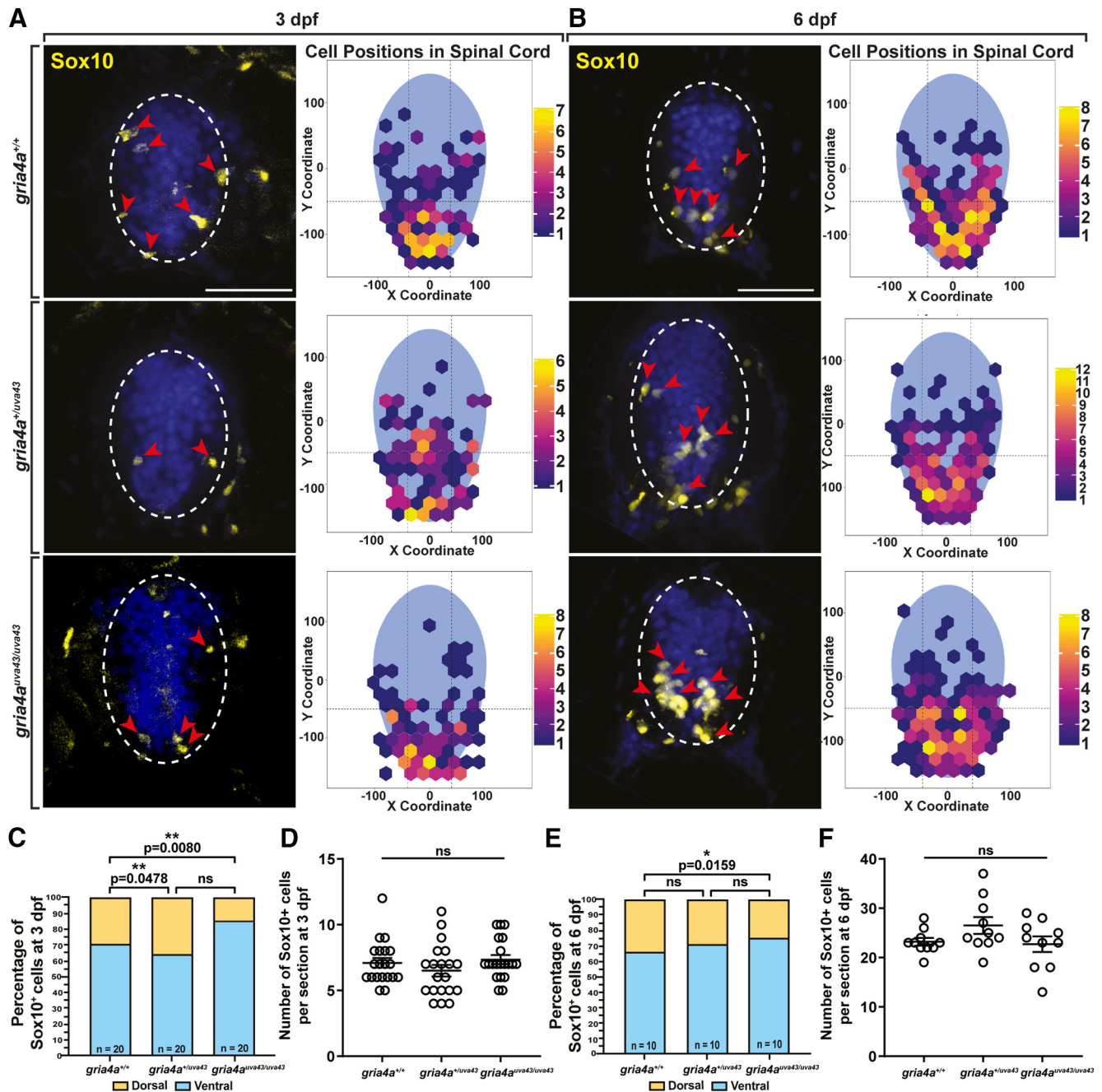


Figure 5. *gria4a* mutants have altered OLC distribution, but not total number. **A, B**, Heatmaps and representative images of Sox10 antibody labeling and DAPI staining in 3- and 6-dpf spinal cord sections from *gria4a*^{+/+} (3 dpf *n* = 20, 6 dpf *n* = 10), *gria4a*^{+/uva43} (3 dpf *n* = 21, 6 dpf *n* = 10), and *gria4a*^{uva43/uva43} (3 dpf *n* = 20, 6 dpf *n* = 10) larvae. Red arrowheads denote Sox10⁺ OLCs. Dashed circle delineates the edge of the spinal cord. In accompanying panels for each genotype and age, we plot the data as a heatmap, with warmer colors representing higher numbers of Sox10⁺ cells. Blue ovals represent an average of the size of all transverse spinal cord sections analyzed, and each hexagon represents a Sox10⁺ cell counted. Caution was taken to only count cells within the spinal cord boundary for each section, and points on the heatmap that fall outside of the average blue oval spinal cord were in fact, located in the spinal cord in the actual section they were quantified. **C, E**, Quantification of Sox10⁺ cells in the ventral and dorsal spinal cord at 3 dpf (*gria4a*^{+/+} vs *gria4a*^{uva43/uva43} *p* = 0.0080; *gria4a*^{+/+} vs *gria4a*^{+/uva43} *p* = 0.0478) and 6 dpf (*gria4a*^{+/+} vs *gria4a*^{uva43/uva43} *p* = 0.0159). **D, F**, Quantification of the number of Sox10⁺ cells per section in each genotype at 3 and 6 dpf. ns = no significant differences between groups. Scale bars: 50 μ m.

into *gria4a*^{uva43/uva43};Tg(*mbp:egfp-CAAX*) host embryos at the same stage and used *in vivo*, time-lapse imaging to analyze the migration dynamics of transplanted OLCs between 56 and 72 hpf. As a control, we also transplanted cells from dextran-injected *gria4a*^{uva43/uva43};Tg(*mbp:egfp-CAAX*) embryos into *gria4a*^{uva43/uva43};Tg(*mbp:egfp-CAAX*) host embryos to control for transplantation effects. At 56 hpf, 66.7% of the transplanted *gria4a*^{+/+} *mbp*⁺/dextran⁺ cells were located in the ventral spinal cord, and the remaining 33.3% were in the dorsal spinal cord,

while all of the transplanted *gria4a*^{uva43/uva43} *mbp*⁺/dextran⁺ cells were in the ventral spinal cord (*p* = 0.0191; Fig. 6F). At 72 hpf, 53.3% of the transplanted *gria4a*^{+/+} *mbp*⁺/dextran⁺ cells were located in the ventral spinal cord, and the other 46.7% were located in the dorsal spinal cord, while 94.1% of the transplanted *gria4a*^{uva43/uva43} *mbp*⁺/dextran⁺ cells were located ventrally and only 5.9% were located dorsally (*p* = 0.0133; Fig. 6G). The greater number of transplanted *gria4a*^{+/+} OLCs in the dorsal spinal cord compared with the transplanted *gria4a*^{uva43/uva43} OLCs is

