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Gnao1 is a molecular switch that regulates the Rho signaling pathway in differentiating neurons

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GNAO1 encodes G protein subunit alpha O1 (G α O). Pathogenic variations in *GNAO1* cause developmental delay, intractable seizures, and progressive involuntary movements from early infancy. Because the functional role of *GNAO1* in the developing brain remains unclear, therapeutic strategies are still unestablished for patients presenting with *GNAO1*-associated encephalopathy. We herein report that siRNA-mediated depletion of *Gnao1* perturbs the expression of transcripts associated with Rho GTPase signaling in Neuro2a cells. Consistently, siRNA treatment hampered neurite outgrowth and extension. Growth cone formation was markedly disrupted in monolayer neurons differentiated from iPSCs from a patient with a pathogenic variant of G α O (p.G203R). This variant disabled neuro-spherical assembly, acquisition of the organized structure, and polarized signals of phospho-MLC2 in cortical organoids from the patient's iPSCs. We confirmed that the Rho kinase inhibitor Y27632 restored these morphological phenotypes. Thus, Gao determines the self-organizing process of the developing brain by regulating the Rho-associated pathway. These data suggest that Rho GTPase pathway might be an alternative target of therapy for patients with *GNAO1*-associated encephalopathy.

Keywords *GNAO1*, Developmental and epileptic encephalopathy, Differentiation, Organoids, Molecular pathway, Rho-associated kinase

G protein subunit alpha O1 (*GNAO1*) encodes the heterotrimer-forming protein Gao^{1,2}. A growing number of reports have indicated that *GNAO1* is associated with developmental and epileptic encephalopathy (DEE), a devastating neurodevelopmental disorder in infancy and childhood^{3–6}. Affected patients present with severe developmental delay, intractable seizures, and involuntary movements^{5–7}. To date, more than 100 patients with DEE have been reported to carry “pathogenic” or “likely pathogenic” variants of *GNAO1*. Trio-based sequencing indicates that these variations occur de novo³.

GNAO1 and its mammalian homologs are highly expressed in the central nervous system, where they play essential roles in neural circuit formation^{8,9}. In differentiating neurons, Gao is enriched in expression at the tip of the growth cone, a dynamic structure that determines the extension or retraction of neurites to organize functional connectivity with adjacent neurons¹⁰. Thus, Gao is considered to organize local molecular signaling in mature and immature neurons so that they correctly guide the growth cone to external cues¹¹. Indeed, as a GTP-binding protein, Gao activates the downstream molecule CDC42 in cooperation with its binding partner GRIN1¹¹. These data support the notion that Gao regulates morphogenic signaling in the developing brain.

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We recently reported that Gao interacts with another DEE-associated protein, spectrin alpha, non-erythrocytic 1 (SPTAN1), in vitro and in vivo¹². A morphological analysis further suggested that Gao is involved in the molecular pathways associated with cytoskeletal remodeling and neuronal connectivity¹². Thus, pathogenic variants in *GNAO1* directly and indirectly affect a broad category of molecular pathways underlying the development of the neural network¹³. However, the mechanism by which Gao regulates downstream molecular events crucial for neuronal differentiation and cytoskeletal remodeling remains largely unknown.

In this study, we report that siRNA-mediated depletion of *Gnao1* (Gao) in Neuro2a cells perturbs the expression of genes associated with the Rho kinase (ROCK) pathway^{14,15}. This study also highlights the aberrant morphology of growth cones in monolayer neurons expressing the p.G203R variant. We further determine the dominant effects of this variant on the ventricle-like structure and phosphorylation pattern of myosin light chain 2 (MLC2) in cortical organoids. Our data provide clues to help understand how Gao regulates morphogenic signaling in differentiating neurons and in the human brain.

Results

RNA sequencing for Gao-depleted Neuro2a cells

To identify the molecular pathways regulating *Gnao1* (Gao) in neuronal cells, we performed RNA sequencing of the mouse neuroblastoma cell line Neuro2a after treatment with control or *Gnao1*-specific siRNA (si*Gnao1*). Quantitative polymerase chain reaction (qPCR) and Western blotting validated the successful (>90%) knockdown of *Gnao1* expression compared to that in control siRNA-treated Neuro2a cells ($p=0.100$ [qPCR, $n=3$] and $p=0.0286$ [Western blotting, $n=4$], Wilcoxon's rank-sum test; Figs. 1a, S1a–c). In si*Gnao1*-treated

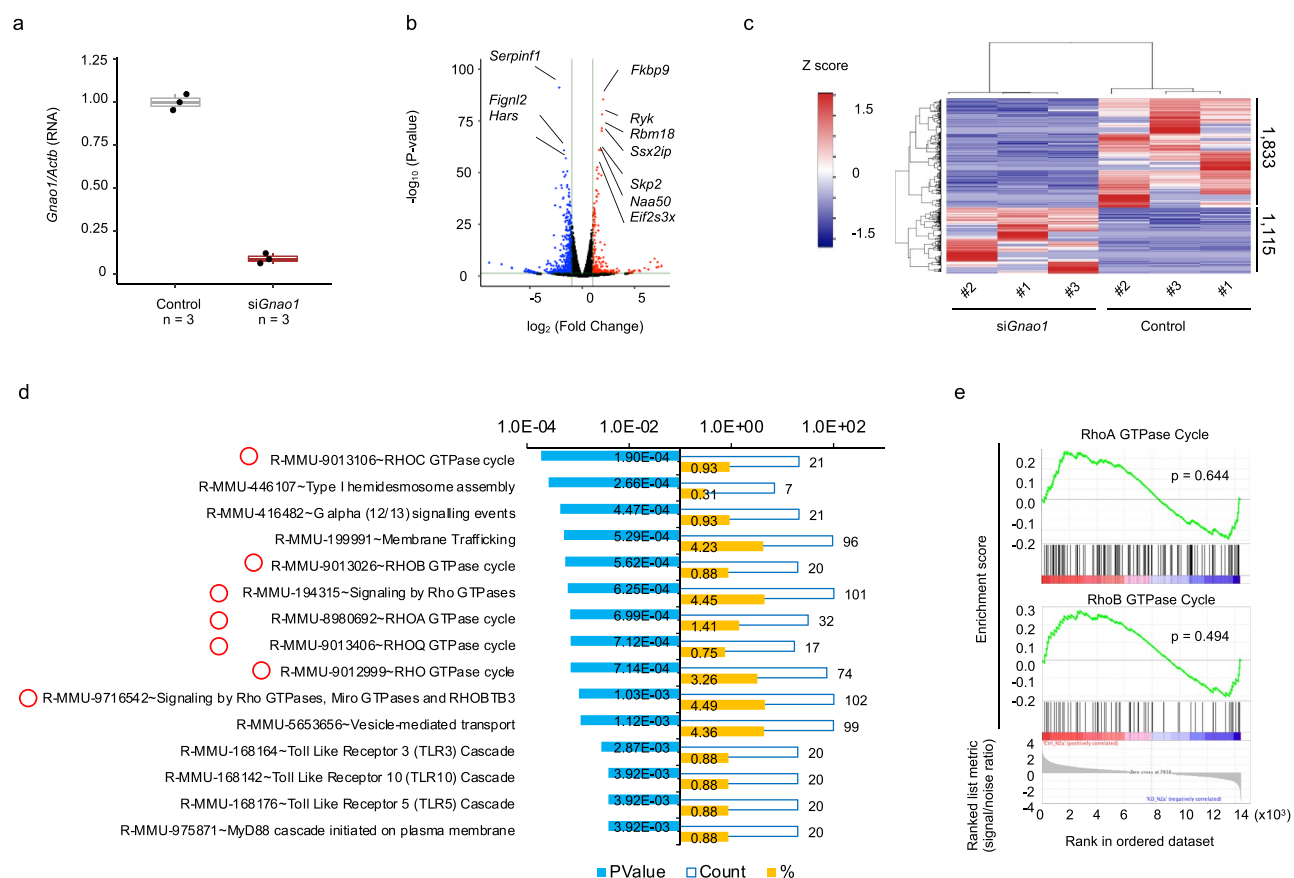


Figure 1. RNA sequencing for Gao-depleted Neuro2a cells. **(a)** Box-dot plot shows the relative expression of *Gnao1* mRNA to actin- β (*Actb*) in Neuro2a cells after treatment with siRNA against *Gnao1* (si*Gnao1*) or control siRNA. $P=0.100$ ($n=3$ each group; Wilcoxon's rank-sum test). **(b)** Volcano plots for genes detected by RNA sequencing. Green lines indicate the cut-off levels: $\log_2$ (Fold Change) > 1 or < -1 ; $p < 0.05$ ($-\log_{10}$ [p -value] > 1.301). Among the 1891 genes with a significant increase (red) or decrease (blue) in expression, the top 10 genes with high $-\log_{10}$ (p -values) were annotated. **(c)** Heat map clustering of rMATS data for the 2948 transcripts differentially expressed in si*Gnao1* and control siRNA-treated Neuro2a cells. The color gradient indicates a Z score > 0 (red: high) and < 0 (blue: low) for the expression of each transcript. **(d)** A Reactome pathway analysis on rMATS data. Bar plots represent p-values for over-representation (blue), the number (white) and percentage (%), orange) of genes annotated in each category. Red circles indicate the categories associated with Rho GTPase signaling. **(e)** GSEA for RhoA and RhoB GTPase cycle pathways in the Reactome. The expression of genes in these categories did not deviate from either the upregulated or downregulated side.

