Molecularly defined cortical astroglia subpopulation modulates neurons via secretion of Norrin

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Despite expanding knowledge regarding the role of astroglia in regulating neuronal function, little is known about regional or functional subgroups of brain astroglia and how they may interact with neurons. We use an astroglia-specific promoter fragment in transgenic mice to identify an anatomically defined subset of adult gray matter astroglia. Using transcriptomic and histological analyses, we generate a combinatorial profile for the in vivo identification and characterization of this astroglia subpopulation. These astroglia are enriched in mouse cortical layer V; express distinct molecular markers, including Norrin and leucine-rich repeat-containing G-protein-coupled receptor 6 (LGR6), with corresponding layer-specific neuronal ligands; are found in the human cortex; and modulate neuronal activity. Astrocytic Norrin appears to regulate dendrites and spines; its loss, as occurring in Norrie disease, contributes to cortical dendritic spine loss. These studies provide evidence that human and rodent astroglia subtypes are regionally and functionally distinct, can regulate local neuronal dendrite and synaptic spine development, and contribute to disease.

Astroglia are the most abundant cell type in the CNS. They have essential roles in the development and homeostasis of the nervous system, maturation and maintenance of synapses, and regulation of neural transmission¹. Disturbances of these essential roles may contribute to various CNS diseases, including amyotrophic lateral sclerosis (ALS)²–⁴. For more than 100 years, astroglia have been broadly defined into two morphologically described subgroups: protoplasmic astroglia, which are localized to gray matter; and fibrous astroglia, which are localized to white matter. However, recent years have witnessed a growing appreciation for potential astroglia diversity beyond simple morphology, with accumulating evidence suggesting the existence of functionally distinct astroglia subpopulations⁵–⁷. Current knowledge surrounding the regional specialization of astroglia populations comes in part from insight provided by the positional identity of astroglia in the developing spinal cord, where defined anatomical locations also define different astroglia subpopulations⁸–¹⁰. Each of these subpopulations displays a unique biological profile that allows the cell to maintain its physiological niche¹¹. In disease, altered function in regional astroglia can contribute to neurotoxic events, such as impaired glutamate uptake by perisynaptic astroglia in the context of neurodegeneration¹². However, very little is known about the molecular identities of different astroglia subgroups in the majority of CNS tissues and how these subgroups might regulate local neuronal function. This limited understanding of astroglia is due in part to the lack of RNA or protein markers to identify different subgroups in the adult CNS, making histological or functional studies of different populations nearly impossible. To begin to develop insight into possible astroglia subgroups, we explored the biology of an astroglia-specific targeted protein, the glutamate transporter excitatory amino acid transporter 2 (EAAT2), known to be focally altered in some disorders¹³,¹⁴.

All astroglia normally express EAAT2, also known as glutamate transporter 1 (GLT1)⁵. Astroglial GLT1 is generally expressed uniformly throughout the CNS, although the levels of GLT1 are approximately tenfold higher in the hippocampus and cortex relative to the spinal cord¹⁵. Dramatic regional dysregulation of GLT1 expression has been observed in a variety of neurological and psychiatric disorders¹⁶–¹⁸. For example, GLT1 is downregulated in a subset of astroglia in the ventral spinal cord and layers of motor cortex in human ALS patients as well as in rodent models of ALS¹⁹. Although the biological basis of this focal dysregulation has

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historically focused on how neurons may focally regulate GLT1 expression\(^1\), in our attempts to define the regulation of GLT1 expression within astroglia specifically, we discovered a cortical layer- specific subpopulation with a unique molecular/protein signature that defines, in part, a functional role in regulating local neuronal dendrite and spine formation. We find that this novel astroglial pathway plays a fundamental role in the childhood neurological disorder, Norrie disease.

**Results**

**Identification and validation of 8.3-astroglia: Glt1 promoter reporter mouse lines.** To examine Glt1 regulation within astroglia, we created multiple mouse lines using DNA inserts that contained fragments of the Glt1 promoter upstream of the Glt1 transcriptional start site (TSS). Downstream of the Glt1 TSS, the insertion of a tdTomato reporter allowed us to visualize cell populations actively transcribing the transgenic constructs. The fragments of the Glt1 promoter ranged from 2.5 kilobases (kb) to 8.3 kb upstream of the transcription start site (Fig. 1a and Supplementary Fig. 1A). These fragment sizes were chosen based on conserved genomic regions with high methylation between the mouse and human Glt1 gene (human nomenclature, EAAT2) (Supplementary Fig. 1A). Multiple founder lines expressing a promoter fragment \(\leq 7.9\) kb showed tdTomato expression that was restricted to neurons (Fig. 1a and Supplementary Fig. 2A-B). Unexpectedly, founder lines with only a slightly larger insert size, 8.3 kb, showed tdTomato fluorescence limited to gray matter astroglia (Fig. 1a–c, Fig. 2a–c, and Supplementary Fig. 3B). Notably, a specific and reproducible subpopulation of gray matter astroglia expressed tdTomato in the cerebral cortex and several other regions unexplored in this study (Fig. 1b–d and Supplementary Fig. 3A; Supplementary Video 1).

To compare this tdTomato-astroglia population (henceforth referred to as ‘8.3-astroglia’) with all other gray matter cortical astroglia, we crossed 8.3-astroglia-labeled mice with a BAC-Glut1-enhanced green fluorescent protein (eGFP) mouse line (Glt1-eGFP), which labels all CNS astroglia with eGFP (Fig. 1a–d and Supplementary Fig. 3B). In the double transgenic mouse line (8.3-astroglia/Glt1-eGFP), all 8.3-astroglia were also eGFP\(^+\), indicating that these cells are indeed astroglia expressing GLT1 (Fig. 2a–c and Supplementary Fig. 3B). In adult cortex gray matter, definable subsets of all eGFP astroglia were also tdTomato\(^+\) (Fig. 1b,c and Supplementary Fig. 3B). Notably, 8.3-astroglia were completely absent from the hippocampus (Fig. 1d; Supplementary Movie 1).

The 8.3-astroglia consisted of approximately 28% of all cortical astroglia as evaluated by the number of 8.3-astroglia/Glt1-eGFP double-positive astroglia in the adult cortex (Fig. 2d and Supplementary Fig. 3C). Layer V of the cortex was preferentially enriched with 8.3-astroglia compared to all other cortical layers, whereas Glt1-eGFP single-positive astroglia were evenly distributed among the cortical layers as assessed by traditional two-dimensional as well as three-dimensional imaging approaches (Fig. 2c; Supplementary Video 1).

