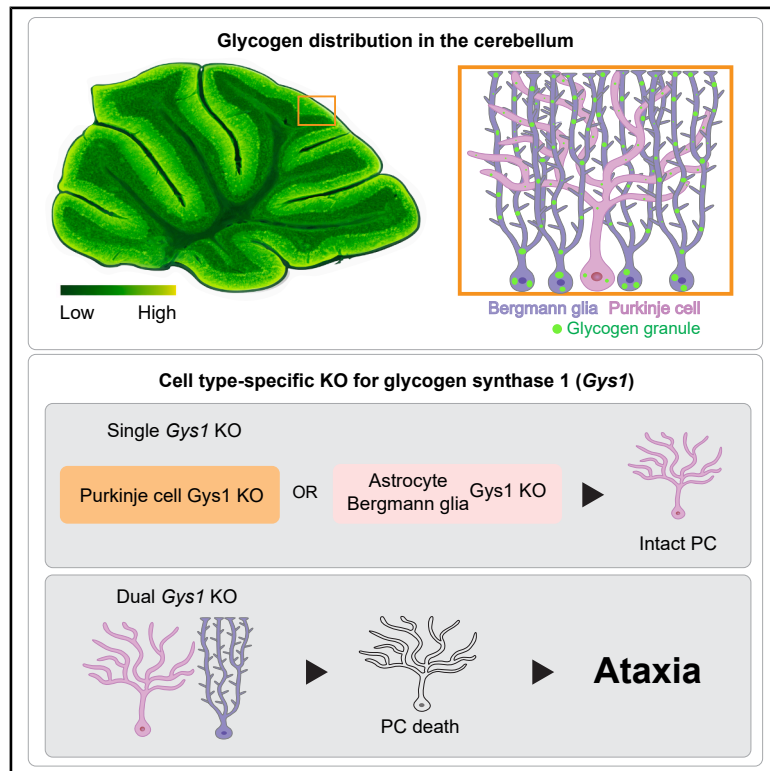


Distribution and functional significance of rodent cerebellar glycogen

Graphical abstract



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In brief

Molecular neuroscience; Cellular neuroscience

Highlights

- Cerebellum contains high amounts of glycogen in the molecular layer
- BG and PCs store glycogen in their somata and processes
- Interfering PC glycogen synthesis by Gys1 conditional KO reduces BG glycogen
- Gys1 KO in PCs and cerebellar GFAP-positive cells induces PC ablation and ataxia



Article

Distribution and functional significance of rodent cerebellar glycogen

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SUMMARY

The mammalian brain stores glucose, the main circulating energy substrate, as glycogen. In rodents, the cerebellum contains relatively high glycogen levels, yet its cellular and subcellular distribution remains poorly defined. Using monoclonal antibodies against glycogen, we examined its distribution in the mouse cerebellar cortex. Glycogen was predominantly localized to Bergmann glia (BG) processes in the molecular layer and was also detected in Purkinje cells (PCs), the principal cerebellar neurons. To assess the functional significance of cerebellar glycogen, we analyzed behavior in mice lacking glycogen synthase 1 (Gys1) in BG or PCs using a floxed Gys1 line. Gys1 deficiency in either PCs or GFAP-positive cells reduced anxiety-like behavior, whereas combined deletion caused PC degeneration and ataxia. These findings reveal a critical role for glycogen metabolism in both astrocytes and neurons in cerebellar function.

INTRODUCTION

Glycogen is a highly branched polysaccharide that stores excess glucose molecules in animal cells to meet immediate energy demands. While glycogen is predominantly localized in the liver and skeletal muscles, it is also stored in the brain, whereby astrocytes have been described to be the predominant cell type that stores glycogen.¹ Nilsson and Hultman^{2,3} showed that during prolonged fasting, brain glycogen levels remain relatively stable, whereas liver glycogen levels decline, suggesting that brain glycogen is under distinct metabolic control. Remarkably, several studies have linked brain glycogen metabolism to synaptic plasticity and memory,^{4–7} implying that lactate from glycogenolysis is transported to neighboring neurons to sustain the plastic nature of neuronal circuit by the astrocyte-neuron lactate shuttle (ANLS).⁸

We have previously described the macroscopic distribution of mouse brain glycogen by immunohistochemistry leveraging

focused microwave (FMW)-assisted fixation,⁹ which instantaneously inactivates enzymatic activities and preserves glycogen.¹⁰ Accordingly, we found that glycogen is enriched in distinct brain regions, including the hippocampus, cerebral cortex, striatum, and cerebellum, with the highest amount.

The cerebellum is a hindbrain structure implicated in motor coordination and has the highest concentration of neurons.¹¹ The cerebellar cortex consists of three distinct layers: the molecular layer, the Purkinje cell (PC) layer, and the granular layer. PCs are the large-sized principal cells that form planar dendrites in the molecular layer and project to the deep cerebellar nuclei. Each PC is innervated by a climbing fiber, and its dendritic arbors form synapses with hundreds of thousands of parallel fibers which arise from exceptionally numerous granule cells.¹² PCs are known for their high discharge activities with “simple” spikes at mean frequencies of 40–60 Hz and “complex” spikes that occur at ~1 Hz by strong excitation by climbing fiber input.^{13–15}



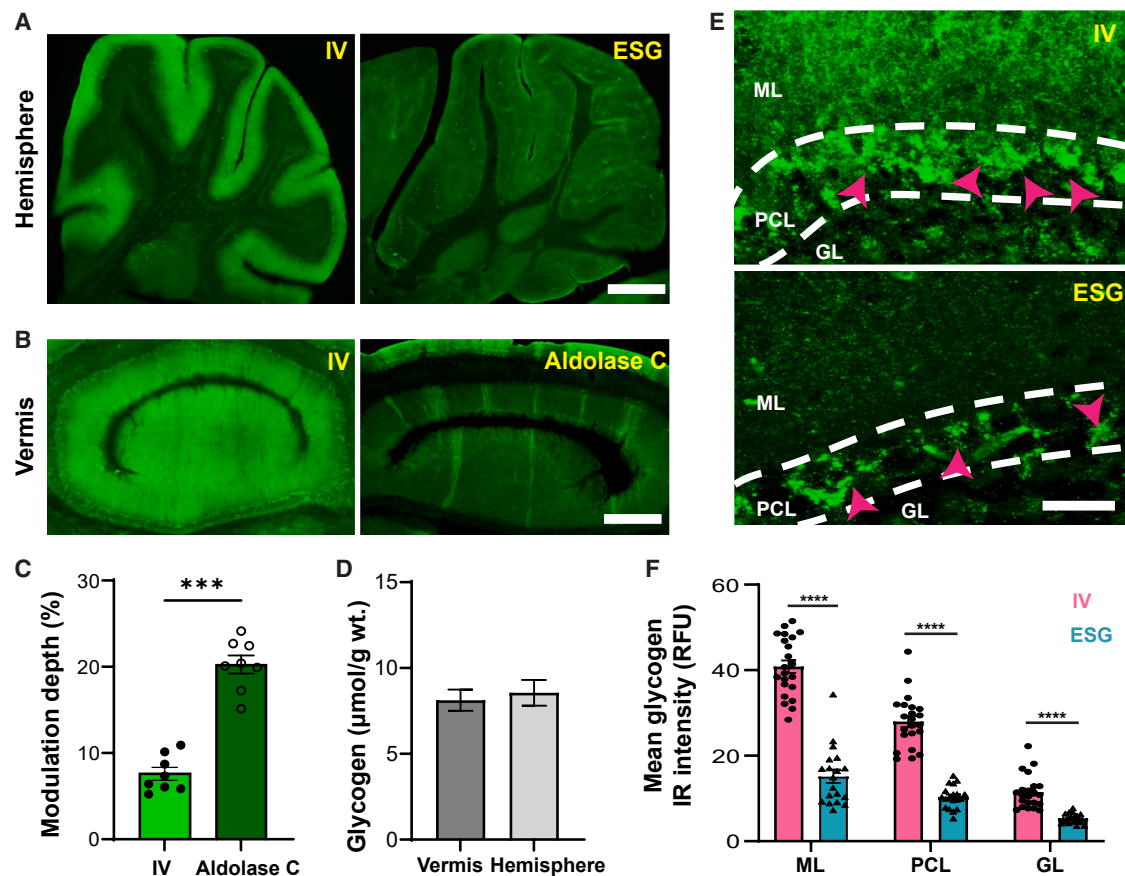


Figure 1. Glycogen immunohistochemistry in the mouse cerebellum

(A) Sagittal cerebellar sections from the hemisphere show glycogen immunohistochemistry with the IV (left) and ESG glycogen (right) antibodies.

(B) Coronal sections of the vermis stained for IV and aldolase C.

(C) Comparison of modulation depth for IV glycogen (left) and aldolase C (right) staining.

(D) Glycogen assay shows that cerebellar vermis and hemisphere have similar glycogen amounts.

(E) Glycogen distribution pattern in distinct cerebellar layers (ML-molecular layer, PCL- Purkinje cell layer, GL-granular layer) detected with IV- (top) and ESG glycogen (bottom) antibodies. Pink arrows point to large glycogen aggregates in the PCL.

(F) Comparison of mean IV and ESG glycogen immunoreactivity (IR) in respective cerebellar layers (mean $\pm$ SEM, $N = 5$ mice, $n = 22$ slices for IV, 19 slices for ESG; All layers differed, as tested by unpaired t-tests. All comparisons between IV and ESG in the ML, PCL, and GL showed, **** $p < 0.0001$). Scale bars, 500 μ m for A and B, 50 μ m for E.

Considering that neuronal energy consumption depends heavily on average firing rate and synaptic input,¹⁶ a reliable energy supply is presumably essential for the sustained operation of the cerebellar cortex.

Despite its rich glycogen storage, cerebellar glycogen distribution has not been described in detail. Here, we first describe cerebellar glycogen immunohistochemistry using two distinct monoclonal antibodies: IV58B6,¹⁷ which detects both small β and large α particles, and ESG1A9,¹⁸ which preferentially detects only the large, aggregated forms of glycogen (or α -particles). We find appreciable amounts of glycogen in astrocytes, including Bergmann glia (BG), and notably in PCs. To understand the functional significance of cerebellar glycogen, we examined mice that lack glycogen synthase 1 (*Gys1*, a major enzyme for glycogen synthesis) in PCs and/or cerebellar astrocytes. Interestingly, PC- and/or cerebellar astrocyte-targeted *Gys1* knockout (KO) mice exhibited reduced anxiolytic behavior

in the elevated plus maze. In addition, the dual (PCs + cerebellar astrocytes) *Gys1* KO caused severe ataxia. Histological analysis revealed pronounced PC ablation in the dual *Gys1* KO mice, suggesting a critical role of glycogen's metabolic interactions between PCs and BG.