Neuro2a cells, 634 genes were upregulated ($\log_2[\text{fold change}] > 1$; $p < 0.05$ [$-\log_{10} > 1.301$]), whereas 1257 genes were downregulated ($\log_2[\text{fold change}] < -1$; $p < 0.05$) (Fig. 1b). Thus, transcriptomic analysis identified 1,891 genes that were aberrantly expressed in Neuro2a cells after treatment with si*Gnao1*. To evaluate aberrantly expressed transcripts through alternative splicing, we applied the rMATS algorithm to the present dataset¹⁶. We found that a total of 2,948 transcripts (1,115 upregulated and 1833 downregulated) were differentially expressed in si*Gnao1*-treated Neuro2a cells compared with control Neuro2a cells (Fig. 1c).

A Gene Ontology (GO) analysis of rMATS data identified enrichment in “actin cytoskeleton organization” (GO: 0,030,036; $p = 1.29 \times 10^{-26}$, $n = 42$) in Biological Process (BP, Fig. S2a) and “cytoskeletal protein binding” (GO: 0,008,092; $p = 3.08 \times 10^{-8}$, $n = 24$) in Molecular Function (MF, Fig. S2b). Consistently, the Kyoto Encyclopedia of Genes and Genomes (KEGG, <https://www.genome.jp/kegg/>) revealed that 35 genes were overrepresented in the category of “axon guidance” (mmu04360; $p = 2.68 \times 10^{-4}$), 23 genes in “neurotrophin signaling pathway” (mmu04722; $p = 4.64 \times 10^{-3}$), and 16 genes in “ErbB signaling” (mmu04012, $p = 0.0205$) (Fig. S2c). These data suggest that *Gnao1* is involved in cytoskeletal remodeling and the molecular pathways associated with neuronal differentiation¹². We further referenced the 2948 transcripts against the Reactome database (<https://reactome.org/>), in which “Rho GTPase cycle” (R-MMU-9012999, $p = 7.14 \times 10^{-4}$, $n = 74$) and Rho-related pathways were recurrently annotated among the top 15 categories of enrichment (7/15, 47%; Fig. 1d, Table S2). We ensured that the 74 genes (transcripts) in “Rho GTPase cycle” category of Reactome were classified into “GTPase regulator activity” and related categories in the GO database (Fig. S3). Thus, we focused on the functional role of Gao in the regulation of Rho-associated molecular pathways. Consistent with this finding, “chromatin organization” (GO: 0,006,325), “DNA repair” (GO: 0,006,281) and “MAPK signaling pathway” (KEGG: mmu04010) were ranked at the top of each category. These data suggest that the depletion of *Gnao1* broadly affected broad molecular pathways downstream to Rho GTPase signaling in nuclear and cytoplasmic compartments of Neuro2a cells^{17–19}.

In contrast, according to a Gene Set Enrichment Analysis (GSEA), genes involved in the Rho GTPase pathway did not show a deviated expression, either to the upregulated or downregulated side (Fig. 1e). Thus, enrichment of Rho GTPase-associated pathways reflected uniquely expressed splicing isoforms but not the summed expression of transcripts in si*Gnao1*-treated Neuro2a cells. Consistent with this notion, the GSEA showed that genes associated with “RNA splicing” and “snRNP assembly” pathways were significantly downregulated in si*Gnao1*-treated Neuro2a cells (Fig. S4).

Impaired neurite outgrowth of *Gnao1*-depleted Neuro2a cells

Based on the assumption that Rho-associated molecular signaling is hyper- or hypoactive in Gao-depleted cells, we examined whether their proliferation rates differed from that of control Neuro2a cells. However, si*Gnao1*-treated Neuro2a cells showed a growth rate similar to that of control cells ($p > 0.05$, Steel–Dwass’ multiple comparison; Fig. S5a). Consistently, a cell cycle analysis did not show significant differences in the proportions of G0/G1, S, G2/M, and subG1 phases between si*Gnao1*-treated and control Neuro2a cells ($p > 0.05$, Steel–Dwass’ multiple comparison; Fig. S5b).

Given the versatile functions of Rho GTPases in cell cycle, epigenetic regulation, and cytoskeletal remodeling, we first tested whether the si*Gnao1* treated cells show disproportionate % of cells in S-phase. Actively growing cells in S-phase are also known to increase the nuclear size and the N/C ratio^{20,21}. Thus, we determined whether si*Gnao1*-treated cells are disproportionately classified into S-phase in comparison with that in Neuro2a cells. Morphologically, si*Gnao1*-treated cells showed a higher nuclear-cytoplasmic (NC) ratio than those treated with control siRNA (median: 0.486 [si*Gnao1*] vs. 0.416 [control], $p = 3.23 \times 10^{-15}$, Wilcoxon’s rank-sum test; Fig. S5c, d). Despite that siRNA-mediated depletion of *Gnao1* did not affect either the proliferation or cell-cycle progression, si*Gnao1*-treated Neuro2a cells showed the higher NC ratio than controls. These data suggested that *Gnao1* plays more important roles in gene expression and morphological regulation than in cell-cycle progression of Neuro2a cells.

Because Rho GTPases and related pathways regulate axonal regeneration in vitro and in vivo²², we tested whether siRNA-mediated depletion of *Gnao1* affects neurite outgrowth in Neuro2a cells. As previously reported¹², the si*Gnao1*-treated Neuro2a cells showed shorter and fewer neurites than those treated with control siRNA ($p = 1.76 \times 10^{-7}$ [length] and 3.32×10^{-5} [number], respectively, Wilcoxon’s rank-sum test; Figs. 2a, b and S6). To investigate the organization of cytoskeletal molecules in si*Gnao1*-treated Neuro2a cells, we quantitatively analyzed the immunofluorescence signals of F-actin (phalloidin) and tubulin $\beta 3$ (Tuj1) by dissecting the structure of neurites into stem and tip regions (Fig. 2c). In both regions, si*Gnao1*-treated Neuro2a cells showed lower Gao and Tuj1 signals than those treated with control siRNA ($p < 2.2 \times 10^{-16}$, Kruskal–Wallis test, $n = 5$ [ROIs] for each group; Fig. 2d, e). Notably, the F-actin signal was highly enriched at the tip (growth cone) of neurites in the control siRNA-treated cells. In contrast, such a gradient pattern of F-actin signals was absent in si*Gnao1*-treated Neuro2a cells, and their neurites showed numerous flossy protrusions expressing F-actin ($p < 2.2 \times 10^{-16}$, Kruskal–Wallis test, $n = 5$; Fig. 2d, e).

A dominant effect of the Gao variant p.G203R on neuronal differentiation

To clarify the functional role of Gao in an experimental model of the human brain, we recently established iPSCs from a healthy volunteer¹². We inactivated the endogenous *GNAO1* gene using the CRISPR–Cas9 system. To further investigate the pathogenic effects of a known variant of Gao, we used iPSCs from a patient carrying a de novo heterozygous variant of p.G203R^{5,12}. When we analyzed the growth of neurospheres during the first 15 days of differentiation, p.G203R-expressing iPSCs, but not *GNAO1*-knockout (KO) iPSCs, showed a significant delay in the growth of neuro-spheres ($p = 8.88 \times 10^{-14}$, Steel–Dwass’ multiple comparison test [factors: day and WT vs. PT; Fig. 3a, b).