To verify that these observations were not due to an artifact of genomic integration, and to provide an alternative approach for studying this astroglia subset without the need for labeled transgenic mice, we introduced the 8.3 kb tdTomato plasmid in vivo in wild-type mice using nanoparticles. Nanoparticles have the advantage of accepting large molecular constructs and have excellent in vivo tissue distribution properties compared to adeno-associated viruses or lentiviruses\(^1\). Cytomegalovirus (CMV)-eGFP control and 8.3 kb tdTomato plasmids were packaged into separate nanoparticles that were approximately the size of nanometer-sized exosomes as visualized by transmission electron microscopy (Supplementary Fig. 3F-G). Following intracortical injection of CMV-eGFP control nanoparticles, eGFP fluorescence was distributed across the entire hemisphere and in all cell types (Supplementary Fig. 3D). However, after intracortical injection of the 8.3 kb tdTomato nanoparticles, tdTomato expression was limited to astroglia in the gray matter and specifically enriched in layers II/III and V, consistent with the 8.3-astroglia transgenic mouse (Fig. 2f and Supplementary Fig. 3E). These data support the hypothesis that the 8.3 kb sequence upstream of the Glt1 TSS is used only by a subset of astroglia.

We next determined the stability of tdTomato fluorescence in this astroglia subset. Stable expression of tdTomato within a subset of astroglia throughout their life span, without the appearance of additional fluorescent signal from other cell populations, would support the use of tdTomato as a fluorescent marker to study the specific subset of cells using the 8.3 kb promoter fragment. To address this possibility, we performed longitudinal multiphoton imaging of the motor cortex (up to 500 \(\mu\)m deep) of 8.3-astroglia-labeled transgenic mice (Supplementary Video 2). We tracked individual 8.3-astroglia over a period of five weeks and saw no changes in fluorescence, nor did we observe the appearance of any additional tdTomato-expressing cells in the cortical regions studied (Fig. 2g; Supplementary Video 2). The cell-specific tdTomato fluorescence was first observed during developmental astrogensis (P5) and remained stable throughout adulthood (not shown). Finally, we assessed the longitudinal expression of tdTomato in vitro using isolated 8.3-astroglia from adult mouse cortex (P60–P90), finding that tdTomato fluorescence continued for up to two weeks before cell passing (Supplementary Fig. 4B-C). The continuous expression of tdTomato in 8.3-astroglia in vivo and in vitro supports the hypothesis that these cells represent a distinct subpopulation of astroglia and that this transgenic mouse model provides a tool to study this cell population in various contexts.

**Molecular properties of 8.3-astroglia.** We next assessed the unique molecular properties of the 8.3-astroglia by determining their transcriptomic profile. Adult mouse cortices (\(n = 3\)) were dissociated into single-cell suspensions and separated by fluorescence-activated cell sorting (FACS) to collect three distinct populations: Glt1-eGFP-only (gray matter astroglia); 8.3-astroglia (gray matter astroglia subset); and the negative-fluorescent cell population (all cells that were not gray matter astroglia) (Supplementary Fig. 4A). During FACS, we noted that tdTomato\(^+\) astrocytes displayed varying levels of tdTomato fluorescence; however, we could only isolate the high expressers, which are continued to be referred to as 8.3-astroglia (Supplementary Fig. 4A). Based on microarray analysis of these populations, each group displayed a unique RNA transcriptome (Fig. 3a). As expected, the two astroglia populations were both enriched in well-established astroglia markers such as Aldh1l1, Acsbg1, and Aqp4 (Fig. 3b). However, when comparing 8.3-astroglia to Glt1-eGFP astroglia and the reporter-negative cell population, the 8.3-astroglia in the cortex displayed consistent and profound enrichment in several candidate markers (Fig. 3c,d; Supplementary Table 1). Some of the highest expressing genes, which are also involved in neurological disorders, include Kcnj10, Norrin, Olig2, Lgr6, and Fndc5 (Fig. 3c,d; Supplementary Table 2); their expression was validated using quantitative PCR (qPCR; Fig. 3d, data not shown).

Hypothesizing that these enriched proteins could provide clues into the unique biological properties of these adult astroglia subpopulations, and to allow us to create a combinatorial profile, we generated a list of markers enriched in single-positive Glt1-eGFP astroglia and double-positive astroglia populations and subjected these modified lists to software-based pathway analytics (Supplementary Tables 1, 3, and 4). We found that these astroglia populations were uniquely enriched in specific pathways, with enrichment of the Wnt/\(\beta\)-catenin signaling pathway in Glt1-eGFP astroglia and enrichment of the Sonic hedgehog pathway in 8.3-astroglia (Supplementary Fig. 4D). Both astroglia populations showed enrichment in pathways common to the function of all
astroglia, such as pathways involved in fat synthesis (Supplementary Fig. 4D and Supplementary Tables 2 and 3). LGR6 and Norrin expression by 8.3-astroglia. We next sought to generate a panel of markers that could be used to uniquely identify 8.3-astroglia. Starting with candidates identified by our transcriptomic analyses, we assessed four genes that were highly enriched in 8.3-astroglia compared to both the GLT1-eGFP and negative-fluorescent cell populations: Kcnj10, Lgr6, Olig2, and Norrin (Fig. 3c,d; Supplementary Table 2). In agreement with our RNA analyses, the cortical distribution of Kcnj10 was enriched in similar regions as the 8.3-astroglia (Fig. 3e and Supplementary Fig. 5B–C). At higher magnification, 8.3-astroglia displayed a higher mean fluorescence intensity of Kcnj10 compared to GLT1-eGFP astroglia (Fig. 3e and Supplementary Fig. 5B–C). To visualize Lgr6-expressing cells, we generated a double transgenic mouse model by crossing LGR6-GFP-ires-CreERT2 mice with the 8.3-astroglia mice. As expected, LGR6-eGFP localized to all 8.3-astroglia in the adult cortex of these double transgenic mice, with varying degrees of fluorescence (Fig. 3f). We also detected nuclear Olig2 expression in 8.3-astroglia, consistent with the microarray data (Supplementary Fig. 4E). We note that although Olig2 is widely used to identify oligodendrocyte lineage cells in the CNS, it is also known to exist in some neuroprotective astroglia subpopulations. In aggregate, the molecular identity and the unique CNS localization of the 8.3-astroglia are useful for elucidating the biological significance of this glial subgroup and allows the study of this subpopulation without the need of transgenic mice.