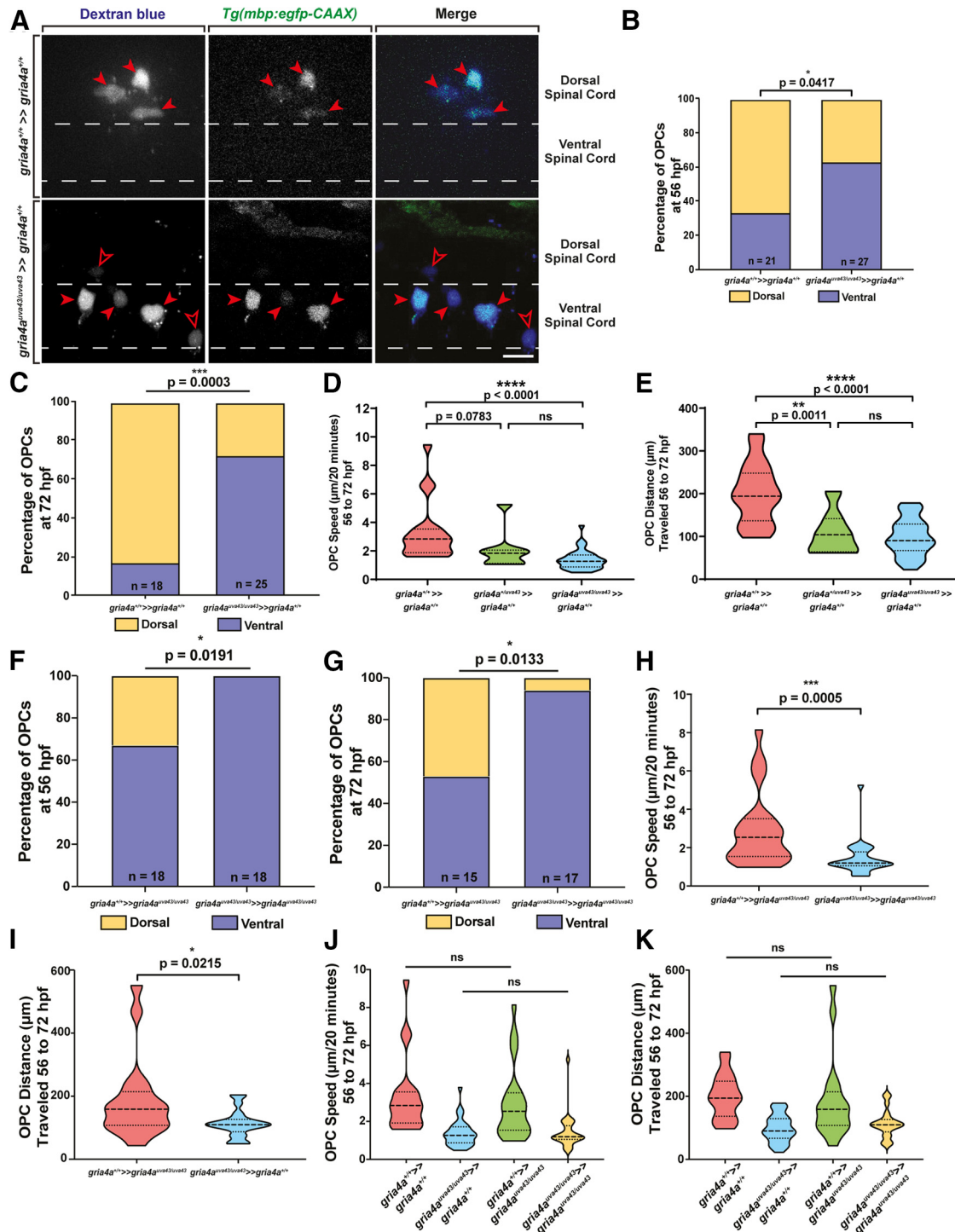


Figure 6. Mutation of *glia4a* alters OLC migration in a cell-autonomous manner. **A**, Genetic mosaic embryos with *Tg(mbp:egfp-CAAX);glia4a*^{+/+} and *glia4a*^{Δ3/Δ3} cells transplanted into *glia4a*^{+/+} embryos, with *mbp*⁺/dextran⁺ OPCs marked with red arrowheads and *mbp*/dextran⁺ cells marked with open red arrowheads. Dashed lines denote the boundary of the ventral spinal cord. **B**, **C**, Quantification of the number of transplanted OLCs in the dorsal versus ventral spinal cord for *glia4a*^{+/+} (n = 21 at 56 hpf and 18 at 72 hpf) and *glia4a*^{Δ3/Δ3} (n = 27 at 56 hpf and 25 at 72 hpf) OPCs transplanted into *glia4a*^{+/+} embryos; p = 0.0417 at 56 hpf, p = 0.0003 at 72 hpf. **D**, **E**, Migration speed (*glia4a*^{+/+} vs *glia4a*^{Δ3/Δ3}, p < 0.0001; *glia4a*^{+/+} vs *glia4a*^{Δ3/Δ3}, p = 0.0783) and distance traveled (*glia4a*^{+/+} vs *glia4a*^{Δ3/Δ3}, p < 0.0001; *glia4a*^{+/+} vs *glia4a*^{Δ3/Δ3}, p = 0.0011) of OLCs transplanted into *glia4a*^{+/+} embryos between 56 and 72 hpf. **F**, **G**, Percentage of transplanted OLCs in the dorsal versus ventral spinal cord when *glia4a*^{+/+} (n = 18) and *glia4a*^{Δ3/Δ3} (n = 18) OLCs were transplanted into *glia4a*^{Δ3/Δ3} embryos at 56 (p = 0.0191) and 72 hpf (p = 0.0133). **H**, Migration speed of OLCs transplanted into *glia4a*^{Δ3/Δ3} embryos between 56 and 72 hpf; p = 0.0005. **I**, Migration distance of OLCs transplanted into *glia4a*^{Δ3/Δ3} embryos between 56 and 72 hpf; p = 0.0215. **J**, Comparison of migration speed of *glia4a*^{+/+} and *glia4a*^{Δ3/Δ3} OLCs transplanted into *glia4a*^{+/+} and *glia4a*^{Δ3/Δ3} embryos between 56 and 72 hpf; p = 0.4745 for *glia4a*^{+/+} into *glia4a*^{+/+} vs *glia4a*^{Δ3/Δ3} into *glia4a*^{+/+}, p = 0.7859 for *glia4a*^{Δ3/Δ3} into *glia4a*^{Δ3/Δ3} vs *glia4a*^{Δ3/Δ3} into *glia4a*^{+/+}. **K**, Comparison of migration distance of *glia4a*^{+/+} and *glia4a*^{Δ3/Δ3} OLCs transplanted into *glia4a*^{+/+} and *glia4a*^{Δ3/Δ3} embryos between 56 and 72 hpf; p = 0.6566 for *glia4a*^{+/+} into *glia4a*^{Δ3/Δ3} vs *glia4a*^{+/+} into *glia4a*^{+/+}, p = 0.2511 for *glia4a*^{Δ3/Δ3} into *glia4a*^{Δ3/Δ3} vs *glia4a*^{Δ3/Δ3} into *glia4a*^{+/+}. ns = no significant differences between groups. Scale bar: 50 μm.