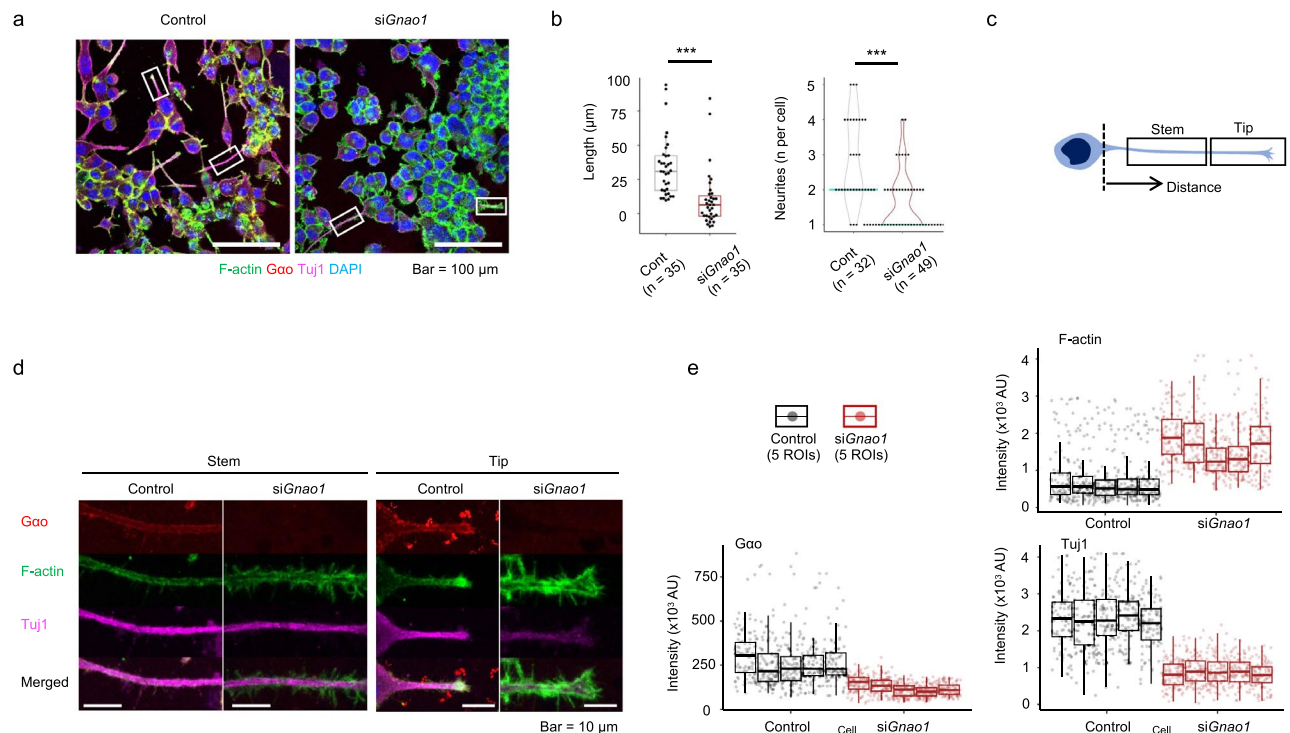


Figure 2. Morphological analyses of Gao-depleted Neuro2a cells. **(a)** An overview of control (left) and Gao-depleted (right: siGnao1) Neuro2a cells. Merged images of F-actin (phalloidin, green), Gao (red), Tuj1 (magenta), and DAPI (blue) are shown. The boxes indicate the regions of interest provided in Panel d. **(b)** Quantitative data on the length (nm) of neurites and number (counts per cell). Median values are shown as horizontal lines (light blue) on the violin plots. *** $p < 0.001$. **(c)** Schematic representation of the stem and tip portions of one neurite. The distance represents the length from the soma-neurite junction to the point of fluorescence measurement. **(d)** Three-channel images of the stem (left) and tip (right) regions of the neurites. **(e)** Quantitative measurements of F-actin, Gao, and Tuj1 signals in panel d. Data from control (black) and siGnao1-treated Neuro2a cells (brown) are shown in box-dot plots. All $p < 0.001$ (siGnao1 vs. control; Kruskal–Wallis test, $n = 5$ [ROIs] for each group). The distance and fluorescence intensity are labelled on the X and Y axes, respectively. AU, arbitrary unit.

To test whether the morphological findings of Neuro2a cells are reproducible in human iPSC-derived neurons, we observed neurite outgrowth in monolayer neurons differentiated from organoids on day 15 of differentiation. We observed neurite outgrowth in monolayer neurons on day 15 of differentiation. Monolayer neurons from a healthy adult's iPSCs (HA) clearly formed lamellipodia and filopodia structures of growth cones at the end of the neurites (Fig. 3c, d). The number of filopodia per growth cone did not differ between HA and KO neurons ($p = 0.085$ [HA vs. KO], $n = 3$ at each point, Steel–Dwass' multiple comparison test; Fig. 3e). However, the patient-derived neurons showed even more dysmorphic lamellipodia with fewer filopodia per growth cone than those in HA neurons ($p = 0.00870$ [HA vs. PT], Steel–Dwass' test; Fig. 3e). These data support the notion that the p.G203R variant of Gao has a gain-of-function and/or dominant-negative effect on neuronal differentiation from iPSCs.

Dysmorphic appearance of cortical organoids expressing the p.G203R variant of Gao

To further characterize the structure of developing neurons, we continued the differentiation of cortical organoids until they formed the laminar structure of the ventricular zone (VZ) and cortical plate (CP) by day 60–65 of differentiation in vitro. At this stage, the HA organoids developed a VZ, which showed a ventricle-like appearance (Fig. 4a, b). In KO organoids, a more round- and ovoid-shaped VZ was more predominant in appearance than in HA organoids. The structures in PT organoids were too dysmorphic to evaluate the laminar structures of the VZ and CP. When we closely examined the VZ and CP regions, Gao-KO organoids showed a comparable thickness of VZ (median 82.4 μm [HA] vs. 69.8 μm [KO], $p = 0.063$, Wilcoxon's rank-sum test; Fig. 4c). However, KO organoids showed a thinner CP than HA organoids (median 134.6 μm [HA] vs. 68.9 μm [KO], $p = 0.014$, Wilcoxon's rank-sum test; Fig. 4c). The thickness of either the VZ or CP was immeasurable in PT organoids. Thus, we evaluated the circularity of the VZ in HA, KO, and PT organoids. We confirmed that PT organoids formed a VZ with lower circularity than KO and PT organoids ($p = 0.009$ [HA vs. PT] and $p = 2.1 \times 10^{-5}$ [KO vs. PT], Kruskal–Wallis test; Fig. 4d, S7).