To determine whether this astroglia subpopulation is also present in the human cortex, we used LGR6 expression as a surrogate marker for the 8.3-astroglia population. Using immunohistochemistry...
Fig. 2 | 8.3 kb tdTomato expression is static and limited to an astroglia subset in the cerebral motor cortex. **a**, 8.3 kb tdTomato does not colocalize with the neuronal marker NeuN. **b**, 8.3 kb tdTomato does not colocalize with the microglial marker Iba1. **c**, 8.3 kb tdTomato does not colocalize with myelin and the oligodendrocyte marker 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase). **d**, 8.3-astroglia comprise about 25% of all gray matter astroglia in the motor cortex. **e**, 8.3-astroglia are heavily enriched in cortical layers II/III and V. GLT1-eGFP-only astroglia are equally distributed across all cortical layers. ***P < 0.001. **f**, 8.3 kb tdTomato nanoparticles are expressed by a subset of GFAP-positive astroglia enriched in layers II/III and V of the mouse motor cortex (scale bars, 15 µM). **g**, Cortical multiphoton in vivo imaging performed weekly for 5 weeks in adult mice tracking individual 8.3-astroglia (N = 5 mice, 100 cells). Astroglia cell counting was performed with sections imaged and subjected to validated automated cell counting of eGFP and tdTomato fluorescence cells. The statistics used to compare eGFP only to 8.3-astroglia per cortical layer included a two-way ANOVA with Tukey post hoc analysis. Error bars represent the s.e.m. For the nanoparticle injections, n = 5 mice were intracortically injected and analyzed. For all experiments, n = 5 mice were analyzed, with 3–5 images per mouse. The red arrowheads indicate the 8.3-astroglia and the white arrowheads indicate non-8.3-astroglia cells.
(IHC) and RNA in situ hybridization on postmortem adult human cortex tissue, we detected LGR6 expression in only a subset of astroglia in the human cortex, consistent with the results of our rodent studies (Fig. 3g,h and Supplementary Fig. 5A). To validate the astroglia-specific expression of LGR6 in humans, we used IHC to visualize the astrocyte-specific marker ALDH1L1. LGR6 colocalized only with ALDH1L1+ cells in the adult human cortex, providing further support that these cells are indeed a subset of human cortical astroglia (Fig. 3h and Supplementary Fig. 5A).

LGR6-positive astroglia subsets could also be reliably identified in vitro in both pure mouse primary cortical cultures and in human induced pluripotent stem cells (hiPSCs) differentiated into astroglia (Fig. 3i,j). We also observed a higher colocalization of KCNJ10 and LGR6 in hiPSC-derived astroglia, supporting the results of our combinatorial profile (not shown). Thus, these studies establish a well-defined and geographically organized astroglia subpopulation in the adult human and rodent cortex and provide reliable markers to study their involvement in normal and diseased adult CNS physiology.

Functional assessment of LGR6 in astroglia. We next aimed to determine the functional significance of our observation that the receptor LGR6 is consistently enriched and labels this astroglia subpopulation. In addition, we focused on LGR6 because it showed the highest enrichment in our RNA analytics and has been widely shown to be astroglia-specific in the CNS32–34. To address this question, we investigated the effects of adding its ligand, R-spondin (RSPO1). RSPO1 has been shown to be neuron-specific in the adult cortex, but little is known about the downstream consequences of its interaction with LGR6 in the CNS. Consistent with published RNA in situ hybridization data, RSPO1 was highly enriched in 8.3-astroglia-dense areas in layer V (Fig. 4a and Supplementary Fig. 6A-D). Furthermore, we performed immunofluorescence for the neuronal marker, NeuN, followed by RNA fluorescence in situ hybridization (FISH) for RSPO1 and noted that RSPO1 colocalized only to NeuN-positive neurons, but only in a subset in the lower cortical layers (Supplementary Fig. 6A-D). This provides additional support that RSPO1 is neuron-specific in the adult motor cortex but that it is also limited to only a subset of neurons in cortical layer V.

Next, we wanted to explore the astroglia response to RSPO1 in vitro. Treatment of primary astroglia cultures with increasing doses of RSPO1 resulted in significant enrichment in the overall numbers of LGR6+ astroglia compared to PBS-treated control cultures (Fig. 4b and Supplementary Fig. 7A-B). To determine whether this increase in LGR6+ astroglia was due to proliferation, we stained astroglia with the proliferation marker Ki67, revealing a significant dose–response increase of Ki67-positive astroglia following RSPO1 treatment (Supplementary Fig. 7A). This increase in proliferation was due to an increase of LGR6+ astroglia (Supplementary Fig. 7B).

These in vitro findings suggest that in postnatal conditions, local neuronal release of RSPO1 could stimulate proliferation of astroglia, suggesting a specific interaction between an RSPO+ neuron subpopulation and LGR6+ 8.3-astroglia.

The stimulatory effect of LGR6 on the proliferation of 8.3-astroglia in vitro suggest that loss of LGR6 could decrease the population of 8.3-astroglia. Indeed, we observed a dramatic loss of 8.3-astroglia in vivo in the heterozygous 8.3-astroglia/LGR6-GFP-ires-CreERT2 mice, which have 50% lower expression of LGR6 compared to wild-type mice; this decrease further correlated with a minimal but significant loss of cortical thickness (Fig. 4c,d and Supplementary Fig. 7C). Since astroglia play a major role in neuronal synapse formation and elimination, we analyzed the overall density of spines on apical dendrites in layer V where 8.3-astroglia are highly enriched. We found a significant decrease in spine density in the heterozygous LGR6-eGFP/8.3-astroglia mouse model, correlating with the loss of 8.3-astroglia (Fig. 4e and Supplementary Fig. 8A-B).

Next, we sought to determine which factor(s) released by 8.3-astroglia could be responsible for the deficits in dendritic spine density, focusing on secreted proteins that were highly enriched in 8.3-astroglia as identified by our transcriptomic analyses. To test for the secretion of neuromodulating proteins, we stimulated LGR6+ astroglia with RSPO1 and used enzyme-linked immunosorbent assay (ELISA) to detect candidate secreted proteins and determine which proteins increased with RSPO1 treatment. One highly abundant protein not enriched in the PBS control or LGR6 knockout primary astroglia was Norrin (norrin cystine knot growth factor NDP (Ndp)), a protein that is expressed in vivo by cortical astrocytes with strong colocalization with 8.3-astroglia in cortical layer V and has been shown to be astroglia-specific in the cortex (Fig. 4f–h)35–37.

Norrin release by 8.3-astroglia regulates dendritic growth and spine formation. Norrin is known to be astroglia-specific in the adult cortex, where it binds to frizzled-4 and several other receptors such as LGR4 to activate the Wnt signaling pathway and thereby induce upregulation of neurotrophic growth factors, including brain-derived neurotrophic factor (BDNF)38–40. Patients with Norrin mutations develop Norrie disease, a CNS and ocular disease that also has strong cognitive and behavioral deficits including mental retardation, psychosis, and early-onset dementia in many but not all patients35,37. Some patients with Norrie disease have a deletion of exon 2, resulting in the possibility of two truncated proteincs from exon 3. To evaluate the effects of increased Norrin on cortical neurons, we treated primary mouse cortical neurons with recombinant Norrin, compared to the PBS vehicle control and the two Norrin truncated proteins from exon 3 (referred to as ‘truncated 1’ and ‘truncated 2’) (Fig. 5a, Supplementary Fig. 9A-D, and Supplementary Fig. 10A-D). We found that treatment with both truncated proteins did not affect dendritic arborization or length;...
However, Norrin significantly affected dendritic arborization and increased dendritic length (Fig. 5b,c; data not shown). These findings show that the truncated Norrin protein translated in Norrie disease patients is inefficient at effecting neuronal dendrites.