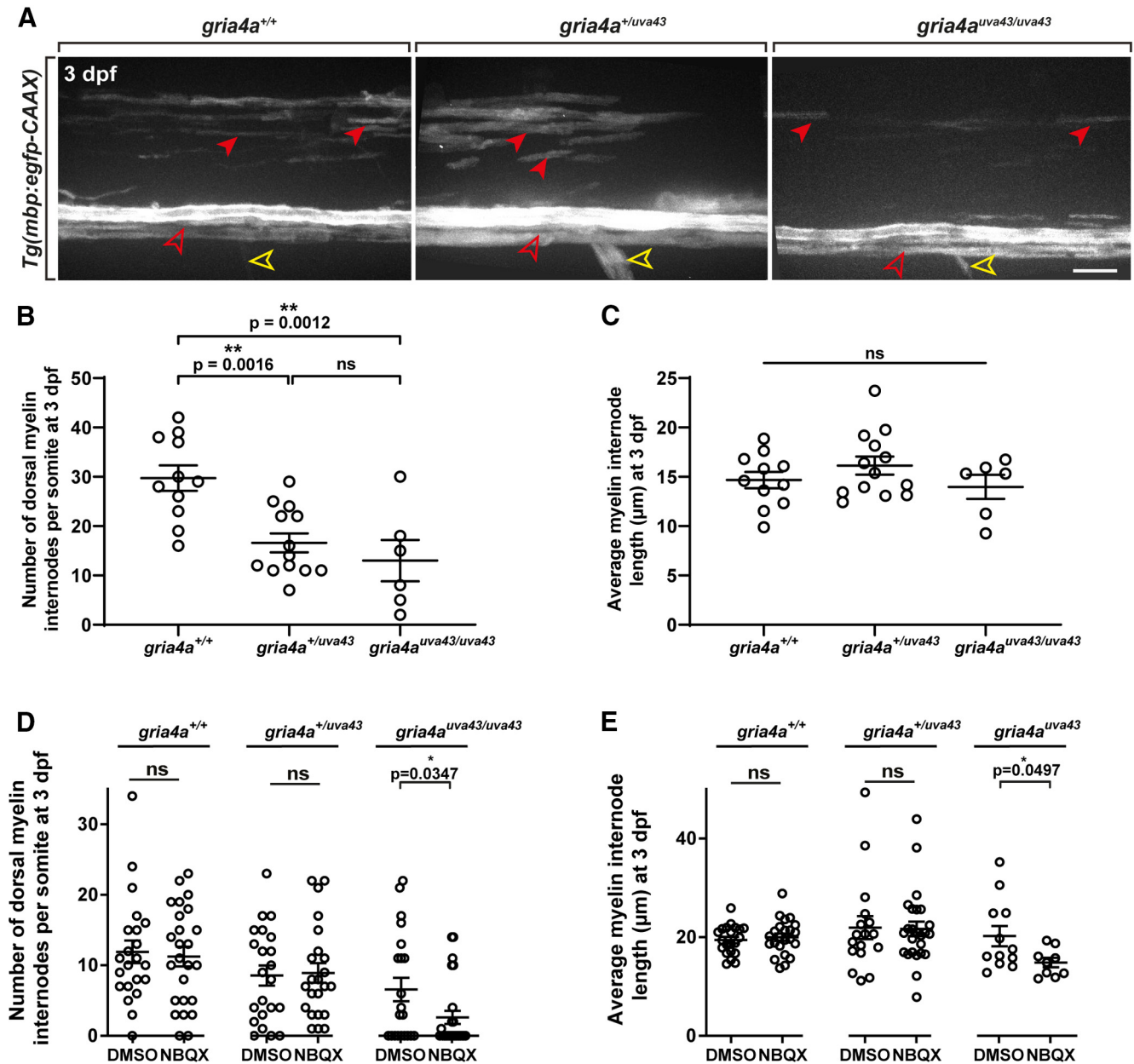


Figure 7. Dorsal spinal cord myelination is reduced in *gria4a*^{uva43/uva43} larvae at 3 dpf. **A**, Myelin expression marked by *Tg(mbp:egfp-CAAX)* in *gria4a*^{+/+} ($n = 11$), *gria4a*^{+/uva43} ($n = 12$), and *gria4a*^{uva43/uva43} ($n = 6$) larvae at 3 dpf, with myelin internodes labeled by red arrowheads. Yellow arrowheads denote normal peripheral myelin along spinal motor nerve roots. **B**, Mean $\pm$ SEM of the number of dorsal myelin internodes per somite at 3 dpf in each genotype, measured in z-stack; $p = 0.0003$ across all groups (*gria4a*^{+/+} vs *gria4a*^{uva43/uva43} $p = 0.0012$; *gria4a*^{+/+} vs *gria4a*^{+/uva43} $p = 0.0016$). **C**, Quantification of the average myelin internode length for each genotype at 3 dpf. **D**, **E**, Mean $\pm$ SEM of the number (**D**) and average length (**E**) of dorsal myelin internodes per somite at 3 dpf in *Tg(mbp:egfp-CAAX)* larvae treated with 40 μ M NBQX in 1% DMSO or 1% DMSO alone from 55 to 80 hpf. *gria4a*^{+/+} $n = 22$ and 25, *gria4a*^{+/uva43} $n = 22$ and 23 and *gria4a*^{uva43/uva43} $n = 21$ and 26, for DMSO or NBQX-treated larvae, respectively. *gria4a*^{uva43/uva43} number of internodes $p = 0.0347$, average internode length $p = 0.0497$. ns = no significant differences between groups. Scale bar: 25 μ m.

consistent with the migration effects we observed both with *gria4a*^{uva43/uva43} OLCs transplanted into *gria4a*^{+/+} embryos and global *gria4a*^{uva43/uva43} embryos, which provides evidence for this altered distribution pattern as a cell-autonomous phenomenon. However, we did observe a greater proportion of dorsal OLCs when cells were transplanted into *gria4a*^{+/+} embryos compared with cells transplanted into *gria4a*^{uva43/uva43} embryos, which indicates that there may be a minor non-cell-autonomous effect also influencing the distribution of OLCs in the developing spinal cord.

To further explore this non-cell-autonomous effect of loss of GluR4 on OLC migration, we analyzed the migration dynamics

of OLCs transplanted into *gria4a*^{uva43/uva43} mutant host embryos from 56 to 72 hpf using the Manual Tracking plugin in ImageJ. Wild-type OLCs transplanted from *gria4a*^{+/+} embryos into mutant hosts migrated faster ($p = 0.0005$; Fig. 6H) and for a greater distance ($p = 0.0215$; Fig. 6I) than mutant OLCs transplanted from *gria4a*^{uva43/uva43} embryos into mutant host embryos between 56 and 72 hpf (Fig. 6H,I). These differences in migration dynamics were similar to those observed in both the global *gria4a*^{uva43/uva43} migration analysis and the transplant into wild-type hosts. To determine the contributions of cell-autonomous versus non-cell-autonomous effects on these migration dynamics, we compared the results from OLCs transplanted into either

wild-type or mutant host embryos. The migration speed of *gria4a*^{uva43/uva43} OLCs transplanted into *gria4a*^{uva43/uva43} embryos was similar to that of *gria4a*^{uva43/uva43} OLCs transplanted into *gria4a*^{+/+} embryos ($p=0.7859$; Fig. 6J), and the speed of *gria4a*^{+/+} OLCs transplanted into *gria4a*^{uva43/uva43} embryos was similar to that of *gria4a*^{+/+} OLCs transplanted into *gria4a*^{+/+} embryos ($p=0.4745$; Fig. 6J). Furthermore, the distance traveled by *gria4a*^{uva43/uva43} OLCs transplanted into *gria4a*^{uva43/uva43} embryos was similar to that of *gria4a*^{uva43/uva43} OLCs transplanted into *gria4a*^{+/+} embryos ($p=0.2511$; Fig. 6K), and the distance migrated by *gria4a*^{+/+} OLCs transplanted into *gria4a*^{uva43/uva43} embryos was similar to that of *gria4a*^{+/+} OLCs transplanted into *gria4a*^{+/+} embryos ($p=0.6566$; Fig. 6K). Together, these results show that *gria4a* cell-autonomously drives OLC migration speed and distance, with some possible minor contributions of non-cell-autonomous effects to the dorsoventral distribution of OLCs.