RESULTS

To investigate cerebellar glycogen distribution, we performed glycogen immunohistochemistry on FMW-fixed cerebellar samples using the IV- and ESG glycogen antibodies. For both antibodies, sagittal sections revealed low glycogen signals in the inner regions of the cerebellar cortex, including the cerebellar white matter (Figure 1A), which is consistent with our prior work reporting relatively low glycogen levels in forebrain white matter.⁹ Immunohistochemistry with the IV glycogen antibody showed that the glycogen-rich portion formed a belt-like

Table 1. Antibodies used for histological experiments

Antibody name	Dilution	Product ID	Vendor	Target cell/molecule type
IV	1:300	IV58B6	Baba et al. ¹⁷	Glycogen
ESG	1:50	ESG1A9	Nakamura-Tsuruta et al. ¹⁸	Glycogen
Aldolase C	1:500	Af1390	Frontier Institute	Zebrin (+) Purkinje cells
Calbindin D-28k	1:1000	CB-38a	Swant	PCs
hdGFP	1:200	–	Nakamura et al. ²³	Heat-denatured GFP
NeuN	1:1000	AB177487	Abcam	Neurons
Parvalbumin	1:1500	PV-28	Swant	Fast-spiking inhibitory neurons (incl. PCs)
GAD65/67	1:1000	G5163	Sigma-Aldrich	GABAergic neurons (incl. PCs)
GFAP	1:1000	G9269, AB7260	Sigma, Abcam	Astrocytes (incl. BG)
s100B	1:1000	S2532	Sigma	Astrocytes
Iba1	1:1000	019-19741	WAKO	Microglia

continuous structure corresponding to the molecular layer spanning along the cerebellar surface. Furthermore, we checked the specificity of our antibody for glycogen by performing amylase treatment. While clear glycogen aggregates were observed with the IV glycogen alone (see [Figure S1](#)), the signals disappeared after amylase treatment.

Glycogen distribution by macroscopic zones

The cerebellum is organized into evolutionarily conserved transverse zones and parasagittal stripes, which correspond to molecularly distinct PC subtypes. One of the most widely used markers of these stripes is aldolase C (also known as zebrin),^{19,20} a key enzyme for glycolytic metabolism expressed in PCs and reported to modulate synaptic plasticity at the parallel fiber,²¹ although its functional significance in cerebellar physiology remains an active area of investigation ([Figure 1B](#)). Of note, we obtained Aldolase C patterns that form continuous and discontinuous vertical stripes across layers and lobes as described in detail in Fujita et al.²² To determine if glycogen distribution forms similar stripe patterns, we examined whether immunohistochemical glycogen signals vary along the medio-lateral direction in coronal sections. Unlike the aldolase C pattern, IV glycogen horizontal patterns appeared continuous, with only weak striping in regions where aldolase C expression is expected to be positive ([Figures 1A and 1B](#)). Consistent with the visual impression, the spatial modulation depth was markedly higher for aldolase C signals ([Figure 1C](#), 20.3%) than for IV glycogen signals (7.6%). Using paired samples dissected from the same individual mice, the glycogen contents of the vermis and the hemisphere were quantified to be similar by glycogen assay on FMW-treated cerebellum samples ([Figure 1D](#), 8.12 ± 0.62 vs. 8.55 ± 0.75 $\mu\text{mol/g wt.}$).

Glycogen distribution by layer

We sought to describe the glycogen distribution by cerebellar layer and cell type using IV and ESG glycogen immunohistochemistry ([Figure 1E](#)). Both antibodies labeled the neuropil of the molecular layer with a fine punctate pattern, whereas large granular aggregates were observed in the PC layer. The granular layer was labeled to a lesser extent. Next, we compared mean glycogen immunohistochemical signal intensities of the cerebellar layers (IV: $N = 5$ mice, $n = 22$ slices; ESG: $N = 5$ mice,

$n = 19$ slices). While we observed that the inner portion of the molecular layer (adjacent to the Purkinje cell layer) tended to exhibit lower IV glycogen signal, we averaged the signal intensity across the entire molecular layer to compute the mean intensity. For both IV and ESG glycogen signals, the molecular layer was the richest in glycogen content, followed by the PC layer, and the granular layer was the least glycogen-containing layer ([Figure 1F](#)).

Molecular layer

The molecular layer glycogen is characterized by a continuous dense pattern that constitutes the highest glycogen signals among the cerebellar layers. The molecular layer encompasses complex dendritic arbors of PCs and fine processes of BG that extensively enwrap parallel fiber synapses. To determine the glycogen localization by cell type, glycogen-immunostained cerebellar slices were co-labeled with cell type-specific markers ([Table 1](#)) and examined with a confocal microscope. First, we expressed GFP in BG and granular layer velate astrocytes by AAV9-GFAP-GFP microinjected into dorsal lobules V, VI, and VII. The cytosolic GFP expression visualized the morphology of these cell types, enabling the evaluation of glycogen signal colocalization more reliably than cytoskeletal GFAP immunohistochemistry. Similar to the forebrain, confocal images of glycogen signal with submicron resolution showed punctate patterns. As expected, punctate IV glycogen patterns colocalized with GFP-expressing BG processes ([Figure 2A](#)).

The punctate pattern appeared uniformly in the molecular layer without any distinct patterns, suggesting that PC dendrites, a major constituent of the molecular cell layer, also contain glycogen. We labeled PCs with calbindin D28k and detected IV glycogen signals in PC dendrites ([Figure 2A](#)). To exclude the possibility that glycogen signals are in the perisynaptic BG processes, which are known for their extensive coverage of PC spines, we examined a single plane confocal image of PC dendrites and confirmed glycogen signals inside the dendritic branches of PCs ([Figure 2B](#), left). To substantiate the presence of glycogen in PC dendrites, we generated conditional KO mice that lack *Gys1*, a major enzyme for glycogenesis, selectively in PCs (PC *Gys1* KO; see [Table 2](#) for the nomenclature of conditional *Gys1* KO mice). In these mice, glycogen signals

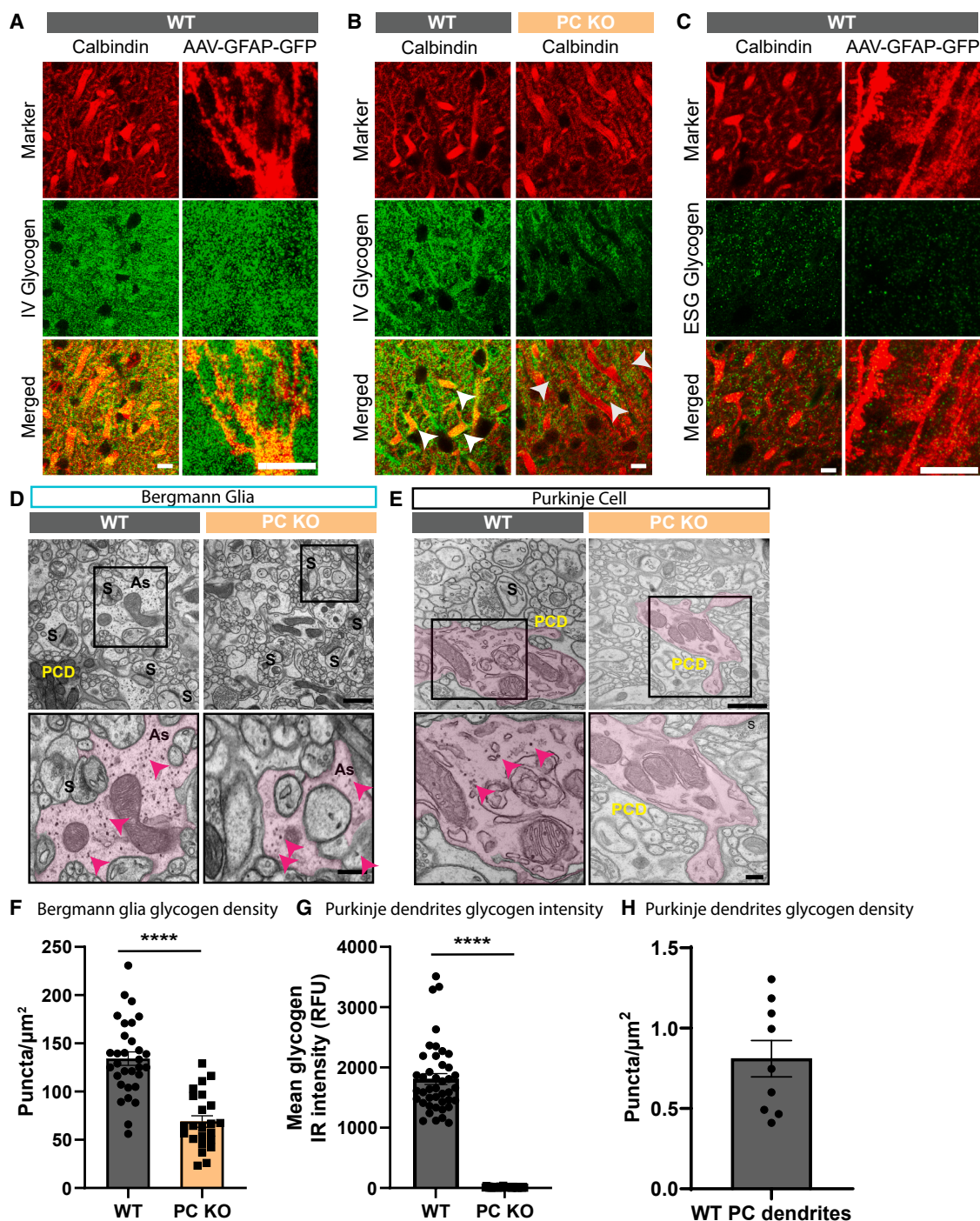


Figure 2. Glycogen distribution in the molecular layer

(A) IV glycogen colocalization with PC dendrites (calbindin D28k, left) and GFP-labeled BG (GFP expressed by AAV-GFAP-GFP, right).
(B) Single plane confocal image of IV glycogen and calbindin immunohistochemistry of a WT mouse (left). White arrowheads indicate glycogen colocalization with PC dendrites and in BG processes. Immunohistochemistry of IV glycogen and calbindin in the PC-selective Gys1 KO mouse (PC KO) did not show colocalization in PC dendrites (right).
(C) ESG signals do not colocalize with PC dendrites.
(D) Electron microscopic examination shows that glycogen puncta (arrows) are present in the perisynaptic BG processes in both WT (left column) and PC KO (right column). BG glycogen puncta are sparser in PC KO than WT, as quantified in Figure 2F.
(E) Representative micrographs demonstrate PC glycogen puncta in WT (left column) and PC KO (right column). In WT, the puncta (arrowheads) are scattered in the dendrites. No glycogen was observed in the PC dendrites from PC KO mice.