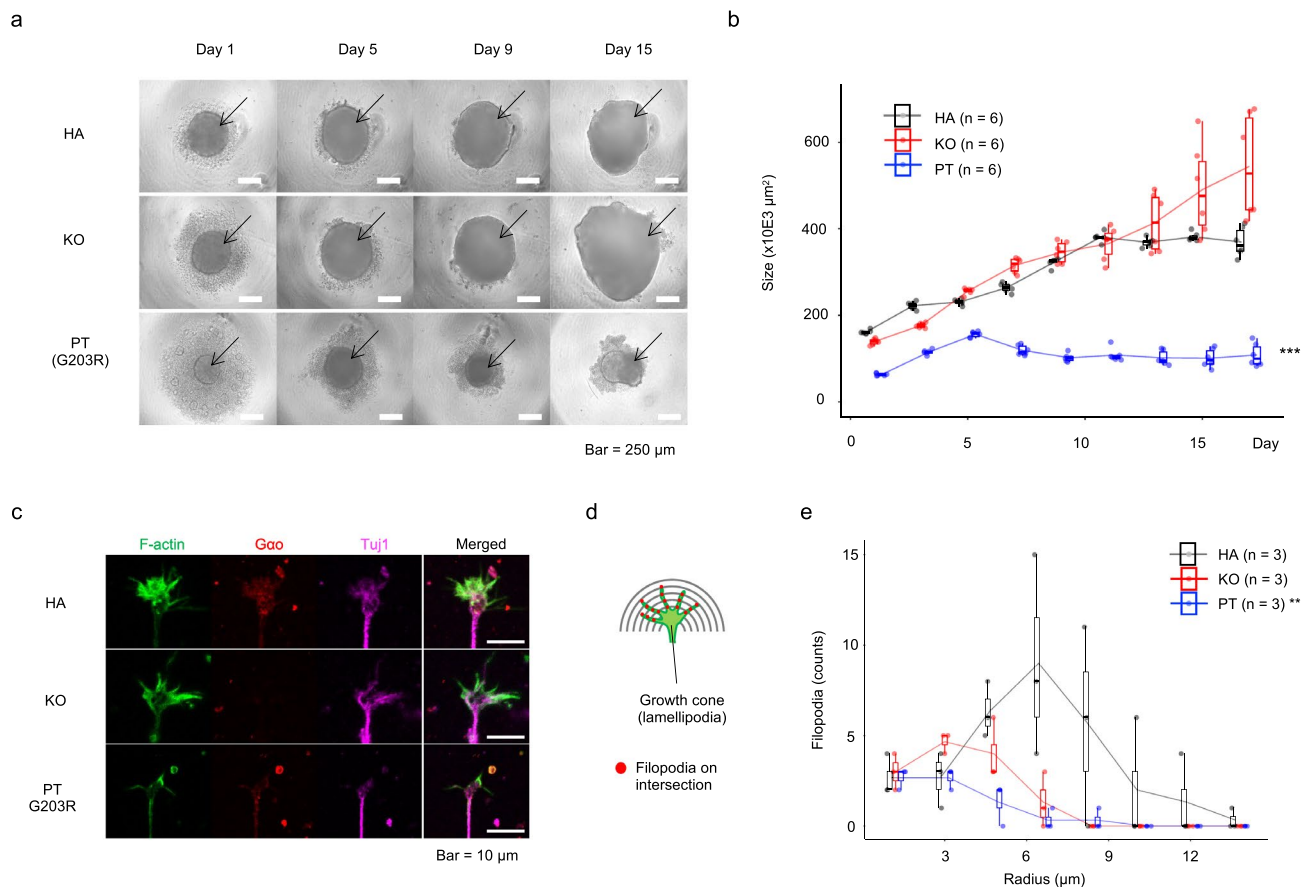


Figure 3. Morphology of neuro-spheroids and neuronal growth cones. **(a)** Phase-contrast images of neuro-spheroids (arrows) from days 1 to 15. HA, iPSCs from a healthy adult; KO, Gao-knockout iPSCs from the same individual as HA; PT, iPSCs from a patient with p.G203R variant. **(b)** Quantitative results of HA, KO, and PT-derived neuro-spheroids. Box-dot plots are line-connected with the mean values of spheroid size at indicated days of differentiation. *** $p < 0.001$ (HA vs. PT) and $p = 0.484$ (HA vs. KO), Steel-Dwass' multiple comparison test ($n = 6$). Six independent spheroids were used for this analysis. **(c)** Confocal images of growth cones in monolayer neurons differentiated from HA, KO, and PT-derived iPSCs. **(d)** A schematic diagram of the Sholl analysis. Segmented filopodia are depicted as red dots on green protrusions, extending from a growth cone (lamellipodia). The red dots are counted as the number of filopodia at a defined radius (μm). **(e)** A Sholl analysis of growth cones in the HA, KO, and PT-derived neurons. Box-dot plots are line-connected with mean values of the number of filopodia at the indicated radius. $P > 0.05$ (HA vs. KO) and ** $p < 0.01$ (HA vs. PT); Steel-Dwass' test ($n = 3$). Three independent organoids were analyzed.

Expression of neuronal lineage markers in organoids

Because we did not perform the microscopic analysis at a single-cell resolution, we were unable to count the number of progenitors and terminally differentiated neurons in the VZ and CP regions. To compensate this information, we quantitatively measured the expression level of molecular markers in the respective regions. We found a contrasting expression of progenitor (FOXG1: a marker for dorsal telencephalon^{23–30}) and mature (NeuN) neuronal markers in the VZ of HA organoids, where FOXG1 was high and NeuN was low (Figs. 4a, b and S8). Consistently, the PAX6 signal (a dorsal forebrain progenitors^{23,28,29,31}) was higher in VZ than in CP of HA organoid. Thus, the NeuN/FOXG1 ratio was as low as 0.515 (range: 0.237–2.76) in the VZ of HA organoids. KO organoids also showed a lower NeuN/FOXG1 ratio than VZ in HA organoids (KO: median 0.503 [range: 0.233–1.28], $p = 0.045$ [HA vs. KO]). In contrast, PT organoids showed a higher NeuN/FoxG1 ratio (median 0.905 [range: 0.312–3.66]) than HA organoids ($p < 2 \times 10^{-16}$ [HA vs. PT], Kruskal–Wallis test; Fig. 4e).

Consistently, HA and KO organoids expressed another progenitor marker PAX6 at the higher level in VZ than in the CP region (Fig. S9a,b). In contrast, they expressed mature neuronal markers, Reelin and Tuj1 (tubulin-β3) in VZ at lower levels than in CP. Reelin showed the strongest signals at the outmost layer of CP (Fig. S9a), supporting the concept that the population of Reelin-expressing (Cajal-Retzius) cells are distinct from those of PAX6-expressing dorsal progenitors^{23,26,28,29,31}. As observed in the FOXG1-NeuN immunofluorescence data, the PT organoids showed a highly disorganized pattern of VZ-CP regionalization. It was therefore suggested that p.G203R-mutant Gao disturbed the process of neurogenesis or neuronal differentiation. These data suggest that the p.G203R variant affects the physiologically regulated process of neuronal differentiation of human iPSCs.