Mutation of *gria4a* reduces myelination in the dorsal spinal cord

Several recent studies demonstrate that oligodendrocyte myelination of CNS axons can be activity dependent (Hines et al., 2015; Koudelka et al., 2016; de Faria et al., 2019; Noori et al., 2020). Therefore, we hypothesized that the combination of reduced dorsal OPC migration and reduced sensitivity of OLCs to neuronal activity because of mutation of *gria4a* would lead to a reduction in dorsal spinal cord myelination in mutant larvae. To investigate this hypothesis, we used the *Tg(mbp:egfp-CAAX)* transgenic line to perform *in vivo* imaging at 3 dpf when myelination is occurring in the developing spinal cord. From the z-stacks of these images, we quantified the number of myelin internodes in the dorsal spinal cord and measured the internode length in both mutant and wild-type larvae (Fig. 7A–C). From these studies, we observed fewer myelin internodes in both *gria4a*^{+/uva43} and *gria4a*^{uva43/uva43} larvae compared with *gria4a*^{+/+} larvae at 3 dpf ($p=0.0016$ *gria4a*^{+/+} vs *gria4a*^{+/uva43}; $p=0.0012$ *gria4a*^{+/+} vs *gria4a*^{uva43/uva43}; Fig. 7A,B), although the average internode length was similar across genotypes ($p=0.3008$; Fig. 7A,C). Notably, the slightly larger number of dorsal OPCs in heterozygous larvae at 3 dpf (Fig. 5A,C) may indicate some compensation in the heterozygous larvae for the decrease in myelination we observed.

Because we observed an interesting migration result when treating *gria4a* mutant larvae with NBQX, we hypothesized that it may also affect myelination in mutant larvae. To investigate this, we treated *Tg(olig2:dsred);Tg(nkx2.2a:megef)* embryos from 55 to 80 hpf, which is after most OLCs have migrated out of the ventral spinal cord, with either 40 μ M NBQX dissolved in 1% DMSO or with 1% DMSO as a control. We then performed *in vivo* imaging and compared dorsal myelination between the different genotypes. Blocking AMPAR signaling with NBQX from 55 to 80 hpf did not significantly change the number of myelin internodes per somite, nor affected the average length per internode, in wild-type and heterozygous larvae (Fig. 7D,E). However, treatment with NBQX decreased the internode length as well as number of dorsal myelin internodes in *gria4a* mutant larvae (number of internodes $p=0.0347$, internode length $p=0.0497$; Fig. 7D,E). Therefore, while NBQX blocks OPC migration in wild-type larvae (Fig. 3), it did not significantly alter myelination. On the other hand, NBQX increased OPC migration in *gria4a* mutant larvae (Fig. 3), while it significantly decreased myelination.

We also imaged myelin at 5 dpf in the *Tg(mbp:egfp-CAAX)* transgenic line, to assess whether the decreased myelination was

caused by a delay in development. Because of increased myelination at this stage, our measurements of myelin internode number and length were conducted in a single z-plane for all groups. At 5 dpf, the number of myelin internodes remained significantly reduced in *gria4a*^{uva43/uva43} larvae compared with *gria4a*^{+/+} larvae ($p<0.0001$ *gria4a*^{uva43/uva43}; Fig. 8A,B), while the number of internodes in *gria4a*^{+/uva43} was now significantly larger than in *gria4a*^{uva43/uva43} larvae ($p=0.0027$; Fig. 8A,B), although still not completely at the same level as observed in *gria4a*^{+/+} larvae. The average internode length was not significantly different between the groups ($p=0.8968$; Fig. 8C). Therefore, we conclude that both the decrease in dorsal OPC migration and myelination in *gria4a*^{uva43/uva43} larvae were not caused by a developmental delay. This is in line with our observation that mutant larvae appear healthy and indistinguishable from wild-type and heterozygous larvae with regard to body length and eye size both at 2 and 5 dpf (Fig. 1F). Interestingly, at both 3 and 5 dpf, the *gria4a*^{+/uva43} myelin phenotypes were either intermediate between the *gria4a*^{+/+} and *gria4a*^{uva43/uva43} phenotypes, or comparable to the *gria4a*^{uva43/uva43} phenotype. However, the intermediate myelin phenotype in *gria4a*^{+/uva43} larvae was independent of a significant change in dorsal OPC distribution compared with *gria4a*^{+/+} larvae. This leads us to hypothesize that GluR4A may affect migration and myelination via distinct mechanisms, which we will address in more detail in the discussion.

In the previous set of studies, we used the stable *Tg(mbp:egfp-CAAX)* line, which makes it difficult to distinguish internodes from individual oligodendrocytes. Therefore, to assess myelin on an individual cell basis, we injected the *mbp:egfp-CAAX* construct into *gria4a*^{+/+}, *gria4a*^{+/uva43}, and *gria4a*^{uva43/uva43} larvae and imaged individual oligodendrocytes at 5 dpf. We observed that although the average length per internode of dorsal oligodendrocytes was comparable between the different genotypes (Fig. 8D,E), *gria4a*^{uva43/uva43} oligodendrocytes made more internodes per cell compared with *gria4a*^{+/+} oligodendrocytes ($p=0.0188$; Fig. 8D,F). Interestingly, there was no difference in the number of internodes or average internode length per cell in ventral oligodendrocytes across the different genotypes (Fig. 8D,G,H).

Glutamate signaling via AMPARs regulates OPC migration and myelination by modulating voltage-gated calcium channels

Several proposed mechanisms for AMPAR-mediated glutamate signaling as a regulator of OPC development involve downstream Ca^{2+} influx via voltage-gated calcium channels (Gudz et al., 2006; Paez et al., 2010; Harlow et al., 2015). To determine whether voltage-gated calcium channel activation plays a role in directing OPC migration, we treated *gria4a* mutant, heterozygous, and wild-type *Tg(olig2:dsred);Tg(mbp:egfp-CAAX)* embryos with either 5 μ M the L-type voltage-gated calcium channel agonist ($\pm$)-Bay K 8644 in 1% DMSO or 1% DMSO alone from 55 to 72 hpf and performed *in vivo*, time-lapse imaging. Both wild-type and mutant larvae treated with ($\pm$)-Bay K 8644 looked healthy and indistinguishable from DMSO-treated larvae (Fig. 9A). At 56 hpf, we observed a similar number of dorsal OPCs across all genotypes and conditions ($p=0.4721$; Fig. 9B). In contrast, at 72 hpf, we observed fewer dorsal OPCs in *gria4a*^{uva43/uva43} embryos treated with DMSO compared with *gria4a*^{+/+} embryos treated with DMSO ($p=0.0008$; Fig. 9C). However, in *gria4a*^{uva43/uva43} embryos treated with ($\pm$)-Bay K 8644, there were more dorsal OPCs at 72 hpf compared with the *gria4a*^{uva43/uva43} DMSO control