(legend continued on next page)

were absent in PC dendrites (Figure 2B, right, quantification in Figure 2G, $p < 0.0001$), strongly supporting glycogenesis in PCs and confirming that Gys1 is the critical enzyme. Unlike the IV glycogen antibody, the ESG glycogen signals appear weaker and less extensive in the molecular layer (ML) (Figure 2C), which is reminiscent of hippocampal astrocytes' ESG pattern that labeled fine processes.⁹ This pattern suggests that large-sized ML glycogen is primarily located in BG processes.

To further investigate the localization of glycogen in the molecular layer, we performed transmission electron microscopy (TEM) analysis. Glycogen puncta were identified by their electron-dense punctate appearance, and astrocytic processes (BG processes in the molecular layers) were characterized by their electron-sparse cytosol. Glycogen puncta were predominantly observed in perisynaptic BG processes, where they were largely scattered, although clusters of a few puncta were observable (Figure 2D, pink arrow). Remarkably, glycogen puncta in PC distal dendrites (PCDs) were also present (Figure 2E, pink arrow). The density of glycogen puncta in PC dendrites was relatively smaller than in BG processes. To confirm the presence of glycogen in PC dendrites, we examined PC Gys1 KO mice. Accordingly, electron-dense puncta were absent in PC dendrites in PC Gys1 KO mice (Figure 2E, right). Interestingly, the density of glycogen puncta in BG processes is significantly lower in PC Gys1 KO than WT (Figures 2D and 2F, PC Gys1 KO vs. WT: 68.99 ± 5.99 vs. 134.10 ± 6.96 puncta/ μm^2). WT PC dendrites had a glycogen puncta density of 0.81 ± 0.113 puncta/ μm^2 (Figure 2H).

Overall, our analysis of the molecular layer showed the presence of glycogen in both BG processes and PC dendrites by both immunohistochemistry and TEM.

Purkinje cell layer

The two major constituents of the PC layer are PC and BG somata. We observed large glycogen clusters surrounding PC somata by both IV and ESG immunohistochemistry (Figures 3A and 3B), making the PC layer the second most glycogen-dense layer in the cerebellar cortex. For IV glycogen, large clusters colocalized with astrocytes labeled by AAV-GFAP-GFP (Figure 3A). On the other hand, smaller glycogen clusters that formed PC soma-like shapes were observed. Indeed, co-immunostaining with calbindin confirmed that the pattern was in PC somata, as indicated by Manders' coefficient. (Figure 3A; Table 3). For ESG, the colocalization of glycogen in PC somata was not observed frequently (Figure 3B), ESG-labeled glycogen was observed mostly in BG soma and BG/velate astrocyte processes surrounding the PC soma (Figure 3B). Further evaluation of glycogen presence surrounding the PC soma by TEM showed dense puncta in astrocytic processes in close proximity to PC somata (Figure 3C). Unlike in the molecular layer, these glycogen

puncta formed large clusters, which is in line with our immunohistochemical observations.

For quantitative analysis, we computed the number of glycogen-immunoreactive puncta per $1 \mu\text{m}^2$ area and the immunolabeled area fraction (the portion of glycogen-occupying area) for both PC and BG/astrocytic somata. On average, 8% of PC somata and 12% of BG somata were occupied with IV glycogen signals (Figure 3D). For ESG, the proportions were 1.3% and 27%, respectively. We sought to further validate the presence of glycogen in PC soma by TEM. While many electron-dense puncta were observed inside PC somata (Figure 3E), they were located within endoplasmic reticula thereby representing ribosomes. Glycogen-like electron-dense clustered puncta in the cytosol were observed (Figure 3E, pink arrow); however, they were difficult to distinguish from free ribosomes in PC soma. To avoid overstatements due to false positives, we did not include quantifications from PC soma in our TEM analysis. In PC Gys1 KO mice, similar to WT, aggregated glycogen signals were found in astrocytic processes near the PC soma (see Figure S2A). However, the density and the prevalence of occurrence were lower than WT, in line with our findings in the molecular layer. Glycogen-like puncta that we observed in WT PCs were absent in these mice (Figure 3E). Moreover, light microscopy images of PC Gys1 KO also showed attenuated glycogen labeling in the PC layer (see Figure S2B). These results collectively demonstrate the presence of glycogen in both BG and PC in the PC layer.

Granular layer

Glycogen distribution in the granular layer was sparse for both IV and ESG. Large-cluster glycogen signals were rare. IV glycogen signals displayed an uneven, scattered pattern (Figure 4A) that did not resemble granule cell morphology. Instead, the IV glycogen pattern resembled velate astrocytic morphology, whose fine processes are difficult to visualize by FMW-fixed glycogen immunohistochemistry.

To visualize velate astrocytes in the granular layer, AAV-GFAP-GFP virus was injected into the layer and co-immunostained with IV glycogen (Figure 4B). As shown in the figure, the sparse IV glycogen signals nicely colocalized with GFP-labeled velate astrocytes. To investigate further, we examined glycogen puncta by TEM. Accordingly, glycogen puncta were observed in the astrocytic processes, whereas glycogen puncta were absent in granule cells (Figure 4C). Moreover, a similar glycogen puncta distribution was observed in velate astrocytes of PC Gys1 KO mice (Figures 4C and S3A).

Apart from the cell types described above, the cerebellar cortex is populated by various other types of interneurons, including basket, stellate, Golgi, and Lugaro cells. While these cell types were not investigated in the current study, we did not observe obvious neuronal somata with high glycogen signals in molecular and granular layers.

(F) Comparison of BG process glycogen puncta density between WT and PC KO showing significantly fewer glycogen puncta in PC KO; (mean $\pm$ SEM, $N = 3$ animals each, $n = 31$ ROIs for WT, $n = 23$ ROIs for PC Gys1 KO, **** $p < 0.0001$).

(G) Quantitative analysis of glycogen intensity in PC dendrites. Graph shows the mean glycogen IR intensity per thick dendritic branch in WT versus PC KO mice; (mean $\pm$ SEM, $N = 5$ animals each, $n = 42$ ROIs for WT, $n = 40$ ROIs for PC Gys1 KO, **** $p < 0.0001$).

(H) Glycogen puncta density in WT PC dendrites (mean $\pm$ SEM, $N = 3$ animals, $n = 9$ ROIs). Scale bars, 25 μm for A, B, and C; 1 μm for D and E (top); 400 nm for D and E (bottom). For D and E, "PCD" is Purkinje cell distal dendrites, "As" is astrocytes, and "s" represents synapses.

Table 2. Mouse groups used in behavioral experiments

Label	Genotype	Gys1 KO target cell type
PC KO	L7-Cre(tg/wt) Gys1(fl/fl)	PC
WT	L7-Cre(wt/wt) Gys1(wt/wt)	None, wildtype littermate control
AB KO	Gys1(fl/fl); AAV9-GFAP-Cre	Cerebellar AS and BG
Dual KO	Gys1(fl/fl); AAV2/9-L7-Cre + AAV-GFAP-Cre	PC, cerebellar AS, and BG

AS: astrocytes, BG: Bergmann glia, PC: Purkinje cell, AB: Astrocytes and Bergmann glia.

Impacts of cell-specific Purkinje cells and Bergmann glia glycogen synthase 1 deletion on mouse behavior

Our histological study demonstrated that both BG and PCs store glycogen. To gain insight into the functional significance of cerebellar glycogen, we examined the behavior of cerebellar astrocyte- and/or PC-selective *Gys1* KO mice as described in Table 2 (nomenclature) and Table 4 (AAV identity and titer). All virus injections were performed directly into the mouse cerebellum only. We use the abbreviation AB since the *GFAP* promoter is activated in both granular layer astrocytes and BG.

First, we confirmed that Dual *Gys1* KO results in a significant decrease of cerebellar glycogen content (Figure 5A, WT vs. Dual *Gys1* KO: 6.73 ± 0.47 μ mol/g vs. 3.44 ± 0.71 μ mol/g). The decrease in glycogen content is unlikely due to glial inflammation, since LPS-treated mice, in which glial cells are highly reactive, show a significant increase compared with saline-injected controls (see Figure S4A, control vs. LPS: 7.04 ± 0.52 μ mol/g wt. vs. 14.79 ± 2.26 μ mol/g wt.).

Ambulatory and anxiety-related behavior

We evaluated ambulatory and anxiety-related behavior using the open field test and the elevated plus maze (Figures 5C–5H). Exposure to a novel open environment did not result in significant differences in all groups (distance traveled – WT: 2461 ± 240.2 cm, AB *Gys1* KO: 2169 ± 283.6 cm, PC *Gys1* KO: 2334 ± 377.1 cm, Dual *Gys1* KO: 1516 ± 322.3 cm, $p = 0.8627$; center time – WT: 98.0 ± 26.4 s, AB *Gys1* KO: 85.6 ± 58.5 s, PC *Gys1* KO: 70.0 ± 25.9 s, Dual *Gys1* KO: 175.1 ± 78.0 s, $p = 0.8720$) (Figures 5C–5E). The elevated plus maze test shows less anxiety behavior in all *Gys1* KO groups compared to WT (Figures 5G and 5H, time in open arm – WT: 6.2 ± 3.2 s, AB *Gys1* KO: 30.9 ± 6.5 s, PC *Gys1* KO: 35.5 ± 7.5 s, Dual *Gys1* KO: 50.3 ± 5.2 s, $p = 0.0008$; time in closed arm – WT: 559.8 ± 12.5 s, AB *Gys1* KO: 537.8 ± 11.6 s, PC *Gys1* KO: 518.0 ± 12.4 s, Dual *Gys1* KO: 489.5 ± 10.3 s). AB, PC, and Dual *Gys1* KO animals tended to spend more time in open arms, and PC and Dual *Gys1* KO animals spent less time in closed arms. These observations suggest that *Gys1* deficiency in PCs and/or astrocytes/BG may result in an anti-anxiety-related phenotype.