Norrin has known effects on retinal biology, but its function in the cortex is unknown. To determine whether absence of Norrin would lead to neuronal deficits, we analyzed Norrin-null mice and quantified cortical neuron dendritic spine density. We found a significant loss of spines in cortical layer V in Norrin-null mice compared to their control littermates (Fig. 5d,e and Supplementary Fig. 8A,C).

Next, we wanted to evaluate the effects of in vivo Norrin treatment on cortical neuronal dendritic spine density. To address this, we created a genetic plasmid with the astroglia subset-specific 8.3 kb promoter followed by Norrin complementary DNA and packaged this plasmid into nanoparticles that were injected into the mouse motor cortex. Norrin secretion driven solely by the 8.3 kb promoter significantly increased dendritic spine density in the Norrin-null mice.
Fig. 4 | Functional and dysfunctional properties of astroglia subpopulation by examination of LGR6 pathways in the cerebral motor cortex. a. Neuron-specific RSPO1 immunoreactive localization is restricted to cortical layer V in the mouse motor cortex. b, RSPO1 treatment in vitro of primary mouse astroglia increases LGR6 immunoreactivity. c, Lgr6 heterozygous mice display reduced numbers of 8.3-astroglia. d, Lgr6 heterozygous mice display a significant reduction in the number of 8.3-astroglia; *P = 0.0003. e, LGR6 alters the neurite properties; Lgr6 heterozygous null mice have reduced spine density compared to control mice as assessed with the Golgi–Cox staining. f, The LGR6 receptor agonist RSPO1 affects astroglial Norrin. Norrin levels are increased following RSPO1 treatment on primary astroglia as assessed by ELISA. g, Norrin mRNA colocalizes with 8.3-astroglia in the mouse motor cortex. h, High magnification of colocalization of Norrin mRNA and tdTomato. N = 5 different mice with 3–5 coronal slices were imaged for IHC and RNA FISH. N = 5 mice were used for spine analysis with 5 neurons analyzed per mouse motor cortex. N = 3 different primary astroglia cultures were treated for 48 h with RSPO1 and repeated 3-5 times for ELISA analysis (control versus RSPO1: *P = 0.0025; RSPO1 versus Lgr6 knockout: *P = 0.031). Two-sided Student’s t-test; values represent means and error bars represent s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.
and LGR6-eGFP-ires-CreERT2 mice compared to the CMV-eGFP control nanoparticle-injected Norrin-null and LGR6-eGFP-ires-CreERT2 mice (Fig. 5f). This provides evidence that Norrin produced solely by 8.3-astroglia is sufficient to restore the dendritic abnormalities and suggests a role for this astroglia-specific pathway in the regulation of neuronal spine and synaptic physiology.
Fig. 6 | Norrin alters the electrophysiological properties of cortical neurons; Norrin-null mice display neurobehavioral abnormalities. 

a. Norrin treatment enhances cortical neuron connectivity and firing rate. 
b. Norrin treatment significantly increases the degree of neuronal firing. 
c. Norrin treatment significantly increases the weight of the neuronal firing strength. 
d. Norrin-null mice are significantly more hyperactive than their wild-type littermate controls. 
e. Total travel distance is significantly higher in Norrin-null mice compared to their wild-type littermate controls. 
f. Norrin-null mice have significantly decreased resting time compared to their wild-type littermate controls. 
g. Norrin-null mice have significantly more arm entries than their wild-type littermate controls. 
h. Norrin-null mice are significantly faster than their wild-type littermate controls. 
i. Norrin-null mice have significantly more rearings than their wild-type littermate controls. 

MEA analyses were performed three times at each time point, each with three different treatments. For the open field assay and Y-maze, n = 15–20 mice were used per genotype. Statistics include a two-way repeated-measures ANOVA and two-sided Student’s t-test. 

b-i, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. The values plotted represent the mean with the error bars representing the s.e.m.
Norrin treatment effects the electrophysiological properties of neurons. Next, we wanted to explore the effects of Norrin on the electrophysiology of cultured neurons. Using multielectrode array (MEA) plates with rat cortical neurons and astrocytes, we monitored basal activity before and 24h after Norrin treatment (Fig. 6a and Supplementary Figs. 11A, 12A-F, and 13A; Supplementary Videos 3–5). Spike and burst rates were normalized to pretreatment activity to control for batch effects in baseline activity. There was a significant increase in the difference in both node degree and connection strength between electrodes, as well as a reorganization of spiking patterns wherein a greater percentage of detected spikes were organized into bursts (Fig. 6b,c and Supplementary Fig. 12A-F). These findings demonstrate that Norrin could function to organize and strengthen cortical neuronal connectivity.

Norrin-null mice display neurobehavioral abnormalities. Norrin-null mice displayed a pronounced loss of dendritic spines in layer V of the cortex and affected both electrophysiology and dendrites in vitro, so we sought to determine if they also displayed altered behavior. To test this hypothesis, we subjected the Norrin-null mice and their wild-type littermates to an array of behavioral assays. Surprisingly, in the open field assay, Norrin-null mice were significantly more hyperactive than their wild-type littermates, as shown by increased exploration and decreased resting time (Fig. 6d–f). In the Y-maze test, Norrin-null mice displayed increased combinations and arm entries, supportive of a hyperactive phenotype (Fig. 6g). Overall, the average speed of Norrin-null mice was significantly higher than the controls (Fig. 6h). When evaluating rearing, Norrin-null mice had significantly more rearing behaviors than their wild-type littermates (Fig. 6i). Furthermore, at this time period (P60–P90), these mice did not display abnormalities in several assays, such as the elevated platform maze, fear conditioning, and rotarod performance tests (data not shown). These findings further support that Norrin-null mice have neurobehavioral abnormalities, such as hyperactivity, which may be the result of their cortical phenotype.