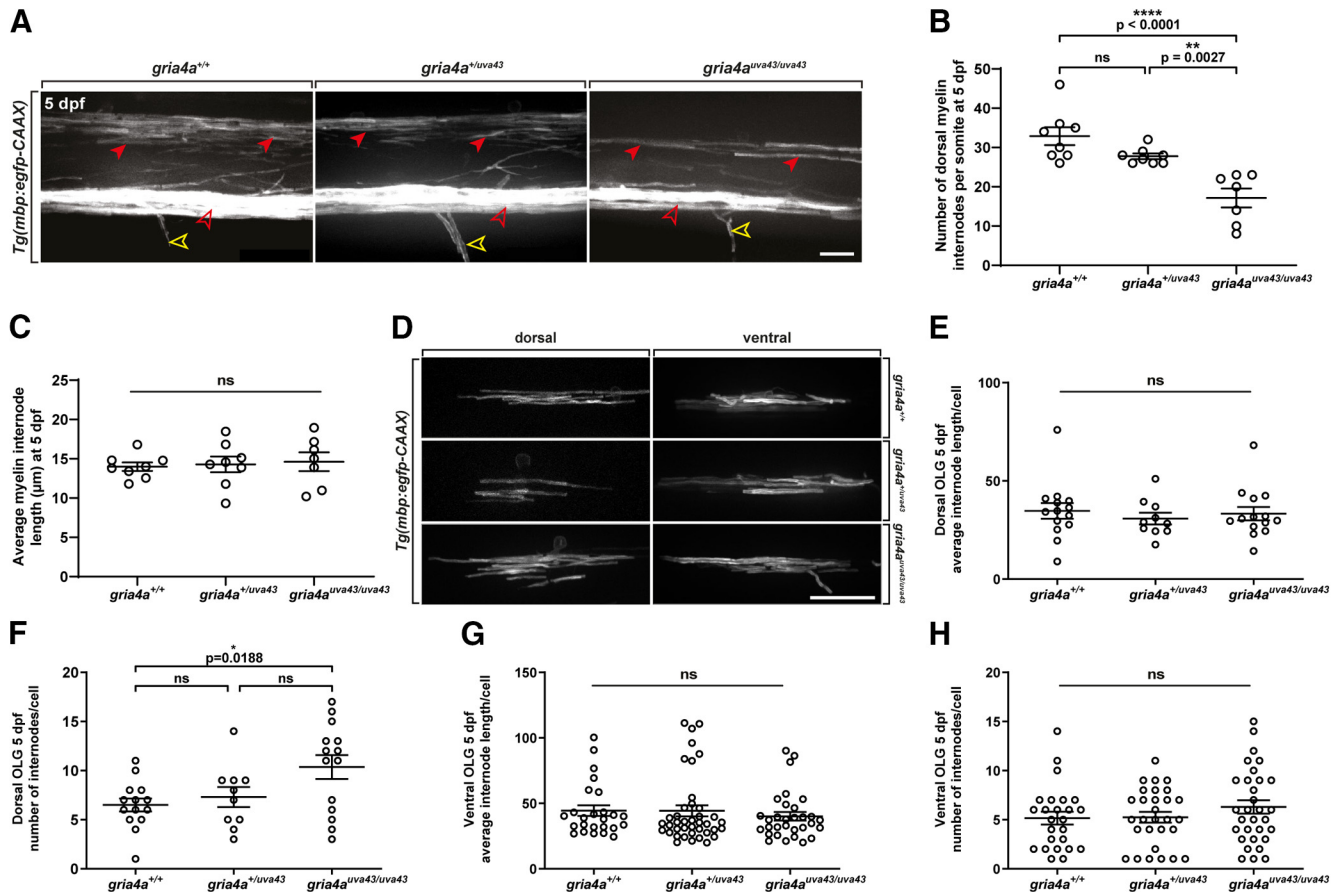


Figure 8. *gria4*^{uva43/uva43} larvae have less dorsal myelin at 5 dpf, but more internodes per cell. **A**, Myelin expression marked by *Tg(mbp:egfp-CAAX)* in *gria4*^{+/+} ($n = 8$), *gria4*^{+/uva43} ($n = 8$), and *gria4*^{uva43/uva43} ($n = 7$) larvae at 5 dpf, with myelin internodes labeled by red arrowheads. Yellow arrowheads denote normal peripheral myelin along spinal motor nerve roots. **B**, Mean $\pm$ SEM of the number of myelin internodes per somite at 5 dpf in each genotype in a single z-plane with the most myelin internodes visible (*gria4*^{+/+} vs *gria4*^{uva43/uva43} $p < 0.0001$; *gria4*^{+/uva43} vs *gria4*^{uva43/uva43} $p = 0.0027$). **C**, Mean $\pm$ SEM of the average myelin internode length for each genotype at 5 dpf. **D**, Representative images of myelin expression by individual oligodendrocytes marked by transient injections of the *mbp:egfp-CAAX* construct in *gria4*^{+/+} (ventral $n = 25$ cells in $n = 8$ larvae; dorsal $n = 14$ cells in $n = 6$ larvae), *gria4*^{+/uva43} (ventral $n = 31$ cells in $n = 13$ larvae; dorsal $n = 10$ cells in $n = 9$ larvae), and *gria4*^{uva43/uva43} (ventral $n = 30$ cells in $n = 9$ larvae; dorsal $n = 14$ cells in $n = 4$ larvae) at 5 dpf. **E**, **F**, Mean $\pm$ SEM of the number (**E**) and average length (**F**; *gria4*^{+/+} vs *gria4*^{uva43/uva43} $p = 0.0188$) of ventral myelin internodes per somite at 5 dpf in each genotype. **G**, **H**, Mean $\pm$ SEM of the number (**G**) and average length (**H**) of dorsal myelin internodes per somite at 5 dpf in each genotype. ns = no significant differences between groups. Scale bars: 25 μ m (**A**) and 50 μ m (**D**).

group ($p < 0.0001$; Fig. 9C), and there was a similar number of dorsal OPCs in both the *gria4*^{+/+} ($\pm$)-Bay K 8644-treated group ($p = 0.5983$) and the *gria4*^{+/+} DMSO control group ($p = 0.9578$). These results indicate Ca^{2+} signaling induces OPC migration downstream of AMPAR activation (Fig. 9D), and that stimulation of voltage-gated calcium channels with ($\pm$)-Bay K 8644 effectively rescues the OPC migration phenotype in *gria4*^{uva43/uva43} embryos. Notably, OPC migration in wild-type larvae did not increase with ($\pm$)-Bay K 8644 treatment, whereas migration of OPCs in *gria4*^{uva43/uva43} larvae did. We hypothesize this is because wild-type OPCs have already reached a maximum threshold of Ca^{2+} influx that drives OPC migration under physiological conditions.

Similar to its effect on OPC migration, we hypothesized that voltage-gated calcium channel activation with ($\pm$)-Bay K 8644 would rescue the myelin phenotype in *gria4* mutant larvae. To assess this, we treated *gria4* wild-type, heterozygous, and mutant *Tg(mbp:egfp-CAAX)* embryos with either 5 μ M ($\pm$)-Bay K 8644 in 1% DMSO or 1% DMSO alone from 55 to 72 hpf or from 55 to 80 hpf, and performed *in vivo* imaging of myelin at 80 hpf. In *gria4*^{+/+} larvae, treatment with ($\pm$)-Bay K 8644 did not alter the number or the length of myelin internodes (Fig. 10A–C). In *gria4*^{+/uva43} larvae, ($\pm$)-Bay K 8644 also did not

significantly change the number of myelin internodes (Fig. 10A, D). However, the average internode length was slightly decreased on a 24-h treatment ($p = 0.0371$; Fig. 10A, E). Interestingly, in *gria4*^{uva43/uva43} larvae treated for 24 h, but not 16 h, with ($\pm$)-Bay K 8644, we observed a significant increase in the number of dorsal myelin internodes ($p = 0.0192$; Fig. 10A, F), while the internode length was not altered (Fig. 10A, G). Therefore, while OPC migration is restored after 16 h of voltage-gated calcium channel activation with ($\pm$)-Bay K 8644 (Fig. 9B), voltage-gated calcium channel activation is also required for efficient myelination, as only a 24-h treatment with ($\pm$)-Bay K 8644 rescued the myelin phenotype in *gria4* mutant larvae.