General motor ability

While the open field behavior measured by mouse position tracking did not show significant differences, we noticed that Dual *Gys1* KO mice appeared to exhibit shivering and balance abnormalities. Therefore, we examined locomotive behaviors in the next set of experiments. First, we tested for motor strength using the inverted mesh test. All three AAV-injected groups

showed similar performances, receiving a score of 3 or 4, and were generally able to hold the inverted screen for 60 s (see Figure S6A). To determine if Dual *Gys1* KO mice express an ataxic phenotype, we performed a ledge test and a hindlimb clasp test. For the ledge test, AB *Gys1* KO mice had a score of zero, whereas the Dual *Gys1* KO mice had an average score of 1.2 (Figure 5I), indicating an ataxic phenotype. On the other hand, the hindlimb clasp test did not reveal noticeable changes in any *Gys1* KO mice compared with WT mice (score of 0) (see Figure S6B). These tests suggested that the ataxic phenotype is expressed when both PCs and cerebellar astrocytes are deprived of glycogen, while muscle strength remains unaffected.

Motor coordination

To further characterize the motor coordination of cerebellar cell type-specific *Gys1* KO mice, we performed the accelerated rotarod test. The ataxia phenotype of Dual *Gys1* KO can originate from PC- or dual cell type *Gys1* KO. To examine the effect of *Gys1* KO in PCs throughout the cerebellum, we examined PC *Gys1* KO mice in the rotarod apparatus. As a result, WT and PC *Gys1* KO mice performed similarly in rotarod (Figure 5J, latency to fall: 146.9 ± 17.26 s vs. 83.27 ± 21.18 s), showing that PC-selective *Gys1* KO is not sufficient to express the ataxia phenotype. Only Dual *Gys1* KO animals showed a distinct ataxic phenotype in the rotarod test and performed poorly.

Purkinje cells are ablated after glycogen deletion from both astrocytes and Purkinje cells

To investigate the cerebellar circuit alterations by the dual cell-type *Gys1* KO, we examined the cerebellar histology of Dual *Gys1* KO mice. First, we find that 10 μ L AAV9-GFAP-GFP injection resulted in GFP expression in wide areas of the cerebellum, although the AAV did not reach distal folia >2 mm from the injection site (see Figure S5A). To evaluate the possible toxicity of Cre recombinase expression, 10 μ L AAV-L7-Cre-GFP was injected into a WT mouse. As a result, normal PC morphology was observed by both GFP (Figure 6A) and calbindin immunohistochemistry (Figure 6C), confirming that neither the AAV vector nor the Cre-recombinase expression alone induces PC degeneration. Next, we examined Dual *Gys1* KO mice. Remarkably, PCs were largely ablated in areas within ~ 2 mm from the AAV injection site (Figures 6B–6E), as observed by the absence of calbindin signals. Intact PCs were observable in distal folia, where AAV presumably did not reach to infect both PCs and astrocyte/BGs to delete *Gys1* (see Figure S5B). To exclude the possibility that PCs are viable but somewhat depleted calbindin, we attempted to visualize PCs and GABAergic interneurons using parvalbumin

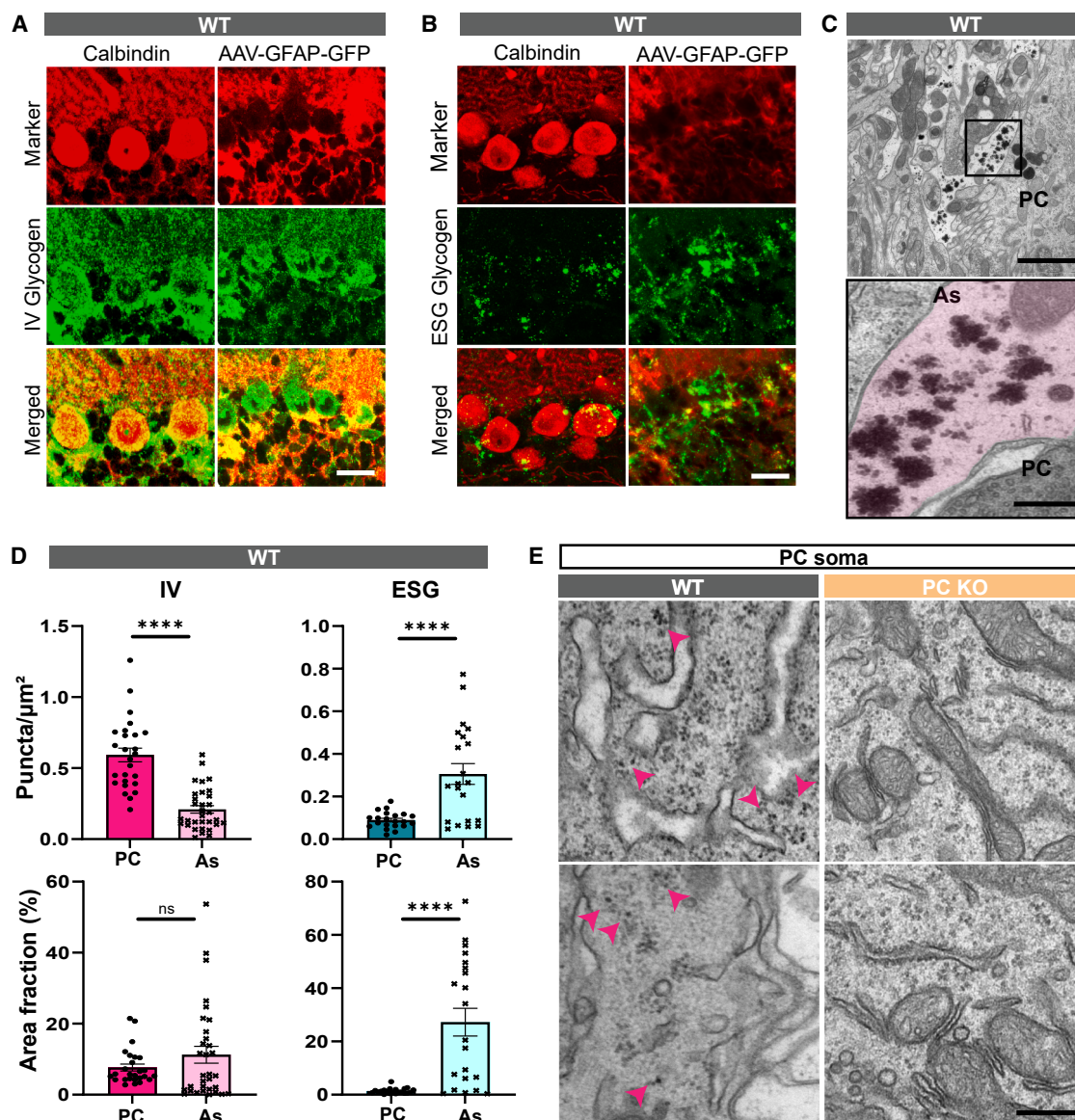


Figure 3. Glycogen distribution in the PC layer

(A) Aggregated IV glycogen signals are visualized with PCs (calbindin) and GFP-labeled astrocytes/BGs (by AAV-GFAP-GFP) immunostaining. (B) ESG glycogen signals visualized with PCs and GFP-labeled astrocytes/BGs as in (A). Most ESG glycogen is contained in BG/astrocytic processes. (C) Confirmation of large glycogen clusters in the astrocytes/BGs by electron microscopy. Large glycogen clusters are seen in the astrocytic process adjacent to PC somata in a WT mouse. The bottom panel shows the area marked by a square in the top panel at the magnification matching with E. (D) Subcellular distribution of glycogen analyzed by glycogen granule density (puncta/ μm^2 ; top) and area fraction of glycogen immunofluorescence (%; bottom). (mean $\pm$ SEM, $N = 5$ animals, $n = 15$ –32 images, **** $p < 0.0001$). (E) Glycogen-like electron-dense puncta (pink arrows) are observed in the PC soma in WT mice (left), whereas no such puncta were observed in the PC soma in PC Gys1 KO mice. Two examples for each genotype. Scale bars, 20 μm for A, B; 2 μm for C (top) and 300 nm for C (bottom); 400 nm for E. For C and D, “PC” is Purkinje cell and “As” is astrocyte.

and GAD65 immunohistochemistry. Similar to calbindin, neither parvalbumin nor GAD65 visualized PCs in Dual Gys1 KO mice, whereas intact PCs were labeled in AAV-L7-Cre-GFP injected mice (Figure 6C). Of note, parvalbumin and GAD65 label molecular layer interneurons.

Besides PCs, we also examined astrocytes and microglia. High GFAP expression was evident in dual Gys1 KO compared

to AAV-L7-Cre-GFP injected mice (Figures 6D and 6F), which is indicative of astrocytic activation accompanying PC ablation. Likewise, reactive microglia were prevalent in PC-ablated areas of Dual Gys1 KO, as evidenced by high Iba1 signals and the lack of fine ramified processes (Figures 6D–6G). Considering normal glial morphology of AB Gys1 KO and PC Gys1 KO mice (see Figure S5C), the astrocytic and microglial activation in Dual

Table 3. Manders' colocalization coefficients for IV glycogen staining with cell-type markers in molecular and Purkinje cell layers

Layer	IV staining with	Mean Manders' coefficient $\pm$ SEM (ROI number)	
		tM1	tM2
Molecular layer	Calbindin	0.45 $\pm$ 0.04 (6)	0.98 $\pm$ 0.00 (6)
	GFAP-GFP	0.54 $\pm$ 0.05 (6)	0.57 $\pm$ 0.05 (6)
Purkinje cell layer	Calbindin	0.92 $\pm$ 0.02 (6)	0.96 $\pm$ 0.05 (6)
	GFAP-GFP	0.52 $\pm$ 0.04 (6)	0.80 $\pm$ 0.02 (6)

Gys1 KO cerebellum was conceivably accompanied by PC death.

Finally, to confirm PC ablation *in vivo*, we chronically imaged PC somata in PC Gys1 KO injected with AAV-GFAP-Cre and PHP.eB-CAG-dlox-jGCaMP8-dlox in the cerebellum vermis. To observe cell ablation in a controlled manner, we injected all viruses at a titer of 10^{11} vg/mL locally in the vermis with 500 nL per site (total of two injection sites per mouse). The control group (PC Gys1 KO mice injected with CAG-dlox-jGCaMP8-dlox virus) does not show any change in Purkinje cell count over time. However, the number of Purkinje cells in Dual Gys1 KO mice injected with AAV-GFAP-Cre decreased over time (Figures 7A–7C) and immunohistochemistry confirmed these *in vivo* findings (Figure 7D).