Discussion

The majority of evidence for astroglia heterogeneity has arisen from developmental studies in the spinal cord or found as a pathologic consequence in neurological disorders. To date, there are few data that molecularly and/or physiologically define the presence of astroglia subsets in the adult nervous system that function to interact and/or regulate regional neuronal populations. Furthermore, no reliable tools exist to accurately and robustly identify these subpopulations in normal and disease states. With the generation of the 8.3-astroglia mouse line, we have discovered and are now able to easily study a specific astroglia subset in the cortex of adult mice. This astroglia subset, 8.3-astroglia, has distinct cortical patterns and molecular profiles compared to other gray matter astroglia; it can be robustly identified using unique markers such as OLIG2, NORRIN, KCNJ10, and LGR6 in rodent and human CNS, as well as in primary mouse and hiPSC astroglia cultures.

Our extensive profiling data have allowed us to generate a protein/RNA profile to molecularly identify 8.3-astroglia. LGR6 colocalizes with 8.3-astroglia in the mouse and human cortex, along with subsets of in vitro astroglia from rodents and in hiPSC-derived astroglia. LGR6 contributes to Wnt signaling, one of the pathways enriched in gray matter astroglia; its ligand, RSPO1, is specifically released by pyramidal neurons. In the cortex of adult mice, we demonstrated that RSPO1 is restricted to a neuronal subset in layer V; these are areas highly enriched in LGR6+ 8.3-astroglia. LGR6 and Norrin have both been shown to be highly enriched and limited to astroglia. The correlation of RSPO1, LGR6, Norrin, and 8.3-astroglia cortical patterning strongly suggests that there may be a cross talk between neurons and astroglia, where extrinsic signaling, including the possible release of RSPO1 from neuronal subtypes, could influence astroglia subtype-specific responses (Supplementary Fig. 14). In support of this model, RSPO1 is largely restricted to layer V of the cortex. Neurons influence astroglial physiology, for example, via astroglia expression of the glutamate transporter EAAT2. Future studies should address the positional identity of specific astroglia and neuron populations where neuronal diversity may influence astroglia diversity and vice versa.

The unique anatomical localization of 8.3-astroglia and their enrichment in LGR6 and Norrin suggest that they may be involved in glial-based pathogenesis of Norrie disease. We document a selective loss of spine density in both the LGR6 heterozygous and Norrin-null mice in cortical layer V, corresponding to the location of 8.3-astroglia and Norrin expression. This is particularly interesting since some Norrie disease patients with genetic deletion of exon 2 of the NDP gene developed epilepsy. Epilepsy patients have been documented to have reduced spine density and altered dendritic arbor. Unfortunately, it is not possible to analyze spine density in Norrie disease patients because there is no available postmortem brain tissue.

The effect of Norrin on cortical neuron dendrites is particularly interesting. We show that treating neurons with Norrin leads to increases in dendritic length and arborization. However, treatment with the two truncated Norrin proteins found in Norrie disease patients with deletion of exon 2 exhibited no effect on normal neuronal dendrites. Loss of spines is widespread in several disorders. In an ALS mouse model, there was a significant loss of spines in pyramidal layer V neurons in the motor cortex. This is similar to the pathology in Alzheimer’s disease, where dendritic spine loss is also involved. In transcriptomic data of Alzheimer’s disease mice, Norrin is significantly downregulated in astrocytes. There is also an observed loss of Norrin levels in the hippocampus in Alzheimer’s disease. Finally, reduced spine density is also observed in mental retardation (another documented phenotype in some Norrie disease patients).

Norrin could serve as a potential therapeutic agent in neurological disorders with a spine density pathology. This is not the first report of astroglia regulating synapse maturation and receptors. Recently, the ability of astroglia to regulate the synaptic AMPA receptors and drive synapse maturation via chordin-like 1 was documented. Now, we show that using the 8.3 kb-specific promoter to drive the expression of Norrin in the mouse cortex is sufficient to restore and improve cortical spine density. These data show that the sole release of Norrin strictly by 8.3-astroglia could serve as a therapeutic agent to modulate neuronal spines and synapses. Additionally, use of a ubiquitous promoter would hypothetically elevate Norrin levels even more and could lead to a more dramatic increase in spine density, altering dendritic arborization and length, and the electrophysiology of cortical neurons. In fact, the multielectrode array studies allowed us to test the effects of Norrin on the electrophysiological properties of neurons; Norrin treatment led to improved neuronal connectivity and strength supporting a possible role for this astroglia subpopulation in regulating neuronal connectivity and as a candidate therapy.

To assess the clinical phenotype of these mutant mice, we performed a wide array of behavioral assays on the Norrin-null mice. We found that Norrin-null mice displayed abnormal behavior in the open field and Y-maze tests. Overall, Norrin-null mice were hyperactive, which is similar to what has been shown in BDNF mutant mice; it has been shown that Norrin can drive the expression of BDNF and other neurotrophic proteins. These neurobehavioral abnormalities might be expected in light of the cortical pathology of Norrin-null mice.

Lastly, the focal localization of the cortical layer of 8.3-astroglia is quite intriguing. Based on morphology, in the cortex different layers exhibit different populations of astroglia. We have now shown that...
the 8.3-astroglia subset is the dominant population of layer V astroglia. Different cortical layers exhibit different neuronal populations. Our findings provide further evidence that suggest a layer-specific cross talk between regional subsets of neurons and astroglia potentially indicating a localized neuron–glia functional specification. Future studies could explore this relationship, especially in disorders where a subset of cells is affected (that is, ALS motor neurons) as well as in paradigms of cortical synaptic plasticity.

Taken together, these findings expand the growing understanding of functional astroglial heterogeneity by uncovering and defining a unique subset of astroglia in the CNS. They have led to new tools to manipulate this astroglia subset in efforts to ultimately discover novel therapeutic avenues for neurological disorders affected by this and other astroglia subsets. This has also led to advances in the understanding Norrie disease, which we now define as also involving astroglia, and the contributions that astroglia may play in its pathophysiology. These studies have set a foundation to build on for understanding astroglial subsets, their powerful role in neuronal spine biology, and the unique neuron–glia pairing in normal and pathological states.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41593-019-0366-7.

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behavioral assay generation and interpretation. Z.C. and Y-C.H. assisted with tissue
dissociation and transferred samples to the Johns Hopkins University FACS and
Sequencing/Microarray cores. N.K. generated the nanoparticles and performed the
transmission electron microscopy, which was overseen by J.S.S. and J.H. M.D. and R.T.
performed the tissue clearing and CLARITY-optimized light-sheet microscopy imaging.
J.T.P. assisted with maintenance and differentiation of hiPSC lines. R.D. performed the
MEA experimentation, which was overseen by N.H. M.B.R., R.S., and D.E.B. contributed
to data interpretation and manuscript review. J.D.R. oversaw project development,
experimental design, data interpretation, data representation, and manuscript writing.

Competing interests
S.I.M. and J.D.R. have filed a patent on the use of Norrin. The remaining authors declare
no competing interests.