Discussion

During development, spinal cord OPCs must migrate extensively from their origin in the ventral spinal cord to distribute evenly throughout the CNS and myelinate axons. Several studies have examined how OPC AMPAR activation may stimulate this migration (Gudz et al., 2006; Paez et al., 2010), but *in vivo* experiments have yielded conflicting results (Harlow et al., 2015; Kougioumtzidou et al., 2017). One study demonstrated *in vitro* that in wild-type mouse

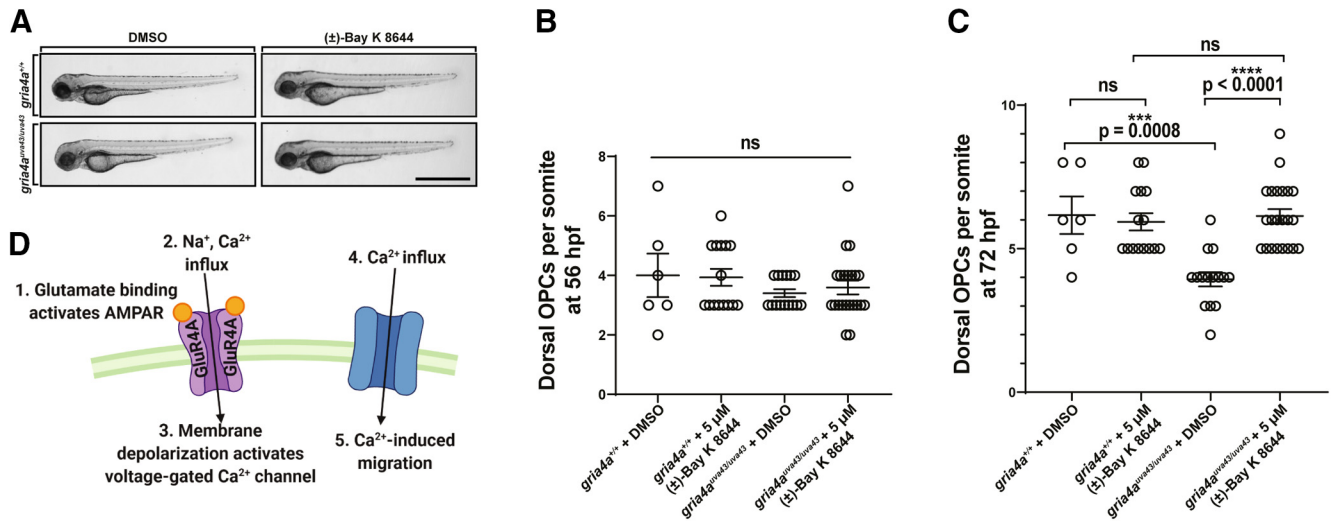


Figure 9. Activation of voltage-gated Ca^{2+} channels restore normal OPC migration in *gria4a*^{Δ43/Δ43} larvae. **A**, Brightfield images of 80-hpf *gria4a* wild-type and mutant larvae after treatment with 5 μM voltage-gated calcium channel agonist (±)-Bay K 8644. **B**, Mean ± SEM of the number of dorsal OPCs per somite in *gria4a*^{+/+} and *gria4a*^{Δ43/Δ43} embryos at 56 hpf following treatment with either 5 μM (±)-Bay K 8644 (*gria4a*^{+/+} *n* = 15, *gria4a*^{Δ43/Δ43} *n* = 22) in 1% DMSO or 1% DMSO control (*gria4a*^{+/+} *n* = 6, *gria4a*^{Δ43/Δ43} *n* = 15); *p* = 0.4721. **C**, Mean ± SEM of the number of dorsal OPCs per somite in *gria4a*^{+/+} and *gria4a*^{Δ43/Δ43} larvae at 72 hpf following treatment with either 5 μM (±)-Bay K 8644 in 1% DMSO or 1% DMSO control. *p* < 0.0001 across all groups, *p* = 0.5983 for *gria4a*^{+/+} + (±)-Bay K 8644 versus *gria4a*^{+/+} + DMSO, *p* = 0.0008 for *gria4a*^{Δ43/Δ43} + (±)-Bay K 8644 versus *gria4a*^{Δ43/Δ43} + DMSO, and *p* < 0.0001 for *gria4a*^{Δ43/Δ43} + (±)-Bay K 8644 versus *gria4a*^{+/+} + (±)-Bay K 8644. **D**, Model of mechanism through which GluR4 works with voltage-gated calcium channels to regulate OPC migration. ns = no significant differences between groups. Scale bar: 1 mm.

OPCs, AMPA accelerates OPC migration. Notably, AMPA treatment resulted in internalization of the calcium-impermeable GluR2 subunit and increased Ca^{2+} influx. However, in *ex vivo* cerebellar slices from GluR2-null mouse, OPCs did not accelerate in response to AMPAR stimulation and resulted in reduced baseline Ca^{2+} influx in OPCs, possibly because of reduced neuronal activity (Harlow et al., 2015). The authors describe this effect on migration as the result of complex formation between PLP, αv-integrin, and the GluR2 subunit, which does not occur in the absence of GluR2 (Harlow et al., 2015). Thus, GluR2 was required for complex formation and initiation of migration followed by internalization of GluR2 and an increase in Ca^{2+} influx. Although not discussed in detail by the authors, GluR4 was also bound by to PLP complex. Our results reveal similar effects on OPC migration following GluR4A disruption, suggesting that these effects are not specific to GluR2 and that they may both act via AMPAR-mediated Ca^{2+} influx, not solely through PLP-integrin complex formation. We conclude from our studies that GluR4A is an important AMPAR subunit for OPC migration and provide evidence for neuron-OPC AMPAR signaling that directly affects OPC migration before myelination *in vivo*.

AMPA subunit composition modulates calcium signaling in OPCs

Several studies have examined the activation of voltage-gated calcium channels as downstream mediators of AMPAR-mediated membrane depolarization in OPCs as a possible driver of developmental processes including membrane extension and myelination (Yuan et al., 1998; Butt, 2006; Gallo and Armstrong, 2008; Giesen et al., 2020). Following voltage-gated calcium channel-mediated Ca^{2+} influx into OPCs, numerous pathways involved in migration are activated, including PKC-dependent process extension, Trk activation, and PKA activation (Paez et al., 2010). In mice, the expression of voltage-gated calcium channels in OLCs varies with developmental stage; OPCs express these channels at a higher level, and have correspondingly higher Ca^{2+} currents than later stages of OLCs (Blankenfeldt et al., 1992).

This developmental variation suggests roles for voltage-gated calcium channel-mediated Ca^{2+} influx at specific points in OPC development, in line with our findings that Ca^{2+} influx stimulates OPC migration.