DISCUSSION

Despite its profound abundance, the functional significance of cerebellar glycogen has remained virtually unknown. In this study, we first characterized the anatomical distribution of glycogen, identifying its presence in BG, velate astrocytes, and PCs. Interference with glycogen synthesis by Gys1 KO in cerebellar astrocytes or PCs alone did not result in a profound behavioral phenotype. Remarkably, Gys1 KO in both cell types resulted in ataxia and ablation of PCs. Since BG are the predominant astrocyte cell type in glycogen storage and directly interface PC somata and dendrites, these findings shed light on the importance of the metabolic interactions between PCs and BG/astrocytes.

Glycogen localization in astrocytes and Purkinje cells

It is well established that astrocytes are the main storage of brain glycogen in the forebrain.^{1,9,24–26}, although active glycogen metabolism in the neurons has been reported.^{27,28} In the cerebellar cortex gray matter, BG and velate astrocytes are two major astrocytic cell types that interact with the neuropil in the molecular layer and granular layer, respectively. While glycogen was detected in both types of astrocytes, the molecular layer contained the highest glycogen levels, indicating the predominant role of BG in glycogen storage. We find that BG perisynaptic processes are enriched with smaller glycogen puncta clusters than those in the soma. Considering the higher breakdown efficiency of small-sized glycogen,²⁹ the molecular layer BG glycogen distribution may reflect the high metabolic activity of the dense array of parallel fiber synapses. In addition to the energy demand arising from synaptic transmission, axonal presynaptic terminals consume significant energy to sustain synaptic vesicle integrity via vacuolar ATPases.³⁰ The

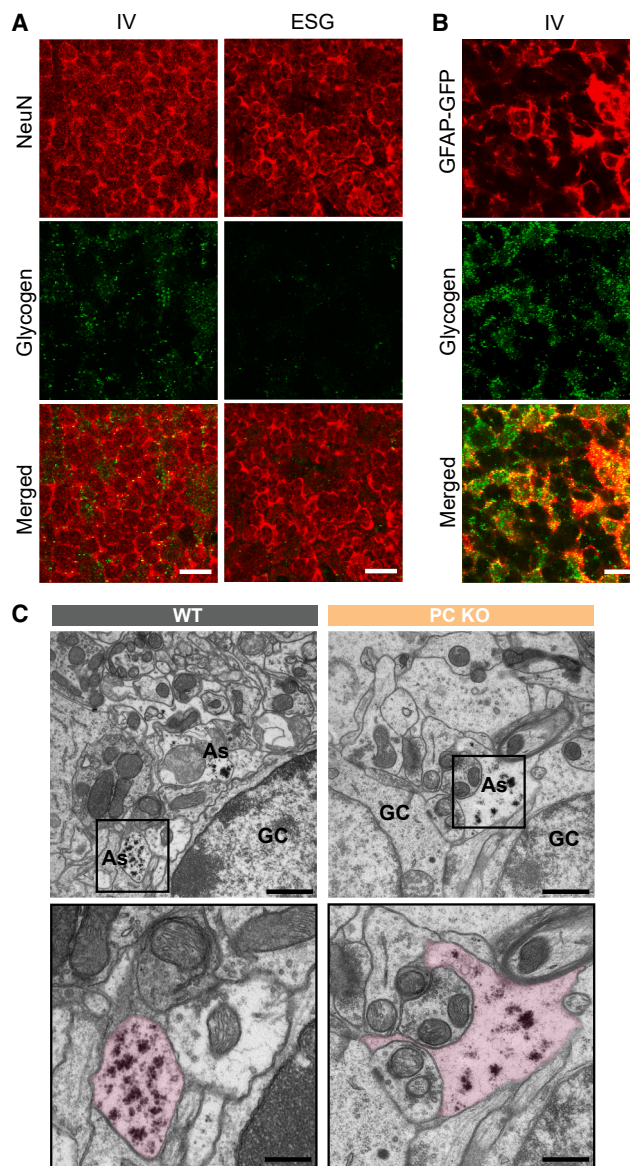


Figure 4. Glycogen distribution in the granular layer

(A) Neither IV nor ESG glycogen signals colocalized with granule cells. IV glycogen signals appeared to surround the granule cells, which resembled astrocytic processes. ESG glycogen signals were not prominent in the granular layer. (B) IV glycogen signal colocalized with AAV-GFAP-GFP-labeled velate astrocytes. (C) Ultrastructural image shows glycogen puncta in the astrocytic processes around granule cells. Both WT and PC Gys1 KO mice exhibited a similar glycogen distribution in the granular layer. As: Astrocytes, GC: granule cell. Scale bars, 50 μ m for A and B, 2 μ m for C (top) and 300 nm for C (bottom).

configuration of BG processes, which completely envelop parallel fiber synapses³¹ conforms to the notion that BG glycogen can act as an energy source for these synapses. Of note, one study suggested that sleep loss leads to the appearance of smaller granules in astrocytes, indicating rapid breakdown and promoted turnover of glycogen during prolonged wakefulness.³²

Table 4. Viral vectors delivered to the cerebellum and their purposes

Viral vector name	Titer (vg/mL)	Purpose
AAV9-GFAP-GFP	1.7×10^{12}	Control to show virus spreading in the cerebellum. Also, to justify AAV mediated inflammation.
AAV9-GFAP-cre	4.4×10^{12}	Delivered to <i>Gys1^{fl/fl}</i> mice to knockout <i>Gys1</i> from astrocytes.
AAV2/9 mL7-6 GFP-P2A-Cre	8.2×10^{11}	Delivered to <i>Gys1^{fl/fl}</i> mice to knockout <i>Gys1</i> from Purkinje neurons. A high titer is required for this virus to achieve <i>Gys1</i> knockout from a significant portion of Purkinje neurons. Also used to justify Cre-mediated toxicity.
PHP.eB-CAG-dlox-jGCaMP8-dlox	1.5×10^{13}	Delivered to L7crex <i>Gys1^{fl}</i> mice to visualize calcium activity from Purkinje neurons.

Remarkably, glycogen was also observed in PCs, unlike in principal neurons of forebrain structures. The presence of neuronal glycogen alludes to a distinct energy metabolism network in the cerebellar circuit. PCs sustain their activities with unusually high discharge rates (average 40–60 Hz) and can retain spontaneous discharge activity in the absence of synaptic input.^{14,33–35} Combined with the large cell size, PCs are energetically demanding^{16,36} as evidenced by their high mitochondrial density.³⁷ Indeed, 36% of energy consumption is estimated to support action potential-related activities in the cerebellar cortex.³⁸ The high vulnerability of PCs to hypoxia or ischemic insult^{39–42} underscores the importance of a reliable energy supply to PCs. For instance, a cell culture study reported that neuronal glycogen metabolism protects cells from hypoxia.²⁷ While PC somata are labeled with the pan-glycogen IV antibody, PC layer astrocytic processes were additionally labeled with the large-glycogen-selective ESG antibody. We propose that astrocytic glycogen provides an extra layer of endurance during compromised energy supply (discussion later in discussion).

Glycogen synthase 1 knockout in Purkinje cells and Bergmann glia causes ataxia and Purkinje cell ablation

Multiple studies have shown the functional role of glycogen metabolism in memory and learning in the forebrain using the glycogenolysis inhibitor DAB^{4–6} or conditional *Gys1* KO.⁷ Despite its abundance, cerebellar glycogen has remained unaddressed. We leveraged cell-type-selective *Gys1* KO to understand the functional role of cerebellar glycogen. Since the cerebellum is widely acknowledged for its role in motor coordination, we performed motor-related behavioral tests. Overall, single-cell-type *Gys1* KO did not result in obvious phenotypes except for attenuated anxiety-related behavior. This was unexpected, given the high amounts of glycogen stored in BG and the unusual neuronal presence of glycogen in PCs. Of note, while AAV-mediated conditional knockout enables local genomic manipulation, off-target recombination in neurons has been reported for AAV-GFAP-Cre vectors.⁴³ However, if such off-target recombination was functionally significant, AAV-GFAP-Cre-injected WT mice (i.e., AB-KO) would exhibit marked impairments in motor-related behavioral tests (Figure 5). Instead, severe motor deficits were observed only in the (AAV-GFAP-Cre-injected) Dual *Gys1* KO mice, supporting the effectiveness and cell-type specificity of the targeted gene deletion.

Surprisingly, Dual *Gys1* KO mice showed profound motor coordination phenotypes. Our results indicate that a deficiency of glycogen synthesis in both PC and cerebellar astrocytes results

in ataxia, demonstrating the essential role of glycogen presence in both cell types. A previous study reported that forced glycogen accumulation in PCs in the *mMGS-9A^{pcp2}* mouse model induces progressive PC ablation and locomotion defects.⁴⁴ Moreover, a recent study showed that astrocyte-selective *Gys1* KO in a Lafora disease mouse model mitigated neurodegeneration.⁴⁵ The previous studies and our study suggest that the balance between glycogenesis and glycogenolysis must be tightly regulated for the integrity of the neural circuit. We propose that the mechanism of PC degeneration is linked to severe energy depletion at local synaptic sites. The synergistic loss of both glycogen pools likely results in failure of local buffering and failure of external supply. This creates a state of chronic energy deficit that impairs essential ATP-dependent processes, leading to excitotoxicity, calcium dysregulation, and eventually triggering an intrinsic apoptotic or necrotic pathway responsible for the observed structural ablation.

While the *Gys1* cerebellum has been implicated in psychiatric disorders, including anxiety disorders, and especially hyperactivity in cerebellar areas, appears to correlate with anxiety behavior.⁴⁶ We were not able to find obvious phenotypes in the open field test. However, we observed that all *Gys1* KO groups, including AB, PC, and dual *Gys1* KO mice, tended to spend longer in the open arms. Given that cerebellar motor function depends on the integrity of the topographically preserved PC-deep cerebellar nuclei (DCN) output modules, the absence of a motor phenotype in these single-cell-type KOs suggests that the phasic function of PC-DCN circuitry remains largely intact. However, compromised glycogen metabolism in a single cell type could still influence the cerebellum-wide tonic output of PCs, e.g., by subtly altering their baseline simple-spike firing rates, which, as a population output, conceivably impact cerebello-limbic circuits. For instance, anxiolytic behavior has been reported to be mediated by the hypothalamo-cerebello-amygdalar circuit.⁴⁷ Of note, the cerebellar cognitive affective syndrome (CCAS) has documented that cerebellar dysfunction leads to a series of non-motor consequences, including deficits in emotion-affect and executive function.⁴⁸ Further experiments are needed to determine the impact of cerebellar energy metabolism on the functional coupling between cerebellar and limbic circuits.