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Methods

General procedures. Investigators were blinded to the behavioral analysis of genetic subgroups of mice and to all quantitative IHC staining of tissue and cultures. Randomization was not relevant to the experiments performed in this study. We compared specific control and test conditions through either treatment or genetic indicators.

Animal models. Wild-type (C57BL/6; The Jackson Laboratory), LGR6-GFP-ires-CreERT2 (The Jackson Laboratory), Norrin-null (gift from J. Nathans), BAC-GLT1-eGFP, 2.5 kb tdTomato, 6.7 kb tdTomato, 7.9 kb tdTomato, and 8.3 kb tdTomato mice were used for the in vivo experiments. GLT1/EAAT2 tdTomato transgenic mice were generated by inserting the tdTomato reporter downstream of a 2.5 kb, 6.7 kb, 7.9 kb, or 8.3 kb EAAT2/GLT1 promoter fragment as detailed in part previously45. Multiple founders were established following pronuclear injection at the transgenic core laboratory of Johns Hopkins University. The care and treatment of animals were in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, the Guidelines for the Use of Animals in Neuroscience Research, and the Johns Hopkins University Institutional Animal Care and Use Committee. Mice were housed at standard temperature (21 °C) in a light-controlled environment with ad libitum access to food and water. BAC-GLT1-eGFP mice were crossed with 8.3 kb tdTomato mice to generate double transgenic mice; 8.3 kb tdTomato mice were also bred with LGR6-GFP-ires-CreERT2 mice. Litters were used as controls and for comparisons between different astroglia populations.

Generation of GLT1/EAAT2 promoter tdTomato transgenic mice. GLT1/EAAT2 (2.5 kb) tdTomato. The fluorescence protein tdTomato was cloned downstream of the human EAAT2 promoter sequence (2.5 kb). This DNA fragment was injected into a mouse pronucleus to produce the transgenic mouse. The promoter sequence was based on the following: chr11:35439159-35449110, 6,752 bp. This DNA fragment was injected into a mouse pronucleus to produce the transgenic mouse. The promoter sequence was based on the following: chr11:35439159-35449110, 7,952 bp.

GLT1/EAAT2 (7.9 kb) tdTomato. The fluorescence protein tdTomato was cloned downstream of the human EAAT2 promoter sequence (7.9 kb). This DNA fragment was injected into a mouse pronucleus to produce the transgenic mouse. The promoter sequence was based on the following: chr11:35439159-35448435, 7,677 bp.

GLT1/EAAT2 (8.3 kb) tdTomato. The fluorescence protein tdTomato was cloned downstream of the human EAAT2 promoter sequence (8.3 kb). This DNA fragment was injected into a mouse pronucleus to produce the transgenic mouse. The promoter sequence was based on the following: chr11:35440759-35449110, 8,352 bp.

Nanoparticle preparation and injection. Brain-penetrating DNA nanoparticles were formulated as previously reported43. Briefly, polyethylenimine (PEI) was formulated by a dropwise addition of 10 volumes of DNA (phosphorylated eGFP or purchased from Clontech Laboratories. Brain-penetrating DNA nanoparticles were encoding plasmid DNA driven by CMV promoter (phosphorylated eGFP) was hydroxysuccinimide (mPEG-NHS, 5 kDa; Sigma-Aldrich) was conjugated to GLT1/EAAT2 (6.7 kb) tdTomato. The fluorescence protein tdTomato was cloned downstream of the human EAAT2 promoter sequence (6.7 kb). This DNA fragment was injected into a mouse pronucleus to produce the transgenic mouse. The promoter sequence was based on the following: chr11:35439159-35443219, 2,461 base pairs (bp).

GLT1/EAAT2 (7.9 kb) tdTomato. The fluorescence protein tdTomato was cloned downstream of the human EAAT2 promoter sequence (7.9 kb). This DNA fragment was injected into a mouse pronucleus to produce the transgenic mouse. The promoter sequence was based on the following: chr11:35439159-35449110, 6,752 bp.

GLT1/EAAT2 (8.3 kb) tdTomato. The fluorescence protein tdTomato was cloned downstream of the human EAAT2 promoter sequence (8.3 kb). This DNA fragment was injected into a mouse pronucleus to produce the transgenic mouse. The promoter sequence was based on the following: chr11:35440759-35449110, 8,352 bp.

Cell treatments. Primary mouse astroglia cultures were treated with 250 ng to 1 μg of mouse recombinant carrier-free RSPO1 (R&D Systems) for 72 h. For the cortical neuronal experiments, primary cortical neurons were isolated, matured for 7–10 d, and then treated with 600 ng to 2 μg of mouse recombinant carrier-free Norrin (R&D Systems) or truncated protein 1 or 2 μg of mouse recombinant carrier-free proteins for 24 h.

FACS. The cortex, spinal cord, or cerebellum from BAC-GLT1-eGFP (8.3 kb) tdTomato double transgenic reporter mice (age P60–P90, n = 3 male mice per experiment) were analyzed with FACS. Mice were anesthetized with either an intraperitoneal injection of ketamine/xylazine. Brain tissue was immediately dissected and cryopreserved. Single celld slices were dissected and spread using a MoFlo Multi-laser Sorter high-speed cell sorter (MoFlo XDP Cell Sorter) and gated based on eGFP and tdTomato fluorescence (Supplementary Fig. 4A).

Microarray. The microarray procedure and analyses were performed as previously described44. Briefly, total RNA was isolated from FACS-sorted populations using an RNeasy kit (Qiagen) and the concentration was determined by NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific) and Bioanalyzer Micro 2 Scanner (Agilent). Only samples with an RNA integrity number > 5 were used. Total RNA was linearly amplified and labeled according to a NuGEN protocol. Sample labeling and hybridization with mouse exon 1.0 ST chips (Affymetrix) were performed at the Johns Hopkins University Deep Sequencing and Microarray Core according to the manufacturer’s protocol. After hybridization, hybridization signals were acquired and normalized with the Partek Genomics Suite software (Partek). Differential gene expression between conditions was assessed by statistical linear model analysis using Partek, TIBCO Spotfire X, and Prism version 7 (GraphPad Software) software. The moderated t-statistic P values derived from the Partek analysis were further adjusted for multiple testing using the Benjamini–Hochberg method to control the false discovery rate. A false discovery rate cutoff of < 10% was used to obtain the list of differentially expressed genes. Tissue from at least three mice were used for each microarray analysis. All RNA array data is available online without restriction from the Gene Expression Omnibus.

Pathway analyses. Gene Ontology and pathway analyses were performed from data obtained from the Partek and Spotfire post-statistical analyses using the Ingenuity Pathway Analysis Genomics Suite software (QIAGEN) as previously described45.