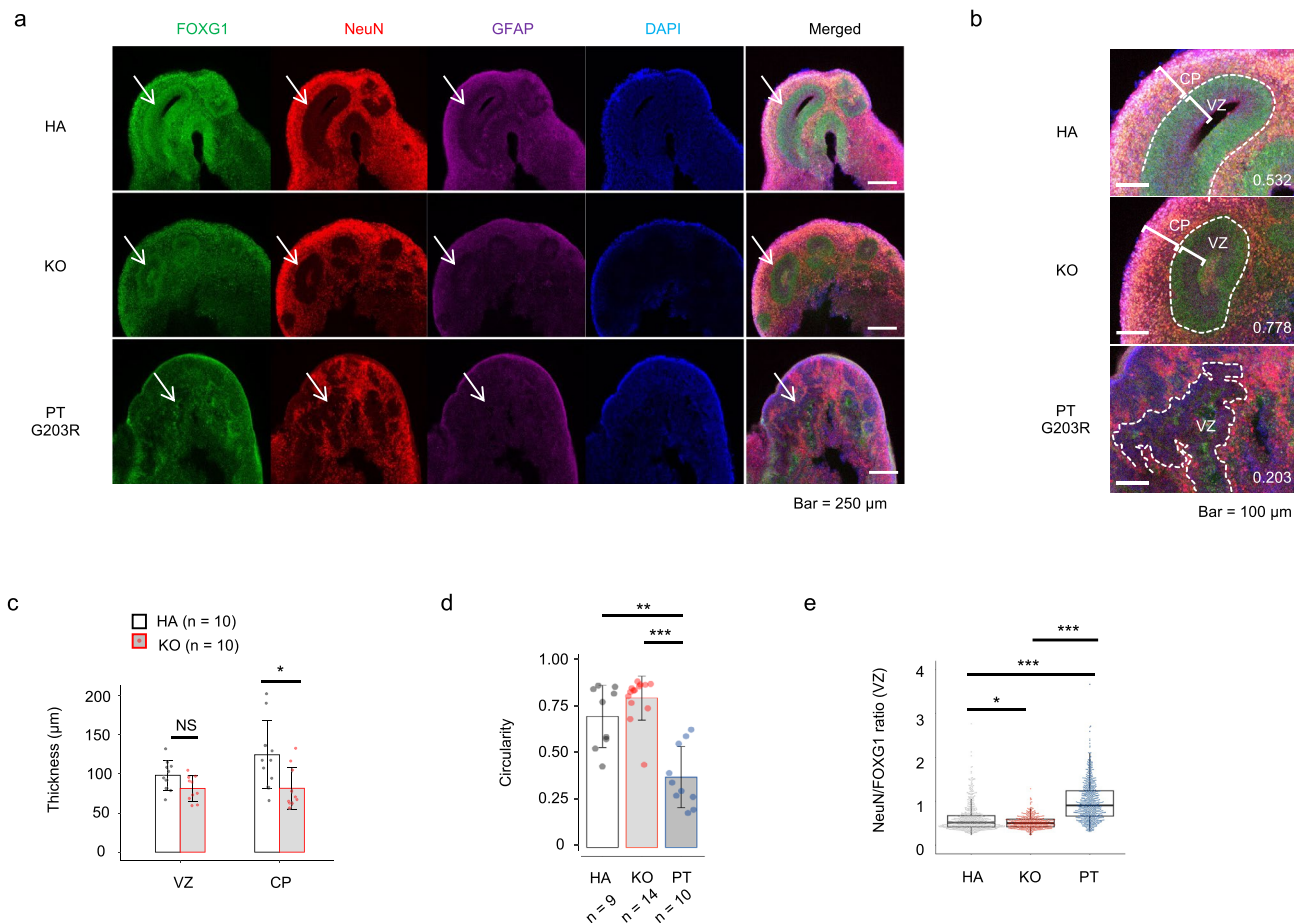


Figure 4. Morphological analyses of cortical organoids. (a) Immunofluorescence images of organoids from a healthy adult (HA), *GNAO1*-knockout (KO), and patient (PT, p.G203R)-derived iPSCs. Organoids on day 60 of differentiation were used in this study. HA and KO organoids clearly developed VZ structures (arrows). PT-derived organoids showed a disordered VZ structure. (b) High-magnification images of the ventricular zone (VZ) and cortical plate (CP) in HA, KO, and PT-derived organoids. The dashed lines indicate the boundaries between the VZ and CP layers. Values indicate the circularity index of each VZ. (c) Quantitative results of VZ and CP thickness. * $p < 0.05$ (Wilcoxon's rank-sum test). The thicknesses of the VZ and CP were unmeasurable in the PT organoids. For C–E, three independent organoids per group were subjected to analysis. (d) A circularity analysis of the VZ in HA, KO, and PT-derived organoids. * $p < 0.05$ (Kruskal–Wallis test). (e) Box-dot plots for the NeuN/FoxG1 signal ratio in the VZ. Small dots represent the value in each ROI ($n = 621$ [HA], 406 [KO] and 628 [PT]). * $p < 0.05$ and *** $p < 0.001$ (Kruskal–Wallis test).

Aberrant phosphorylation of myosin light chain in PT organoids

To determine the relationship between Rho GTPase signaling and the dysmorphic phenotype of KO and PT organoids, we evaluated the immunofluorescence signals of phospho-myosin light chain (pMLC2), an activation marker of the Rho kinase pathway³². In HA organoids, pMLC2 signals were highest in the outer layers of the CP, where the dendrite marker MAP2 was co-expressed (HA, Fig. 5a). Similarly, higher pMLC2 and MAP2 signals were observed in the CP layer than in the VZ layer of the KO organoids (KO, Fig. 5a). However, MAP2-positive neurons were localized in the outermost layer of the CP in KO organoids. In contrast to these findings in HA and KO organoids, PT organoids showed significantly lower signals of pMLC2 in the outer layer of the CP, resulting in the loss of the “CP-high and VZ-low” gradient in pMLC signals ($p = 1.47 \times 10^{-13}$ [HA vs. KO], 3.34×10^{-14} [HA vs. PT], and $< 2.2 \times 10^{-16}$ [KO vs. PT]; $n = 6$ each; Steel–Dwass' multiple comparison test; Fig. 5a,b). PT organoids also expressed lower levels of MAP2 than in KO organoids ($p = 3.33 \times 10^{-14}$ [HA vs. KO], 0.196 [HA vs. PT], and 2.19×10^{-12} [KO vs. PT], $n = 6$ each; Fig. 5a, b).

These results suggested that the gradient pattern of neuronal differentiation (MAP2 and NeuN signals) might reflect the regional difference in the activity of Rho GTPase signaling in HA organoids. We also wondered whether the correlation might be disturbed in KO and PT organoids. To test this possibility, we statistically analyzed the correlation between MAP2 and phosphorylated MLC2 (pMLC2). When we set 140 regions of interest (ROIs) in CP, the expression of MAP2 was correlated with pMLC2 signals in HA ($p < 2.2 \times 10^{-16}$, Spearman's rank correlation, KO ($p < 2.2 \times 10^{-16}$), and PT organoids ($p < 2.2 \times 10^{-16}$; Fig. S10a, b). Notably, the correlation of pMLC2 and MAP2 signals in HA organoids was distributed over a wider range than that in the KO and PT organoids

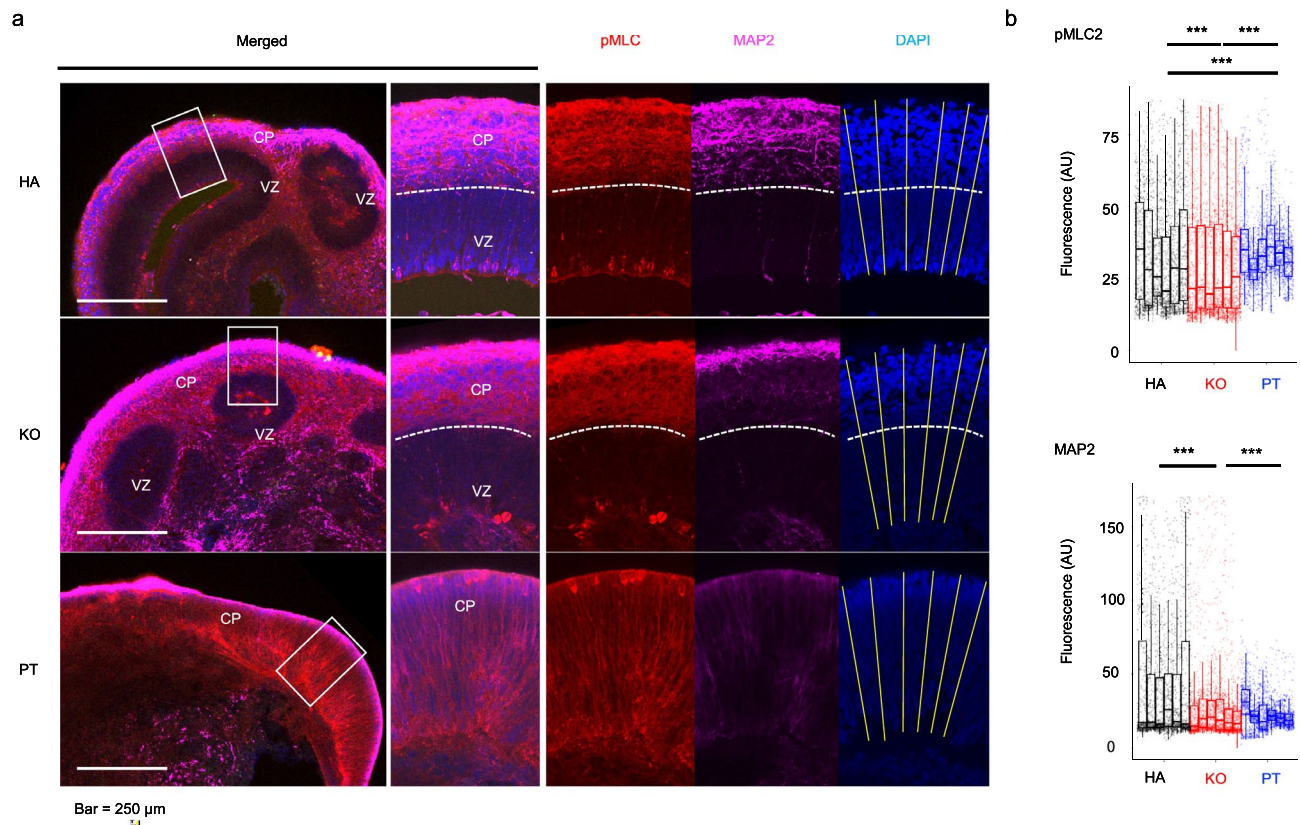


Figure 5. Aberrant phosphorylation of MLC2 in patient-derived organoids. **(a)** Immunofluorescence signals of pMLC2 (red) and MAP2 (magenta) in healthy adult (HA), *GNAO1*-knockout (KO), and patient-derived (PT) organoids after 60 days of differentiation. Nuclei were counterstained with DAPI (blue). The rectangles in the left panels indicate the regions selected for higher magnification (right panels). Solid lines indicate the positions of the optical sections for the quantitative measurement of pMLC2 and MAP2 signals across the CP and VZ layers. Dashed lines denote the boundaries of the CP and VZ layers. **(b)** Box-dot plots for pMLC2 (upper) and MAP2 (lower) signals in HA, KO, and PT organoids. *** $p < 0.001$ (Steel–Dwass’ multiple comparison, $n = 6$ for each group). Six optical sections are shown with yellow lines (DAPI, panel a), from which 360–390 ROIs (single pixel) were randomly selected. AU, arbitrary unit. Three independent organoids per group were subjected to this analysis.