The types of AMPAR subunits expressed in each cell affect its Ca^{2+} influx. At 72 hpf, OLCs in the zebrafish spinal cord predominantly express two AMPAR subunits: *gria2b* and *gria4a* (Fig. 1A; Hoppmann et al., 2008). A posttranscriptional Q/R modification which makes mammalian GluR2 calcium impermeable is genetically encoded in the *gria2b* gene in zebrafish (Kung et al., 2001; Sobolevsky et al., 2003). Like other AMPAR subunits, *gria4a* is calcium-permeable, and the calcium permeability of each heterotetrameric AMPAR is dependent on its constituent subunits (Burnashev, 1998). In embryos harboring a mutation in *gria4a*, OPCs would likely have increased levels of AMPARs with a higher degree of calcium impermeability because of the relative increase in calcium-impermeable GluR2B subunits. In line with this, the loss of GluR4 in mature mouse oligodendrocytes leads to a decrease of Ca^{2+} influx (Evonuk et al., 2020). While in zebrafish some genetic compensation of other calcium-permeable subunits may occur, it is likely that *gria4a*^{Δ43/Δ43} OPCs would have reduced Ca^{2+} influx following AMPAR stimulation compared with their wild-type counterparts. Notably, both in *gria4a* mutant and heterozygous larvae, we detected increased expression of the calcium impermeable *gria2b* subunit gene. Therefore, *gria4a*^{+/Δ43} OPCs would likely also have reduced Ca^{2+} influx following AMPAR stimulation compared with their wild-type counterparts, although more than in *gria4a*^{Δ43/Δ43} OPCs as they express one wild-type copy of *gria4a*. This is in line with our observation that *gria4a*^{+/Δ43} OPCs exhibit an intermediate phenotype with regard to their migration speed and distance traveled.

GluR4A cell autonomously drives OPC migration through glutamate sensing

In our studies, transplanted *gria4a*^{Δ43/Δ43} OPCs in wild-type larvae exhibited altered migration dynamics similar to those

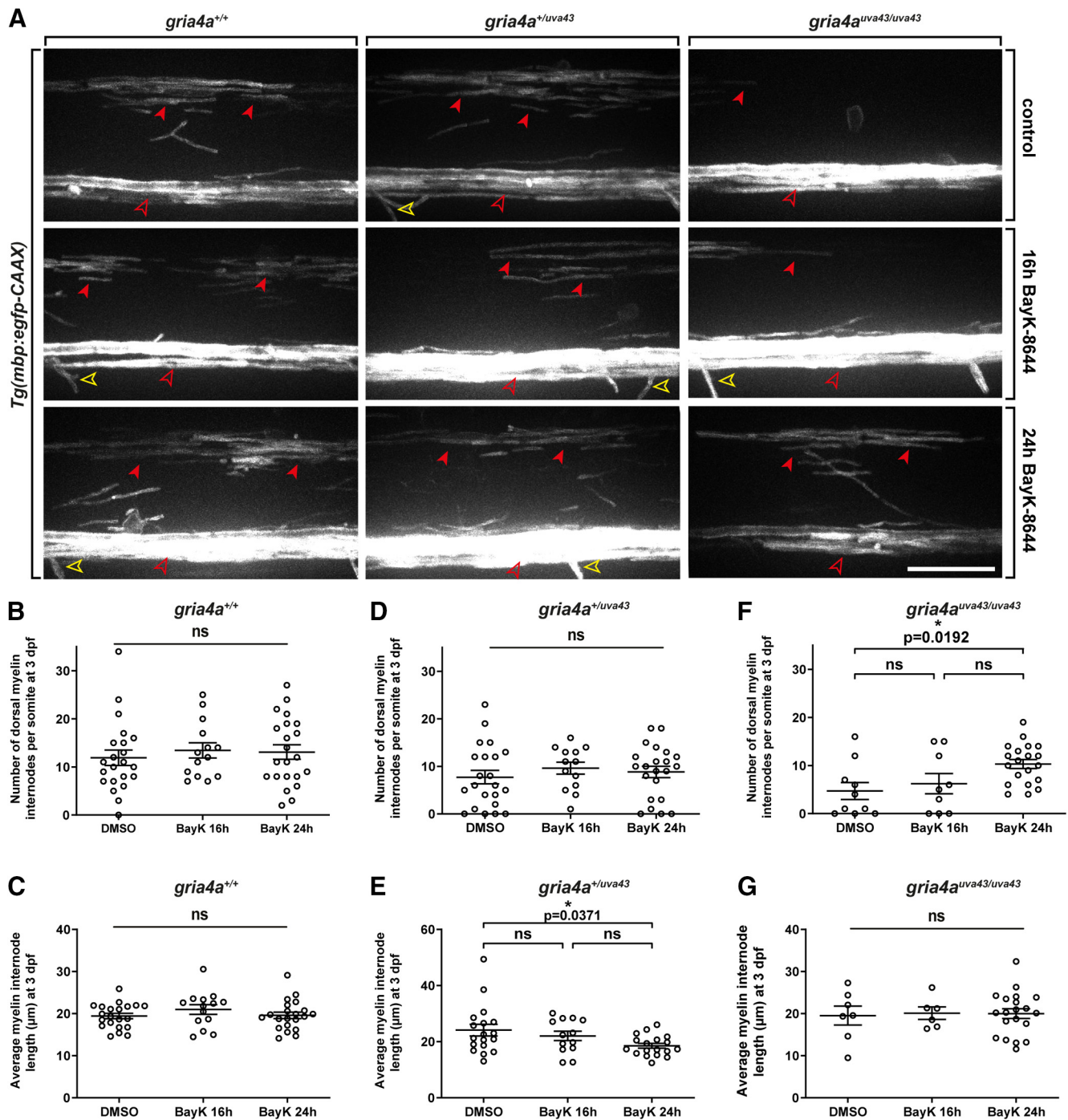


Figure 10. Activation of voltage-gated Ca^{2+} channels restore myelination after in *gria4a*^{u43/u43} larvae. **A**, Representative images of myelin expression marked by *Tg(mbp:egfp-CAAX)* in *gria4a*^{+/+} ($n = 14$ – 22), *gria4a*^{+/u43} ($n = 13$ – 22), and *gria4a*^{u43/u43} ($n = 14$ – 20) larvae at 3 dpf. Myelin internodes are labeled by red arrowheads. Yellow arrowheads denote normal peripheral myelin along spinal motor nerve roots. **B**, **D**, **F**, Mean $\pm$ SEM of the number of dorsal myelin internodes per somite at 3 dpf in *gria4a*^{+/+} (**B**), *gria4a*^{+/u43} (**D**), and *gria4a*^{u43/u43} (**F**). DMSO vs BayK 24 h $p = 0.0192$. **C**, **E**, **G**, Quantification of the average myelin internode length for at 3 dpf in *gria4a*^{+/+} (**C**), *gria4a*^{+/u43} (**E**; DMSO vs BayK 24 h $p = 0.0371$), and *gria4a*^{u43/u43} (**G**). ns = no significant differences between groups. Scale bar: 25 μm .