Robust energy supply system to Purkinje cells

Confirming the notion that ataxia is a hallmark of malfunctioning PCs,^{49–52} PCs in Dual *Gys1* KO mice were found to be ablated. Our histological observation revealed notable signatures of inflammation in areas of PC degeneration (Figure 6D). It remains

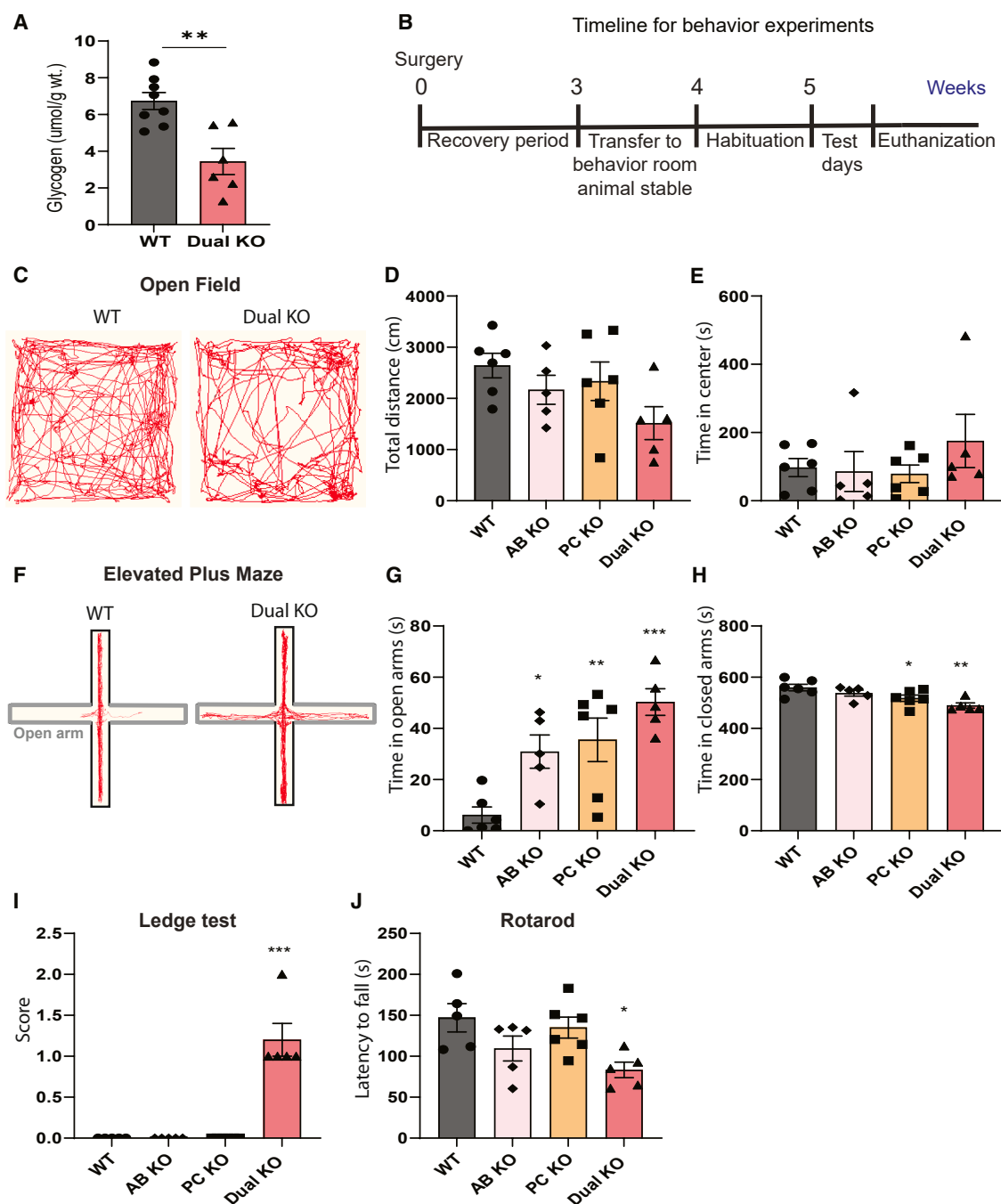


Figure 5. Physiological and behavioral phenotype of Gys1 deletion from either PCs or cerebellar astrocytes

(A) Cerebellar glycogen levels in WT and dual Gys1 KO.

(B) Timeline of the behavior experiments.

(C) Open field trajectory of WT and Gys1 KO mice.

(D) Total traveled distance (E) time in the center area.

(F) Trajectory of WT and Dual Gys1 KO mice in the elevated plus maze. The horizontal arms are the open arms (G). Time spent in the open arms.

(H) Time in the closed arms.

(I) Ledge test shows a significant impairment in motor coordination in Dual Gys1 KO mice, while AB and PC Gys1 KO and WT performed well.

(J) Rotarod test showed dual Gys1 KO animals have an ataxic phenotype. Data are presented as mean $\pm$ SEM, *t* test for A; one-way ANOVA followed by Tukey's post hoc test (C, D, E); $N \geq 5$ for WT, AB, PC, and Dual Gys1 KO (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

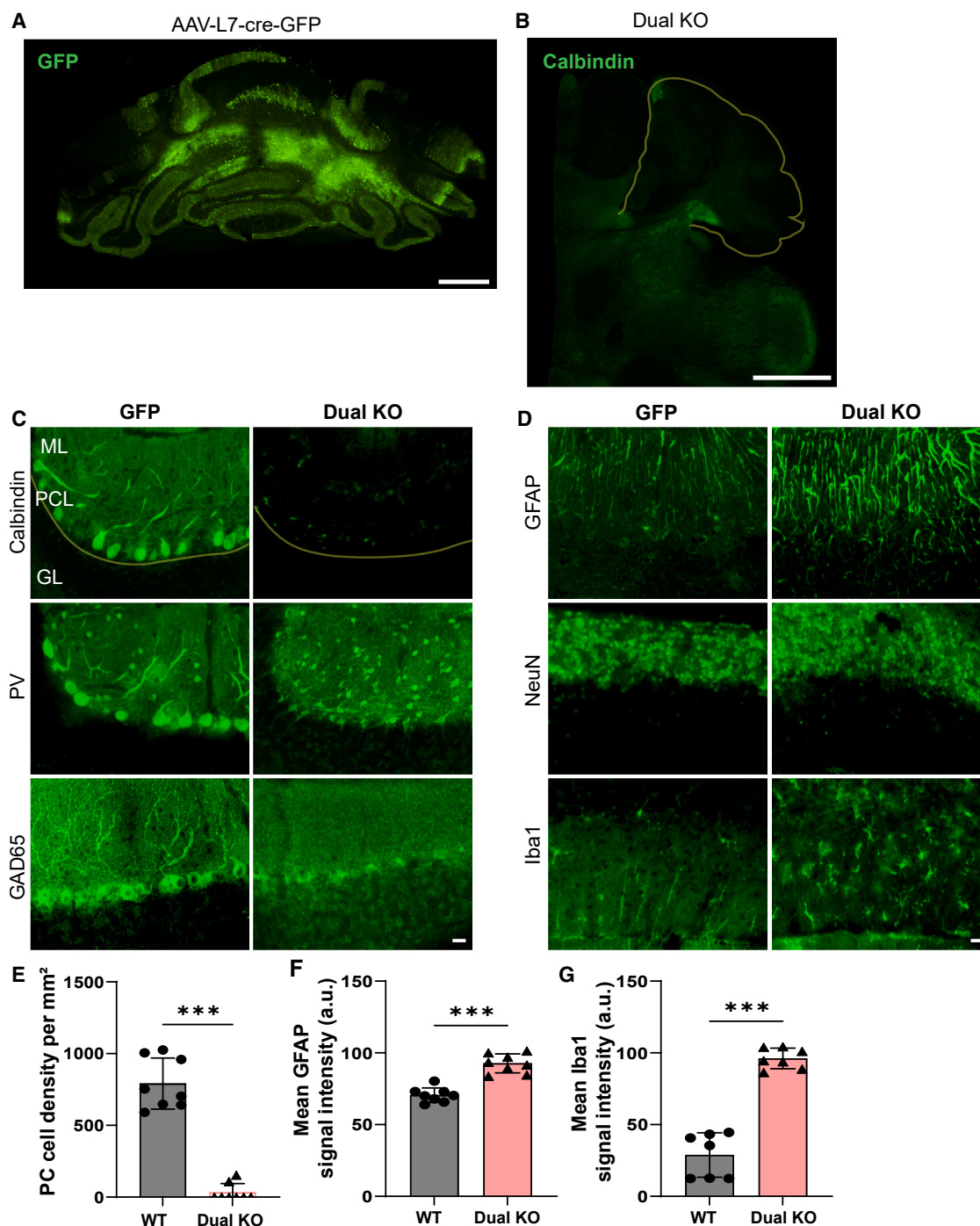


Figure 6. Histological changes in the cerebellum after Gys1 deletion from both PCs and astrocytes

(A) No obvious difference in PC morphology is observed following a high volume of AAV-L7-Cre-GFP injection in WT mice.

(B) PC ablation is observed by calbindin staining in dual Gys1 KO mice.

(C) While AAV-L7-Cre-GFP mice displayed normal PC morphology by three interneuron markers (calbindin, parvalbumin, and GAD65), Dual Gys1 KO mice showed either no or significantly reduced expression.

(D) Comparisons between GFP and Dual Gys1 KO are shown using cell type-specific markers GFAP (astrocytes), NeuN (neurons), and Iba1 (microglia). Enhanced expression of GFAP and Iba1 is evident in Dual Gys1 KO. No obvious changes were noted in NeuN expression in the granular layer.

(E) Quantification of viable PCs.

(F) Comparison of GFAP signal strength.

(G) Comparison of Iba1 signal strength. (mean $\pm$ SEM, $N = 7$ animals for WT, $N = 7$ for Dual Gys1 KO, *** $p < 0.001$) Scale bars, 1 mm for A and B, 20 μ m for C and D.