Histological analysis. Brain IHC. Mice were injected with a lethal dose of ketamine/xylazine and immediately perfused with 1x PBS followed by 4% paraformaldehyde (PFA). Post-perfusion, the brain, cerebellum, and spinal cord tissues were collected and cryoprotected in 30% sucrose. Tissue samples were prepared for cryostat sectioning and sectioned at a thickness of 20 μm. Sections were stained with blocking buffer (5% donkey serum and 0.1% Triton X-100 in PBS) for 60 min at room temperature. The following primary antibodies were used: GFP (1:100; Rockland); OLG1 (1:250; EMD Millipore); glial fibrillary acidic protein (GFAP; 1:250; EMD Millipore); LGR6 (1:100; Abcam and R&D Systems); KCN1 (1:200; Alomone Labs); RPSO1 (1:100; Abcam); Norrin (1:100; Abcam); RFP (1:250; Abcam); and ALDH1L1 (1:100; Abcam). Secondary antibodies were: Alexa Fluor 488 donkey anti-rabbit (1:500; Abcam); Alexa Fluor 488 donkey anti-mouse (1:500; Abcam); donkey anti-rabbit Cy3 (1:500; Abcam); and donkey anti-mouse Cy3 (1:500; Abcam). The use of the antibodies and their protein specificity was determined by each commercial vendor as provided on their website or in their catalogs. Labelled cells were manually counted or overall fluorescence was calculated with the Zeiss Zeiss 2012 software or using spot detection in the Bitplane Imaris software version 8.3.1. All images were captured with a Zeiss LSM 700 confocal microscope, Zeiss Axio Imager, or Zeiss LSM 800 confocal microscope. A minimum of three mice per group were used for each experiment at ages consistent with the microarray experiments (P60–P90).

Cell culture immunocytochemistry. Cells were stained as previously described45. Briefly, cells were grown until the appropriate time point or confluency followed by fixation in 4% PFA and 0.3% Triton X-100 permeabilization. Cells were then stained as described earlier.
Tissue clearing. A passive CLARITY method was used for tissue clearing. The hydrogel monomer solution included 1% (wt/vol) acrylamide, 0.0125–0.05% (wt/vol) acrylamide, 1X PBS, 1% PFA, 1X PBS, deionized water, and 0.25% of the hydrogel monomer solution recipe included 1% (wt/vol) acrylamide, 0.0125–0.05% (wt/vol) bis-acrylamide, 4% PFA, 1X PBS49. After cross-linking for 3–4 h for hydrogel polymerization, tissue was cleared in a 37 °C shaking water bath. The clearing solution was washed off with a 0.2 M boric acid buffer (pH 8.5)/0.1% Triton X-100. For imaging, tissues were mounted in 60% 2,2′-Thiodiethanol with 1X PBS49.

RNA in situ hybridization. Human CNS tissue (Supplementary Table 5) was fixed in 4% PFA, cryoprotected in 30% sucrose, and samples were serially sectioned onto coverglass slides. RNA in situ hybridization was performed using the RNAscope duplex and chromogenic protocol as suggested by the manufacturer (Advanced Cell Diagnostics). Slides were imaged with a Zeiss Axio Imager brightfield microscope. A minimum of three different brain samples were subjected to RNA in situ hybridization analyses. Hybridization probes were designed and purchased from RNAscope (Advanced Cell Diagnostics). Patient demographics are provided in Supplementary Table 5. Human tissue was obtained from Johns Hopkins and University of California, San Diego Target ALS Autopsy Bank. The use of human tissue and associated decedent demographic information was approved by the Johns Hopkins University institutional review board and ethics committee (HPAA Form 5 exemption, application 11-02-10-01RD) and from the Target ALS Consortium.

In vivo imaging and surgical procedure. Adult (P60–P90, n = 5 mice, 100 astroglia) mice were obtained from crosses of the BAC-GLT1-eGFP and 8.3 kb tdTomato reporter mice. For repeated in vivo imaging, a cranial window was prepared as previously described43. In brief, mice were anesthetized with ketamine/xylazine and a craniotomy (3 mm diameter) was placed above the motor cortex. The craniotomy was covered with a permanent glass cover slip (4 mm diameter) and sealed with dental acrylic. For multiphoton imaging, mice were anesthetized with a mixture of oxygen and isoflurane. A metal head-bar was fixed over the craniotomy and placed into a head-bar holder for imaging. Mice were imaged weekly up to 500 µm below the dura using the Zeiss LSM 710 confocal microscope at the Johns Hopkins Neuroscience Imaging Core.

Mean intensity fluorescence analysis. 8.3-astroglia/GLT1-eGFP mice were stained with Kir4.1/Kcnj10 and analyzed with ImageJ (NIH). Briefly, the entire astroglia and processes were traced with the freehand tool to generate a region of interest. Next, the region of interest was measured for mean pixel density and recorded.

Cortical thickness calculation. The motor cortex from multiple mice (n = 5) was imaged on the same reference slice; gross cortical thickness was measured by following the reference brain slice available from the Allen Brain Institute Reference Coronal Atlas.

Sholl analysis and dendritic length. Sholl analysis and dendritic length was determined with ImageJ as described previously44.
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

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☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

☐ Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

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☐ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | Prism 7, Microsoft Excel for Mac 2016 |
|-----------------|--------------------------------------|
| Data analysis   | All software used was from publicly available commercial computer software vendors in our lab or university used to collected data and image analysis: Prism 7, Microsoft Excel for Mac 2016, GenScript, Partek Genomics Suite, Tibco Spotfire, Ingenuity Pathway Analysis, Zen Zeiss 2012, Bitplane Imaris 8.3.1, ImageJ, MC RACK, MDS DataManager, MEAnalyzer |

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- A description of any restrictions on data availability

All microarray data will be uploaded to GEO for readers to access. Otherwise, all information has been stated in the manuscript.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**

Sample sizes were chosen based on previous expertise, knowledge from past experiments and statistical considerations using similar model systems (neuron iPS cultures, mouse studies) and current accepted standards based on literature review. No statistic analysis was used to predetermine sample size. Sample sizes were chosen to be similar or exceed those reported in the previous literatures in the field. Each experiment was repeated 3-6 times, and the statistical analysis demonstrates that our sample sizes revealed significant differences between groups.

**Data exclusions**

No data were excluded from the study.

**Replication**

All experiments were done with a minimum number of replicates based on previous expertise in statistical analyses of similar experimental datasets. Samples sizes were chosen based on previous expertise and knowledge from past experiments using similar rodent cultures, human CNS tissue, mouse in vivo studies and iPSC-derived systems and current accepted standards based on literature review. The findings in this study were collected from multiple independent experiments, and were reliably reproduced.

**Randomization**

Not relevant to this study. When appropriate, animals were randomly chosen for analytics form each genetic subgroup.