observed in global *gria4a*^{u43/u43} larvae, indicating that OPC specific perturbation to GluR4A is sufficient to alter OPC migration in a cell-autonomous manner. Interestingly, *gria4a*^{+/u43} OPCs that were transplanted into wild-type larvae also migrated slightly slower than when in global heterozygous larvae (Fig. 6). However, in *gria4a*^{+/u43} larvae, we observed a trend of reduced OPC migration speed, although it was not significant (Fig. 2). Therefore, it is not completely unexpected that we would see an intermediate phenotype when heterozygous OPCs were

transplanted into wild-type hosts and were surrounded by a wild-type environment, which may cause the migration perturbations to heterozygous OPCs to become more obvious. Additionally, we found that wild-type OPCs transplanted into *gria4a*^{u43/u43} embryos demonstrated the presence of mild, non-cell-autonomous effects of the loss of GluR4A on OPC migration, which may be a result of reduced neuronal signaling and subsequent reduced neurotransmitter sensing by OPCs. With the uncaging of the MNI-glutamate, we directly assessed

the ability of OPCs to sense glutamate. With this method, we created a local burst of available glutamate. In control conditions, there appeared to be two migratory *gria4a*^{+/+} OPC populations: a slow-migrating and fast-migrating population. However, after a focal increase in glutamate availability after MNI-glutamate uncaging, we observed a shift in migration speed in these populations, with the previously slow-migrating *gria4a*^{+/+} OPCs increasing their speed to be comparable to the fast-migrating OPCs. Almost all *gria4a*^{+/uva43} and *gria4a*^{uva43/uva43} OPCs, however, were slow migrating and did not significantly increase migration speed, nor distance, in response to glutamate uncaging. We hypothesize that this lack of response to uncaged glutamate is because of the presence of the mutated GluR4A receptor. Therefore, the speed and distance of OPC migration appears to be regulated by glutamate signaling and not the inherent ability of OPCs to migrate.

Regulation of myelination by AMPAR signaling

While myelination can occur in an activity-independent manner (Barres and Raff, 1999; Bechler et al., 2018; de Faria et al., 2019), it can also be highly regulated by neuronal signaling (Barres and Raff, 1993; Hughes et al., 2013; Gautier et al., 2015; Hines et al., 2015; Mensch et al., 2015; de Faria et al., 2019). Activity-dependent myelination can be mediated by multiple mechanisms, including those involving vesicle release (Gautier et al., 2015) and NMDA receptor signaling (Lundgaard et al., 2013), and Ca²⁺ transients are known to stabilize myelin sheath formation and promote sheath elongation (Hines et al., 2015; Baraban et al., 2018; Krasnow et al., 2018). Because numerous interrelated pathways influence myelination, it is not surprising that we observed that *gria4a*^{+/uva43} oligodendrocytes produced less dorsal myelin than wild-type controls, as GluR4A may affect migration and myelination through distinct mechanisms. This also became apparent in our experiments with NBQX.

Previous studies have demonstrated that in rodents, AMPAR signaling regulates OPC differentiation (Gautier et al., 2015; Chen et al., 2018) and remyelination (Gautier et al., 2015). Interestingly, *in vitro* studies demonstrate that before the expression of MBP, there is a change in the AMPAR subunits expressed in OLCs (Itoh et al., 2002). Rat OLCs express GluR4 at an early stage, but in mature oligodendrocytes, the expression of GluR4 is decreased, hence resulting in relatively more calcium impermeable AMPAR containing GluR2.

Based on this, we hypothesize that calcium permeable AMPAR signaling enhances migration and/or blocks differentiation into mature oligodendrocytes, while calcium impermeable AMPAR signaling inhibits migration and/or stimulates differentiation. We were especially fascinated by the results obtained after treatment with NBQX, which has an opposite effect on *gria4a*^{+/+} and *gria4a*^{uva43/uva43} OPC migration and myelination. In *gria4a* mutant larvae, where there are more calcium impermeable AMPARs because of a mutation in *gria4a* and increased expression of *gria2b*, OPC migration, differentiation, and myelination are regulated differently than in wild-type larvae. Because NBQX is a generic AMPAR antagonist, it can inhibit all AMPA subunits. In wild-type larvae, NBQX treatment from 30 to 72 hpf would block GluR4A function, thus blocking OPC migration. However, in our *gria4a*^{uva43/uva43} larvae, NBQX treatment from 30 to 72 hpf would also have a significant block of GluR2B function because of the increased expression levels of this subunit, which could therefore, result in increased migration and also affect myelination. These data demonstrate that fine-tuned, temporal control of expression of distinct AMPAR subunits is

required for both efficient dorsal OPC migration and myelination, and that this balance between calcium-permeable and calcium-impermeable subunits determines when OPCs migrate and differentiate.

Downstream of glutamate sensing, Ca²⁺ influx drives OPC migration and initiation of differentiation, as evidenced by the rescued OPC migration and myelination in *gria4a*^{uva43/uva43} embryos treated with L-type voltage-gated Ca²⁺ channel agonist (±)-Bay K 8644. This is in line with a previous studies that revealed that voltage-operated Ca²⁺ channels affect OPC migration and that voltage-gated Ca²⁺ influx in oligodendroglial cells is critical for normal myelination (Cheli et al., 2016) and Ca²⁺ transients are known to stabilize myelin sheath formation and promote sheath elongation (Hines et al., 2015; Baraban et al., 2018; Krasnow et al., 2018). In another study using primary cultures of mouse OPCs, L-type voltage gated channel signaling increased OPC morphologic differentiation as well as the expression of mature oligodendrocyte markers (Cheli et al., 2015). In our work, we do not observe any increase in the number of myelin internodes or the average internode length in response to (±)-Bay K 8644 in *gria4a*^{+/+} larvae, and the average myelin internode length in *gria4a*^{+/uva43} larvae even slightly decreased. Interestingly, a recent study in zebrafish demonstrated that some OPCs participate extensively in signaling involving Ca²⁺ transients, but preferentially proliferate rather than differentiate into myelin-producing cells (Marisca et al., 2020). However, in our study we did observe a positive effect of (±)-Bay K 8644 on myelination in *gria4a*^{uva43/uva43} larvae. Notably, while OPC migration in *gria4a*^{uva43/uva43} larvae is restored after 16 h of voltage-gated calcium channel activation with (±)-Bay K 8644, there is only a rescue of myelination on a 24-h treatment with (±)-Bay K 8644. Therefore, it seems that Ca²⁺ influx via voltage-gated calcium channels is especially important for initiation of myelination.

AMPA signaling affects multiple processes in OLCs

The effects of inhibiting AMPAR signaling in OLCs are context-specific and diverse. One recent study reported that an induced, mature oligodendrocyte-specific knock-out of *gria4* in mice ameliorated EAE symptoms by reducing oligodendrocyte vulnerability to excitotoxicity (Evonuk et al., 2020). In multiple sclerosis (MS), insufficient migration of OPCs to lesions results in poor remyelination (Boyd et al., 2013). NBQX inhibits the early phases of remyelination *in vivo* in toxin-induced demyelinated lesions in the rat brain, which was attributed to a direct effect on OPCs (Gautier et al., 2015). Inhibition of neuronal activity increased the numbers of OPCs in these demyelinated lesions, while OPC differentiation was reduced (Gautier et al., 2015). Additionally, the loss of GluR4 in rat brain inhibits OPC survival (Kougioumtzidou et al., 2017). Our findings show that GluR4A AMPAR activation drives OPC migration, which raises the question of whether GluR4A-deficient zebrafish would have enhanced remyelination capacity because of reduced vulnerability to excitotoxicity, or would fail to remyelinate MS-like lesions because of a defect in OPC migration, as seen in early myelination of the dorsal spinal cord. The expression of AMPAR subunits in OLCs is tightly regulated; changing one subunit can control migration, proliferation, differentiation, and/or myelination depending on the spatiotemporal environment, which makes OPC AMPAR signaling an exciting area for future research.

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