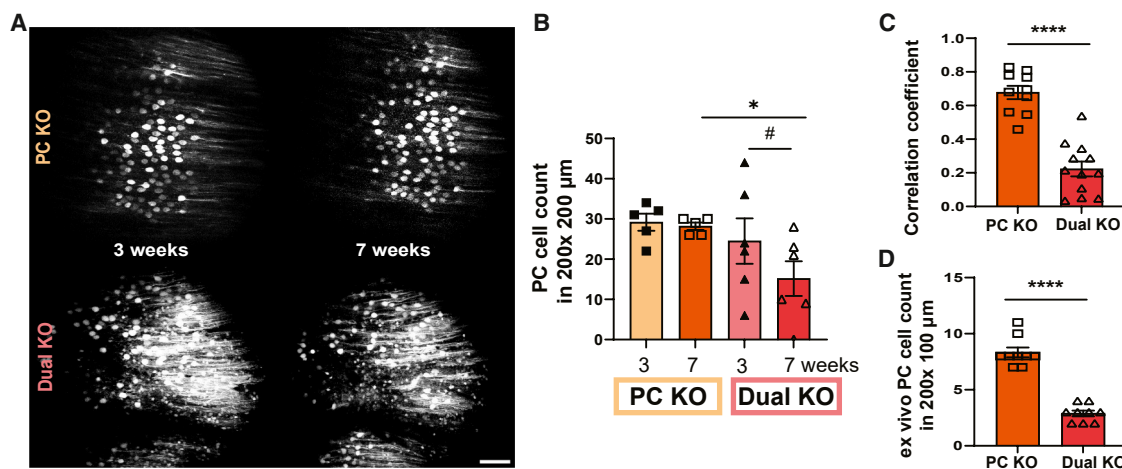


Figure 7. PC ablation is observable *in vivo* in dual-cell-type Gys1-deficient mice

(A) jRCaMP8 expression in PCs from PC Gys1 KO and Dual Gys1 KO animals 3 and 7 weeks after virus injection. jRCaMP8 expression in PC Gys1 KO remained similar across time (upper). PCs were ablated when Gys1 was knocked out in both PC and AB (lower). Scale bars, 100 μm.

(B) Number of PCs in a 200 μm × 200 μm from *in vivo* PC cell images.

(C) Longitudinal ROI tracking assessed by correlation analysis.

(D) *Ex vivo* validation of PC cell death by calbindin immunohistochemistry. Data are presented as mean ± SEM. For B, an aligned rank transform (ART) with repeated measures was used to identify a significant effect in time ($F(1, 9) = 16.20, p = 0.003$), and genotype × time interaction ($F(1, 9) = 9.86, p = 0.012$). The Wilcoxon signed rank test was used for comparisons between two time points within the same group ($V = 21, \#p < 0.05$), and the Wilcoxon rank-sum test was used for comparisons between two different groups at 7 weeks ($W = 2, *p < 0.05$); For C and D, t test was used ($****p < 0.0001$). $N = 5$ and 6 for PC Gys1 KO and dual Gys1 KO, respectively.

unclear whether the observed inflammatory signal is caused by PC death or by impaired glycogen synthesis. Our current study cannot distinguish between these possibilities, including whether pro-inflammatory cytokine production contributes to Purkinje cell death.^{53–55} Considering the large extent of PC neurodegeneration, we attribute the motor function deficits observed in the dual cell-type Gys-1 KO to the ablation of PCs, as reported in other models of PC neurodegeneration.^{56–58} Our study suggests that in the cerebellum, glycogen metabolism in both PCs and astrocytes is critical for the viability of PCs.

The resilience of BG or PC single-cell-type Gys1 KO suggests a robust fueling system that provides a constant energy supply to support PCs' sustained high-frequency discharge activities.¹⁶ For comparison, neurons in glycogen-rich forebrain regions (i.e., hippocampus, cortical superficial layers, and striatum) do not have high discharge rates except for some GABAergic interneurons. The relatively low proportion of energy-demanding neurons is possibly the reason that the forebrain energy metabolism does not critically rely on glycogen. Of note, using neuronal glucose transporter knockout mice, a recent article showed that neurons metabolize glucose through glycolysis, and this neuronal glucose metabolism is required for proper neuronal function.⁵⁹

The high baseline energy demand of PCs conceivably underlies the necessity of a robust fueling system. Upstream of the energy substrate supply is the vascular networks. The vasculature supplies glucose to the parenchyma via glucose transporter 1. Unlike the cerebral cortex, BG endfoot coverage on the capillary networks has not been described in great detail, and Aqp4 immunohistochemistry does not appear to delineate the vasculature organization.⁶⁰ This configuration might allow for the uptake of glucose by both neurons and glia in the cerebellar cortex. In fact, PCs ex-

press an appreciable amount of *Glut3* (*Slc2a3*) according to a recent single-nucleus transcriptomics study.⁶¹ Glucose in PCs can be metabolized via at least two pathways (Figure 8): (a) the classic oxidative glycolysis pathway, which produces pyruvate by enzymatic breakdown, or (b) the glycogen shunt pathway, whereby glucose is first incorporated into glycogen, which in turn is enzymatically processed to produce pyruvate. Glucose is also taken up by BG and (c) processed to produce lactate via the lactate shuttle pathway, or (d) stored in glycogen and broken down to lactate in an on-demand manner. Thereafter, BG lactate is shuttled to neighboring PCs via monocarboxylate transporters.

While the present article did not exhaustively explore all these pathways, we describe decreases in BG glycogen in PC Gys1 KO, suggesting increased utilization of BG glycogen to compensate for the compromised PC glycogen shunt pathway (b). Furthermore, pharmacological blockade of the cerebellar lactate transporter MCT2 (*Slc16a7*), which is highly expressed in PCs, has been reported to severely compromise Rotarod performance.⁶² Considering the functional significance of lactate transport to PCs, it is tempting to postulate that the exceptional glycogen storage in BG serves as a buffer for a seamless energy supply to highly active PCs. Interference with glycogen synthesis in PCs and BG (i.e., (b) and (d)) is sufficient to ablate PCs. It is noted that the acute topical application of a glycogen phosphorylase inhibitor, DAB, does not impact climbing fiber-induced extracellular lactate increases in the cerebellar cortex in anesthetized rats.⁶³ However, anesthesia depletes liver glycogen and elevates blood glucose levels,⁶⁴ hence the balance of energy supply routes may have been altered. Moreover, PC ablation is possibly a slow process that takes weeks. While the gradual nature of protein diminishment by the conditional

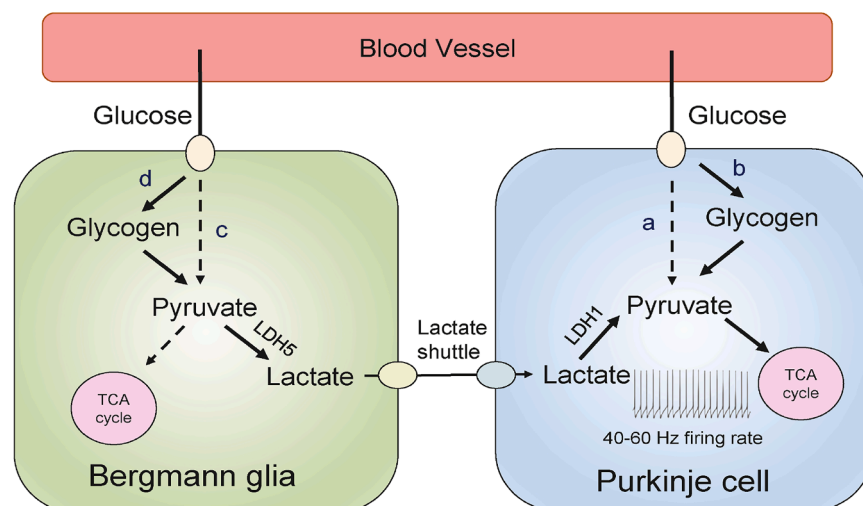


Figure 8. Energy metabolism in the BG-PC circuit

Glucose enters PC through GLUT and follows the classic oxidative pathway to produce energy (a). Some of the glucose is incorporated into glycogen, generating energy via the glycogen shunt pathway (b). BG lactate is produced and transferred to PC via the lactate shuttle (c). BG takes up glucose and stores it as glycogen, which is converted back to glucose to produce ATP during sudden energy demand (d).

knockout approach does not critically address the time course of PC ablation, our *in vivo* observation reports PC ablation a few weeks after Cre AAV injection.

In conclusion, the current study demonstrates that glycogen metabolism plays a central role in the normal operation of PCs.

Limitations of the study

This study demonstrated predominant glycogen localization in cerebellar astrocytes and Purkinje neurons (PCs) but did not assess glycogen content in other inhibitory neuronal subtypes or distinguish between astrocyte populations such as Bergmann glia and velate astrocytes, which may differ functionally. The preservation of glycogen during electron microscopy posed a technical challenge: glycogen was consistently detected in astrocytes, consistent with their storage role, whereas PC glycogen was difficult to preserve due to rapid consumption under anoxic conditions, contributing to discrepancies between immunohistochemistry and electron microscopy. Finally, although the simultaneous suppression of glycogen synthesis in PCs and astrocytes caused PC loss and cerebellar ataxia, single cell-type-specific deletions had minimal effects, leaving the temporal dynamics of glycogen utilization and compensation unresolved, particularly under metabolic stress or sustained firing.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Hajime Hirase (hirase@sund.ku.dk).

Materials availability

This study did not generate new materials or reagents.

Data and code availability

- All data supporting the findings of this study are available from the [lead contact](#) upon reasonable request.
- This study did not generate any new code.
- Additional raw data can be obtained from the [lead contact](#) upon reasonable request.