**Blinding**

Investigators were blinded for behavioral analysis of genetic subgroups of mice and for all quantitative IHC staining of tissue and cultures.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| x   | Antibodies            |
| x   | Eukaryotic cell lines |
|     | Palaeontology         |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |

### Method

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChIP-seq              |
|     | Flow cytometry        |
|     | MRI-based neuroimaging |

**Antibodies**

The following primary antibodies were used: (NEED CATALOG numbers)

- GFP (Rockland), #600-401-215L; 600-401-215S
- OLG2 (Millipore), #AV31464
- GFAP (Millipore), SAB2500462, MAB360, ab5320
- GFAP (Abcam), ab53554, ab5541
- GFAP (Abcam), ab240030
- LGR6 (Abcam, R&D Systems), ab240030
- KCNJ10 (Alomone Labs), APC-035-AG
- KCNJ10 (Abcam), ab240876
- RSP01 (Abcam), ab106556, ab231125
- Norrin (Abcam), ab185175, ab90690
- ALDH111 (Abcam), ab87117, ab56777
- RFP (Abcam), ab62341

Secondary antibodies were all tested to be specific for the species identified.

- donkey anti-rabbit Alexa Fluor 647 (Abcam), ab150073, ab175651
- donkey anti-mouse Alexa Fluor 647 (Abcam), ab150107
- donkey anti-rabbit 488 (Abcam), ab150061
- donkey anti-mouse 488 (Abcam), ab150105
Validation

The use of the antibodies and their protein specificity was determined by each commercial vendor as provided by their websites:

- GFP (Rockland): ELISA, WB, IHC, IF, FC
- OLIG2 (Millipore), IHC, WB
- GFR (Millipore), ELISA, IHC, WB
- LGR6 (Abcam, R&D Systems), flow, ICC, IHC, WB
- KNCJ10 (Alomone Labs), IHC, ICC, WB
- KNCJ10 (Alomone Labs), ICC, WB
- RSPO1 (Abcam), IHC, WB
- Norrin (Abcam), IHC, WB
- ALDH1L1 (Abcam), ICC, IHC, WB

Secondary antibodies were all tested to be specific for the species identified.

- donkey anti-rabbit Alexa Fluor 647 (Abcam), ELISA, flow, ICC, IHC
- donkey anti-mouse Alexa Fluor 647 (Abcam), IHC, flow, ICC, ELISA
- donkey anti-rabbit 488 (Abcam), ELISA, flow, ICC, IHC
- donkey anti-mouse 488 (Abcam), ELISA, flow, ICC, IHC
- donkey anti-rabbit Cy3 (Jackson, Abcam), IHC
- donkey anti-mouse Cy3 (Jackson, Abcam).

Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | Primary mouse cortical neurons and astrocytes and primary rat cortical neurons were dissected and obtained based on the methods stated in the manuscript. |
|---------------------|----------------------------------------------------------------------------------------------------------------------------------|

Authentication

Authentication was performed by validating with known pan-markers for the respective cell lines.

Mycoplasma contamination

All cell lines tested negative for mycoplasma contamination.

Commonly misidentified lines

No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Wild type (C57/BL6), LGR6-GFP-ires-CreERT2 (Jackson Laboratory), Norrin-null (gift from Jeremy Nathans), BAC-GLT1-eGFP, 2.5 kb-tdTomato, 6.7 kb-tdTomato, 7.9 kb-tdTomato, and 8.3 kb-tdTomato mice were used for in vivo experiments. GLT1/EAAT2-tdTomato transgenic mouse were generated by inserting the tdTomato reporter downstream of a 2.5kb, 6.7kb, 7.9 kb or 8.3kb EAAT2/GLT1 promoter fragment as detailed in part previously. Multiple founders were established following pronuclear injection at the transgenic core laboratory of Johns Hopkins University. Mice were housed at standard temperature (21°C) in a light-controlled environment with ad libitum access to food and water. BAC-GLT1-eGFP mice were crossed with 8.3 kb-tdTomato mice to generate double transgenic mice. 8.3 kb-tdTomato mice were also bred with LGR6-GFP-ires-CreERT2 mice. Littermates were used as controls and for comparisons between different astroglia populations.

Wild animals

Please state that the study did not involve wild animals.

Field-collected samples

Please state that the study did not involve field collected samples.

Ethics oversight

The care and treatment of animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, the Guidelines for the Use of Animals in Neuroscience Research, and the Johns Hopkins University IACUC.

Human research participants

Policy information about studies involving human research participants

Population characteristics

Human control CNS tissues were available from the Johns Hopkins Autopsy Tissue Bank maintained in our lab and the Target ALS postmortem core. For Target ALS specimens, a web-based searchable database of the postmortem tissue inventory provides estimates of fixed and frozen postmortem tissues available that meet basic demographic criteria. Induced Pluripotent stem cell lines (iPS) were generated in our lab, under NIH-NINDS supervision. These line were deposited in the NIH-NINDS cell repository and are publicly available thru NIH. Additional iPS cell lines are available thru the NeuroI correlated iPS cell consortium and obtained via the Cedars Sinai cell repository (https://www.cedars-sinai.edu/Research/Research-Cores/Induced-Pluripotent-Stem-Cell-Core/)

Recruitment

Eligible patients and/or family members are recruited from our ALS clinic for voluntary participation in autopsy donations.
Flow Cytometry

Plots

Confirm that:
☑️ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑️ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☑️ All plots are contour plots with outliers or pseudocolor plots.
☑️ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Cortex from BAC-GLT1-eGFP/8.3 kb-tdTomato double transgenic reporter mice (age P60-P90, n = 3 male mice per experiment) were analyzed with FACS. Mice were anesthetized with an intraperitoneal injection of ketamine xylazine. Brain tissue was immediately dissected and dissociated as previously described [16]. Single cells were then sorted using a MoFlo MLS high-speed cell sorter and gated based on eGFP and tdTomato fluorescence.

Instrument
MoFlo MLS high speed cell sorter

Software
After hybridization, hybridization signals were acquired and normalized using Partek Genomics Suite software (Partek). Differential gene expression between conditions was assessed by statistical linear model analysis using Partek, Tibco Spotfire, and Prism 7 (GraphPad) software. The moderated t-statistic p-values derived from the Partek analysis above were further adjusted for multiple testing by Benjamini and Hochberg's method to control false discovery rate (FDR). An FDR cutoff of < 10% was used to obtain the list of differentially expressed genes.

Cell population abundance
A minimum of 100,000 cells were obtained per isolated cell population.

Gating strategy
Gating parameters are shown in the supplemental figures. Briefly, we validated proper gating of different cell populations by placing an allotted amount of cells on slides and imaged for corresponding fluorescence and cell specific markers. The Johns Hopkins FACS core was used.

☑️ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.