(Fig. S10b, lower). Thus, Rho kinase-associated pathways were profoundly deregulated in PT organoids and to a lesser extent in KO organoids than in PT organoids.

Causal effect of Rho signaling pathways on the dysmorphic structure of organoids

To validate the causal effect of Rho kinase activity on the anomalous development of organoids, we further investigated whether the Rho kinase inhibitor Y27632 restored the dysmorphic phenotype of PT organoids. During the first 15 days of differentiation, an increasing Y27632 concentration (50–250 μM) assisted the assembly and growth of PT neuro-spheroids ($p = 5.18 \times 10^{-9}$ [100 μM vs. 50 μM], 1.61×10^{-3} [250 μM vs. 50 μM], $n = 6$, Steel–Dwass’ multiple comparison; Fig. 6a, b). More specifically, PT spheroids treated with 100 μM Y27632 (light blue) gained significantly larger size after day 5 of differentiation compared to control (50 μM: gray) (Fig. 6a, b). When treated with a higher concentration of Y27632 (250 μM: blue), PT spheroids showed delay in aggregate formation during the first week, but they caught-up in growth by day 15 of differentiation.

Immunofluorescence data showed that the round-shaped structure of the VZ inside the CP layer of PT organoids began to recover by day 15 of treatment with Y27632 (100 μM, Fig. 6c). Of note, treatment of PT organoids with a higher concentration (250 μM) of Y27632 failed to restore the dysmorphic structure of the VZ (Fig. 6c). However, treatment with 100 and 250 μM Y27632 redeemed the “FoxG1-high and NeuN-low” gradient pattern to VZ in PT organoids. Quantitative measurement of immunofluorescence signals revealed that the NeuN/FoxG1 ratio was lower in PT organoids treated with 100 and 250 μM Y27632 than that in organoids treated with 50 μM Y27632 ($p < 2 \times 10^{-16}$ [100 μM vs. 50 μM] and $p < 2 \times 10^{-16}$ [250 μM vs. 50 μM], $p = 5.51 \times 10^{-150}$, Kruskal–Wallis test; Fig. 6d).

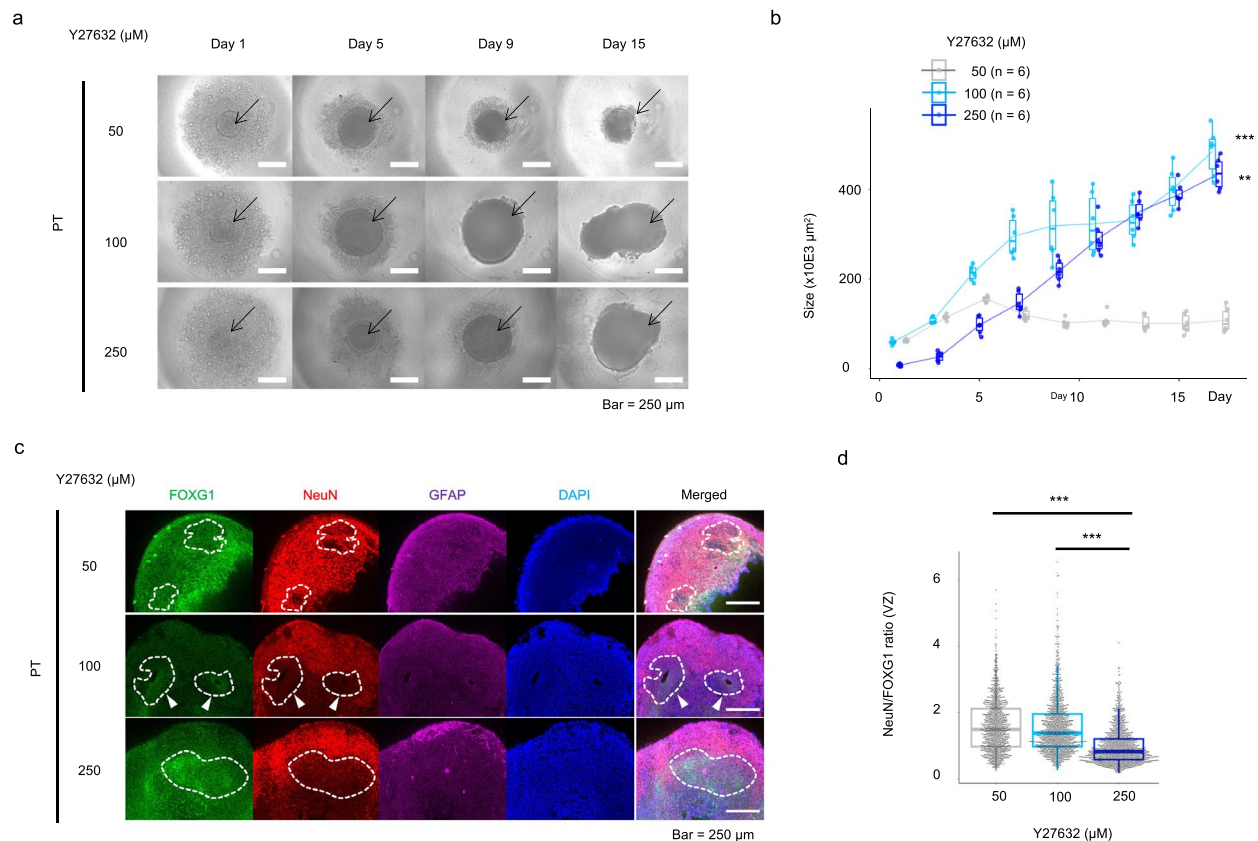


Figure 6. Restoring the growth and differentiation of patient-derived organoids by Rho kinase inhibitor. (a) Phase-contrast images of patient (PT)-derived neuro-spheroids (arrows) after treatment with 50, 100 and 250 μM Y27632. Microscopic images were captured at the indicated time points (days 1–15). (b) Area size of spheroids (μm^2) on the indicated days of treatment. Box-dot plots are line-connected with the mean values of spheroid size at indicated days of differentiation. $**p < 0.01$, $***p < 0.001$ ($n = 6$, Steel–Dwass' multiple comparison [50 μM vs. 100 μM] and [50 μM vs. 250 μM]). Six independent spheroids were used for this analysis. (c) Immunofluorescence images of PT organoids treated with 50, 100, and 250 μM Y27632. Organoids were cryo-sectioned 15 days after treatment with Y27632. The dashed lines indicate the VZ region. Arrowheads indicate that round-shaped VZ structures were recovered after treatment with 100 μM Y27632. (d) NeuN/FoxG1 ratio in the VZ of PT organoids treated with 50, 100, and 250 μM Y27632. Small dots represent the value in each ROI ($n = 1,106$ [50 μM], 1,357 [100 μM] and 1438 [250 μM]) $***p < 0.001$ (Kruskal–Wallis test). Three independent organoids were analyzed.

Discussion

This study provided experimental data to rationalize the regulatory role of Gao in Rho GTPase-associated signaling using the mouse neuroblastoma cell line Neuro2a and human brain organoids. The results of unsupervised transcriptomic analyses led us to link the distinct phenotypes of Gao-depleted Neuro2a cells to the deregulation of Rho GTPase signaling. Morphologically, Neuro2a cells treated with siRNA against *Gnao1* showed shorter and fewer neurites, which were also characterized by the loss of polarized F-actin assembly at their far-end and the presence of fluffy protrusions in the stem portion, than control Neuro2a cells. In agreement with such findings in Neuro2a, Gao-KO cortical organoids presented an altered morphology of neuronally differentiating cells. Furthermore, the Gao-mutant (p.G203R) organoids showed hardly any identifiable VZ and CP layers in their internal structures. These dysmorphic features were associated with aberrant patterns of pMLC2 signals in the Gao-KO and mutant (p.G203R) organoids. The ROCK inhibitor Y27632 partly rescued the dysmorphic phenotypes of the Gao-mutant organoids. Thus, the pathogenic variant (p.G203R) of Gao was considered to induce hyperactivation of the Rho kinase pathway, which deregulates the proliferation and differentiation of neuronally committed progenitors. Specifically, p.G203R-expressing iPSCs might have lost the appropriate timing of differentiation from GFAP-expressing neural stem cells to FOXG1 and/or PAX6-expressing progenitors. In this regard, aberrant Rho GTPase signaling in patient (p.G203R) organoids might have driven the GFAP-expressing stem cells to exit prematurely to neuronal progenitors. Thus, the population of FOXG1-expressing progenitors might be increased in the patient organoids. Consequently, neuronal progenitors in patient (p.G203R) organoids might have missed the physiological condition of gene expression to differentiate themselves into NeuN and Reelin-expressing mature neurons.

Recent studies have shown that the p.G203R variant causes a *neomorphic* effect on GTPase activities of Gao1². In other words, pathogenic variants in *GNAO1*, including p.G203R, exhibit opposing features of loss-of-function,

gain-of-function, and dominant-negative profiles in their GTPase functions. In agreement with this concept, we found that the growth cone formation and Rho GTPase-related pathway were more profoundly affected in p.G203R-expressing neurons than in *GNAO1*-knockout neurons. Collectively, these data suggested that the p.G203R variant might render Gao to exert the neomorphic functions in Rho GTPase-related molecular pathways. According to the Bgee database (<https://www.bgee.org/gene/ENSG00000087258>), a bulk-RNA sequencing supported evidence that *GNAO1* is expressed in the fetal cortical plate at 14 weeks of post-fertilization. Thus, as recently reported³³, *GNAO1* is likely to regulate the process of differentiation and maturation of neuronal progenitors.

Given the higher NeuN/FOXG1 ratio in the patient's organoids than control and KO organoids, one could hypothesize that the patient's organoids gained the phenotype of accelerated neurogenesis, but not impairment in the process of neuronal differentiation. We previously reported that the patient's organoid showed higher signals of GFAP-expressing neurons on the surface layer than KO and control organoids¹². From these data, however, we cannot still conclude that the p.G203R variant Gao promotes neurogenesis or neuronal differentiation. To address this issue, we need to clarify the amount of proliferating GFAP-expressing stem cells with immunolabelling the BrdU incorporation. It will be also informative to characterize when GFAP-positive neurons start expressing the differentiation markers (FOXG1, PAX6, Reelin, and NeuN) during 60 days of organoid development.

The Rho family of GTPases consists of 20 subclasses in mammalian species, all of which belong to the Ras superfamily³⁴. Among them, the three most common Rho GTPases (Cdc42, Rac1, and RhoA) have been extensively studied³⁵. As small GTP-binding proteins (21 kDa), Rho GTPases play versatile roles in cell mobility, the cell cycle, adhesion, neurite outgrowth, and synaptic plasticity^{14,15,36}. Specifically, local activation of Rho GTPases stimulates regional actin polymerization and cytoskeletal remodeling by stimulating numerous downstream effectors³⁷. In excitatory synapses, RhoA and Cdc42 relay the transient CaMKII activation to synapse-specific, long-term signaling required for spinal structural plasticity^{14,15}. Thus, the phosphorylation status of potential targets of Rho GTPase must be further investigated. For the same reason, it is worth studying the gene expression profiles of p.G203R-expressing neurons before and after treatment with Y27632 and other ROCK inhibitors.

Recent studies have focused on Rho GTPases as potential biomarkers and therapeutic targets for schizophrenia^{38–40} and epilepsy⁴¹. For example, rare copy number variations (exonic deletions and duplications) and a single nucleotide variant (p.S490P) have been recurrently identified in *ARHGAP10* among Japanese patients with schizophrenia³⁸. Animal studies also indicated that ROCK is a promising target of treatment for emotional and cognitive dysfunctions of *Arhgap10*-mutant mice^{39,40}. Furthermore, pathogenic variants in *Rho-related BTB domain-containing protein 2 (RHOBTB2)* have been identified in patients with DEE, the most severe form of epilepsy syndrome in childhood^{42,43}. Intriguingly, RHOBTB2 is a substrate of the ubiquitin ligase, Cullin-3 (CUL3), another DEE-associated protein⁴⁴. In this study, we found that HA organoids developed MAP2-expressing neurons in association with the activity of ROCK (pMLC2 signal), one of the molecular markers for the activity of Rho GTPase signaling. In contrast, KO and PT organoids appeared to have impairments in efficiently mediating the Rho GTPase signals to the downstream pathways and conversion of ROCK-dependent signaling to neurogenesis or neuronal differentiation. Given the regulatory role of Gao in the Rho-kinase pathway, a certain group of DEE-associated proteins may share a convergent molecular function, enabling them to cooperatively organize neural circuits in the developing brain.

Clinically, *GNAO1*-associated DEE is characterized by severe developmental delays, uncontrollable seizures, and various types of involuntary movements. Most patients show only minor features on brain MRI; however, a thin corpus callosum with dilated ventricles is commonly observed^{1,45}. Hypoplastic caudate nuclei have also been identified in multiple patients^{1,45,46}. Thus, both regional and interspherical neural connectivity are likely to be affected in patients with *GNAO1*-associated DEE. Our previous data also suggested that the expression of the pathogenic variant of Gao in differentiating neurons disturbs the process of neurite extension required for neural network formation¹². Consistent findings were also shown with human iPSC-derived neurons³³. Progressive choreoathetosis and dystonia have been reported in patients carrying *GNAO1* variants^{47–49}. These findings suggest a pathogenic link between Gao and Rho-kinase in degenerative changes in dopaminergic neurons^{50,51}.

An MRI study identified a right frontal T2-hyperintense lesion, which was pathologically diagnosed as diffuse astrocytoma (WHO grade II), in 1 patient at 16 years old⁴⁵. It might be reasonable to hypothesize that hypomorphic variants of *GNAO1* cause aberrant differentiation of neurons or tumorigenic conditions as a consequence of hyperactive Rho GTPase signaling. Although no other patients have been reported to develop brain tumors, the pathogenic variant p.R209C in *GNAO1* was identified as a driver of infantile-onset acute lymphoblastic leukemia⁵². Thus, the number of patients with low-grade astrocytoma or glioma may be further determined through long-term observation of children and adults with *GNAO1*-associated DEE.

In our bioinformatics analysis, Rho families and related molecular pathways were recurrently annotated in Reactome but not in the KEGG database. These dissociated results stemmed mainly from the difference in the number of “hit” genes that were successfully retrieved in these databases. Of the 2948 differentially expressed transcripts, 1280 (43.4%) genes were listed in the Reactome, whereas only 845 (28.7%) were identified in KEGG. Thus, our experimental data were evaluated in more detail by submitting the dataset to Reactome rather than to KEGG. In addition, we used the rMATS algorithm to attribute the read depth of the transcripts to their expression¹⁶. This approach detected splicing isoforms that were differentially expressed among samples at a higher resolution than that of the conventional method for measuring the expression of transcripts at the gene level. Because ROCK is known to regulate alternative splicing in the brain⁵³, our data suggest that the loss of Gao expression induces an unbalanced expression of splicing isoforms.

Several limitations remain to be addressed before this conclusion can be confirmed. First, we did not perform a single-cell transcriptome analysis using *GNAO1*-KO or patient-derived organoids expressing the p.G203R mutant of Gao. A comparative analysis of their RNA expression with that of HA organoids will clarify the neuronal populations and the timing at which they start showing hyperactive Rho-associated kinase signaling.

Thus, it also remains to be elusive whether ROCK inhibitor (Y27632) shows therapeutic effects on morphological phenotype of patient's organoids only at the early stage (within the first week) of differentiation. For this purpose, we must test whether Y27632 might be also effective when administered at 2 weeks of differentiation or later. Second, we established only classical (cortical) organoids using a conventional method⁵⁴. Directing the patient-derived iPSCs to differentiate into striatal and inhibitory neurons will further clarify the distinctive roles of Gao in different subtypes of brain organoids and in "assembloids"⁵⁵. Third, we cannot exactly characterize the molecular features of the patient-derived organoids until we establish those expressing the wild-type Gao on the isogenic backgrounds. For this purpose, it will be worth investigating whether the neuronal phenotypes of the patient's cortical organoids might be mitigated by the CRISPR/Cas9-based gene editing system or the knock-down of p.G203R-mutant Gao expression. The ectopic expression of the mutant Gao in the control iPSCs may further identify molecular pathways that might be specifically altered in neurons expressing p.G203R-mutant Gao, but not in those expressing other mutants. Thus, the combined analysis of single-cell RNA sequencing for patient-derived organoids with the current dataset will provide further insight into the unique role of Gao in Rho GTPase signaling.

Conclusions

We first demonstrated that *GNAOI* (Gao) regulated gene expression associated with Rho GTPase signaling in Neuro2a cells. In combination with the findings in human iPSC-derived organoids, our data suggest that Gao acts as a molecular switch for Rho-associated kinase, which plays versatile roles in the developing brain. Further analyses of how Gao interacts with Rho GTPases and their effector molecules will facilitate the identification of molecular targets of therapy for patients with *GNAOI*-associated DEE.

Methods

Reagents

Key reagents including antibodies and oligonucleotide primers are summarized in Table S1. Details in experimental procedures are described in *Supplementary Materials*.

Real-time quantitative PCR

Total RNA was extracted using an RNeasy Mini Kit (#74,104; Qiagen, Germantown, MD, USA). Complementary DNA was synthesized using a High-Capacity RNA to cDNA Kit (#4,387,406; Thermo Fisher Scientific) according to the manufacturer's protocol. Assays were performed using the SYBR Green system (#438,561; Thermo Fisher Scientific)¹². The delta-delta cycle threshold algorithm was applied for the quantitative measurement of mRNA expression. Human or murine homolog of actin beta (*Actb*) was used as an internal control (Table S1).

RNA sequencing

The Neuro2a control group (n = 3) was compared with the siGnao1-treated group (n = 3). Sequencing was performed on an Illumina platform (Illumina Inc., San Diego, CA, USA) with a read length of 150 bp pair-ends and 50–60 million reads per sample. The resulting FASTQ files were quality-checked using FastQC version 0.11.9 and trimmed with trimmomatic version 0.38. The quality-checked data were then mapped to the mouse reference genome (GRCm39) using the HISAT2-2.1.0 software program. Reads were counted using Bowtie2 and RSEM 1.3.0. Normalized counts per million (CPM) were then obtained using the EdgeR program⁵⁶. Transcripts containing at least one sample with CPM < 0.1 in each group were filtered. The rMATS algorithm¹⁶ was further applied for an in-depth analysis of splicing isoforms that were differentially expressed in Neuro2a cells with or without Gao deletion. Differentially expressed genes (DEGs) and transcripts were defined as those with a *p*-value < 0.05, false discovery rate (FDR) < 0.05, and fold change (FC) > 2 in the two-group comparison. Transcriptomic data are available at the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE241258.

Cortical organoids and monolayer neurons

Cortical neurons were differentiated from iPSCs using the serum-free floating culture of embryoid body-like aggregates with the quick reaggregation (SFEBq) method^{12,31,54}. On day 18, cell aggregates were transferred to a non-adhesive dish (EZSPHERE, #4020–900; IWAKI, Baibara-gun, Shizuoka, Japan) and cultured in the following organoid medium: DMEM/F12-GlutaMAX (#10,565–018; Thermo Fisher Scientific) with 1% Chemically Defined Lipid Concentrate, 1% N-2 supplement, and 1% antibiotic–antimycotic (#11,905–031, #17,502,048, and #15,240,062, respectively; Thermo Fisher Scientific) at 37 °C under 40% O₂ and 5% CO₂. The medium was replenished every 3–4 days. On day 35, organoids were cultured in the following neuronal maturation medium: 10% FBS, 1% Matrigel Growth Factor Reduced (#356,230; Corning, Gkendale, AZ, USA), and 5 µg/mL heparin was added to the organoid medium. The medium was replaced every 3–4 days. For the morphological analysis of single neurons and growth cones, cerebral organoids were dissociated on day 15 using the enzyme solution of a Neuron Dissociation Solution (#291–78,001; Wako) and plated on poly-D-lysine, fibronectin and iMatrix-511 coated glass-bottom dish at a density of 75,000 cells/cm² in the neural maturation medium. The medium was refreshed every 2–3 days.

Neuronal differentiation from iPSCs

Karyotyping and the quality check of iPSCs were completed as previously reported¹². iPSCs were pretreated for 1 h with 50 µM Y27632 (#036–24,023; FUJIFILM, Wako, Japan), dissociated with TrypLE Express (#12,605–010; Thermo Fisher Scientific), and quickly reaggregated using low cell-adhesion 96-well culture plates with

V-bottomed conical wells (#MS-9096VZ; Sumitomo Bakelite, Tokyo, Japan) in the following differentiation medium (10,000 cells/well, 100 μ L): Glasgow-MEM (#G5154; Sigma-Aldrich) containing 20% Knockout Serum Replacement (#10,828-010; Thermo Fisher Scientific), 0.1 mM MEM Non-Essential Amino Acids Solution (#11,140,050; Thermo Fisher Scientific), 1 mM pyruvate (#S866; Sigma-Aldrich), 0.1 mM 2-mercaptoethanol (#21,417-52; Nacalai Tesque, Inc., Kyoto, Japan), 5 μ M SB431542 hydrate (#S4317; Sigma-Aldrich), 3 μ M IWR1e (#13,659; Cayman Chemical, Ann Arbor, MI, USA), and 1% P/S at 37 °C under a normoxic, 5% CO₂ condition. The medium was replenished every 3–4 days. Y27632 (50 μ M) was added to the differentiation medium from day 0 to 3 unless otherwise stated.

Morphological analyses

The ImageJ software program, version 2.9.0 (<https://imagej.nih.gov/ij/index.html>) was used for the quantitative analysis of neurite outgrowth, morphology of growth cone, and fluorescence signals of immunolabelled proteins. Sholl analyses were performed to identify the number of intersections at radial intervals of 30 μ m starting from the central point of the growth cone (lamellipodia) using the ImageJ plug-in, Sholl Analysis 4.1.12⁵⁷. The circularity index was obtained with the conventional formula 4π [area size] / [perimeter]^{2,58}.

Statistics

All statistical analyses were performed using R version 4.2 (<https://R-project.org>). The R package clusterProfiler version 4.6.2⁵⁹ and DAVID (<https://david.ncicrf.gov/summary.jsp>) were used for enrichment analysis. The collected data are presented as the median (range: min–max) unless otherwise stated. *P* values < 0.05 (*) were considered significant (***p* < 0.01; ****p* < 0.001).

Ethical approval

All experimental procedures were performed in strict compliance with the institutional guidelines and protocols approved by the Institutional Review Board of Kyushu University for clinical studies and experiments with human samples (#28–88, 29–393 and 678–01). The establishment of iPSCs was based on the consent of a healthy donor and the patient's parents. Informed consent was obtained from all subjects and/or their legal guardians of the patient.

Data availability

Clinical data are not publicly available due to privacy or ethical restrictions. RNA-seq data are available at Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>) with accession number GSE241258.

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R.T.: Conceptualization; Formal analysis; Writing—original draft; Writing—review and editing. S.A.: Data curation; Funding acquisition; Writing—review and editing. S.O.: Investigation; Writing—review and editing. F.F.: Formal analysis; Data curation; Writing—review and editing. E.H.: Investigation. K.Y.: Investigation. R.T.: Investigation. H.K.: Investigation. K.M.: Investigation. T.A.K.: Data curation; Supervision; Methodology. R.K.: Resources; Supervision. K.T.: Resources; Supervision; Methodology. K.Y.: Formal analysis; Supervision; Methodology. N.O.: Resources; Supervision; Methodology. S.O.: Investigation; Resources; Supervision; Funding acquisition; Writing—review and editing. Y.S.: Conceptualization; Formal analysis; Supervision; Data curation; Funding acquisition; Writing—original draft; Writing—review and editing.

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Competing interests

The authors declare no competing interests.

Additional information

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