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AUTHOR CONTRIBUTIONS

S.A. and A.B.L.: conceptualization, investigation, writing – original draft, writing-review, and editing. A.K., H.H., C.F.M., J.D., J.G., H.A., T.M., and O.B.: resources and methodology. R.S.: resources, supervision, and investigation. A.A., M.V., and T.M.: investigation. M.N.: conceptualization, funding acquisition, and supervision. H.H.: conceptualization, funding acquisition, investigation, writing – original draft, writing – review and editing, and supervision.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- Animal behavior
- Cranial window preparation
- *In vivo* imaging of PCs
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● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse IV58B6	Baba et al. ¹⁷	IV58B6 https://doi.org/10.5357/koubyou.60.264
Mouse ESG1A9	Nakamura-Tsuruta et al. ¹⁸	ESG1A9 https://doi.org/10.1016/j.carres.2011.12.029
Rabbit polyclonal anti-Aldolase C	Frontier Institute	Cat#MSFR100140; RRID: AB_2571658
Rabbit polyclonal anti-Calbindin D-28K	Swant	Cat#CB38a; RRID: AB_3107026
Mouse anti-hdGFP	Nakamura et al. ⁶⁵	N/A https://doi.org/10.1369/jhc.2008.950915
Rabbit monoclonal anti-NeuN	Abcam	Cat#ab177487; Clone number: EPR12763; RRID: AB_2532109
Rabbit polyclonal anti-parvalbumin	Swant	Cat# PV-28; RRID: AB_2315235
Rabbit polyclonal anti- Glial Fibrillary Acidic Protein	Sigma-Aldrich, Abcam	Cat#G9269; RRID: AB_477035; Cat#ab7260; RRID: AB_305808
Rabbit polyclonal anti- Glutamic Acid Decarboxylase 65/67	Sigma-Aldrich	Cat#G5163; RRID: AB_477019
Mouse monoclonal anti-s100B	Sigma-Aldrich	Cat#S2532; RRID: AB_477499
Rabbit polyclonal anti-Iba1	Wako	Cat#019-19741; RRID: AB_839504
Bacterial and virus strains		
AAV9-GFAP-GFP	Viral Vector Core, Gunma University, Initiative for Advanced Research	N/A
AAV9-GFAP-cre	Viral Vector Core, Gunma University, Initiative for Advanced Research	N/A
AAV2/9 mL7-6 GFP-P2A-Cre	Viral Vector Core, Gunma University, Initiative for Advanced Research	N/A
PHP.eB-CAG-dlox-jGCaMP8-dlox	Viral Vector Facility, University of Zurich	v619; https://www.vvf.uzh.ch/en.html
Chemicals, peptides, and recombinant proteins		
Amyloglucosidase	Sigma-Aldrich	Cat#A7095
Lipopolysaccharides	Sigma-Aldrich	Cat#L4391
Epon (Embed 812)	Electron Microscopy Sciences	Cat#14900
Glutaraldehyde	Sigma-Aldrich	Cat#G5882
Critical commercial assays		
Glycogen Assay Kit	BioVision	Cat#K646-100
Experimental models: Organisms/strains		
Mouse: L7-Cre(tg/wt)	Jackson Laboratory	RRID: IMSR_JAX:004146
Mouse: Gys1(fl/fl)	Duran et al. ⁷	Gys1tm1a(EUCOMM)Wtsi
Software and algorithms		
ImageJ/Fiji	Schindelin et al. ⁶⁶	https://imagej.net/software/fiji/
Graphpad Prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Adult male and female mice of C57BL/6 background were used (age: 2–6 months, weight: 21–35 g). Conditional KO of *Gys1* was achieved using the *Gys1* flox mouse⁷ and expressing Cre recombinase in the target cell types. The L7-Cre line⁶⁷ was used for PC-specific Cre expression. Cre-recombinase activity is observed in PCs as early as embryonic day 17 and the parasagittal domains of L7-Cre activity becomes extensive by postnatal day 2.⁶⁸ Cerebellar astrocyte and Dual *Gys1* KO were achieved by respective Cre-expressing adeno-associated viral vector injection (Table 2 for AAVs and Table 4 for mouse group nomenclature). Mice were housed in a vivarium in 12 h light/dark cycles with *ad libitum* access to food and water.

The procedures involving animal care, surgery and sample preparation performed at RIKEN were approved by the Animal Experimental Committee of RIKEN Brain Science Institute and performed in accordance with the institutional guidelines. All experiments conducted at the University of Copenhagen were approved by the Danish Animal Experiments Inspectorate and were overseen by the University of Copenhagen Institutional Animal Care and Use Committee (IACUC), in compliance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) legislation governing the protection of animals used for scientific purposes.

METHOD DETAILS

Surgery procedure

Mice were anesthetized with isoflurane (induction: 4%, maintenance: 1.5%) and fixed in a stereotaxic frame. An incision was made on the scalp to expose the caudal part of the skull. A small hole was made on the skull above the cerebellar vermis using a dental drill (~7 mm from Bregma, ~1 mm diameter). For large-volume cerebellar AAV injection, a Hamilton syringe (10 μ l, 33 G needle) was inserted into the cerebellum to a depth of 1.6 mm from the brain surface using a fine manipulator attached to the stereotaxic frame. A total volume of 10 μ l of AAV was injected at an infusion rate of 330 nl/min using a syringe pump.⁶⁹ For local expression, either a glass capillary micropipette or a Hamilton syringe (35 G needle) was used to inject 0.5 μ l AAV solution at a depth of 300–600 μ m with a 100 nl/min infusion rate. The syringe was kept in place for another 7–10 min after the injection. After gently withdrawing the syringe, the scalp was sutured and fixed with surgical glue. All mice were given a shot of carprofen (5 mg/kg per day) for three days post-surgery.

Histology

For glycogen immunohistochemistry, mice were euthanized by FMW irradiation using a microwave fixation system (MMW-05, Muromachi Kikai, Tokyo, Japan) as described in Oe et al.⁹ Briefly, an unanesthetized mouse was confined in a specialized tubular animal holder with a hollow wall filled with water (WJM-24 or WJM-28, Muromachi Kikai) and the mouse's position was secured by a plunger. After the holder was placed inside MMW-05, high-energy microwave (5 kW) was applied to the head of the mouse for 0.94–1.10 s depending on its body weight (18–25 g: 0.94 s, 26–30 g: 1.00 s, >31 g: 1.10 s), which induced instant euthanasia. Thereafter, the brain was removed from the skull and incubated in a fixative (4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4)) overnight at 4°C. For other immunohistochemistry, mice were fixed by standard transcardial perfusion with saline (0.9% NaCl, 25 mL) for 5 min, followed by fixative (as above, 50 mL) for 10 min. Perfusion-fixed brains were postfixed in the same fixative overnight at 4°C.

Brain sections of 60 μ m thickness were prepared in PB using a micro slicer (Pro-7 Linear Slicer, DSK or VT1200S, Leica). After washing in phosphate-buffered saline (PBS), the sections were incubated in PBS containing primary antibodies and 0.1% Triton X-100 for 24 h at 4°C while gently shaking. The concentrations of primary antibodies are described in Table 1. The sections were washed three to five times in PBS and incubated with fluorescent secondary antibodies (1:1000 in PBS, Alexa Fluor 488 or 594, Life Technologies; 1:1000 in 0.1% Triton X-100 PBS for IV and ESG stained sections). Brain slices were mounted on slides and coverslipped with PermaFluor mounting medium (Thermo Scientific). For the experiment assessing glycogen antibody specificity, slices were treated with amyloglucosidase (A7095, Sigma-Aldrich; 10U amyloglucosidase in pH = 5.0 phthalate buffer) for 24 h at 45°C, which is the optimal condition for amyloglucosidase activity.

Electron microscopy

Deeply anesthetized mice were rapidly perfused with a fixative containing 2% PFA and 2–2.5% glutaraldehyde in 0.1 M PB without prior saline perfusion. The cerebellum was dissected from the brain and cut into a ~1 mm³ block and subjected to 24-h post-fixation (2% PFA, 2.5% glutaraldehyde, 0.1 M PB; one exchange of the fixative solution). Cerebellar blocks were sliced into 60 μ m sections. After three washes in 0.1 M PB, the sections were incubated in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M PB for 1 h at room temperature. After incubation, the sections were washed three times with ddH₂O. The osmium-treated sections were dehydrated in a series of ethanol solutions with increasing concentrations (50%, 70% and 90%; 5 min each) followed by 100% ethanol (10 min) and propylene oxide (15 min). The sections were embedded in Epon (Embed 812, Electron Microscopy Sciences) according to standard procedures. The region of interest (ROI) was cut under a light microscope and transferred into the resin mold, which was incubated in an oven at 60 °C for 24–48 h. Ultra-thin sections were cut using an Ultracut 7 (Leica) at 60 nm and collected on copper grids with Formvar-supported membranes. The ultra-thin sections were then stained with 1% uranyl acetate and 3% lead citrate (Ultrastainer, Leica). Electron microscopy was performed with a transmission electron microscope (CM100, Philips) operated at an accelerating voltage of 80 kV. Digital images were acquired with an OSIS Veleta 2k $\times$ 2k pixel CCD camera and the ITEM software package.

Biochemical assay of glycogen

Biochemical quantification of cerebellar glycogen was performed using a glycogen assay kit (K646-100, BioVision) following the protocol described previously.⁹ After FMW irradiation, each cerebellum sample was weighed and homogenized using a homogenizer (PT1200E, PT-DA 03/2EC-E050, KINEMATICA) to prepare the final concentration of 10 mg/200 μ l in distilled water. Following centrifugation (18,000 $\times$ G, 10 min), the supernatant and glycogen standard (10 μ l) were transferred to a 96-well plate and incubated with the hydrolysis enzyme mix (1 μ l, 30 min, room temperature) and hydrolysis buffer. The samples were then incubated with the

development enzyme mix (1 μ L) and the OxiRed probe (0.3 μ L) for 30 min in room temperature. During this incubation, the plate was kept in the dark to prevent bleaching. The fluorescence signals of the samples were measured using a microplate reader (SpectraMax iD3, Molecular Devices, Ex/Em 535/587 nm). Glycogen concentration was calculated from the glycogen standard curve followed by background value subtraction (the signal without hydrolysis enzyme mix). For the comparison between normal and inflammatory cerebellum, young adult (6–8 weeks) C57BL6 mice were injected with either saline or lipopolysaccharides (LPS) (L4391, Sigma, 1 mg/mL, 1 mg/kg of body weight) intraperitoneally for three days.

Animal behavior

For the behavioral experiments, all mice followed similar timelines. Briefly, the virus-injected mice were allowed to recover from surgery and kept in the animal room for 3 weeks. After 3 weeks, the mice were moved to the behavioral room and acclimated for one day before the experiment. Depending on the experiment, they were habituated (as described in each experimental method), and after the final test day, they were euthanized. During the habituation and experimental phases, all mice were kept in the behavioral room. Experimenters were blind to the genotypes of the mice during the behavioral experiments.

Open field

Open field consists of a square area of 40 x 40 cm². The box was made of wood covered with white foam polyvinyl chloride (PVC) sheets. The inner area of 20 x 20 cm² was considered as the arena center. Mice were transferred to the room one day before the experiment and tested at the end of the light cycle (5–7 pm). Mouse movement was recorded with a video camera placed above the arena. The test was initiated by placing a single mouse in the center of the box and letting it explore freely for 10 min. Four open field setups, apart from each other by 40 cm and separated by a white board, were used simultaneously. The box was cleaned with alcohol and water after each session. The trajectory of each mouse was analyzed using Ethovision.

Elevated plus maze

Elevated plus maze consists of two open arms (30 cm each) and two closed arms (30 cm each) surrounded by a wall of 20 cm arranged to form a cross, in which the center area is 5 x 5 cm². The floor and the wall are made with foam PVC. Mice were transferred to the experiment room one day before the experiment. All experiments were conducted between 5–8 pm. To initiate the test, a single mouse was placed on the center of the maze facing one open arm. The mouse was allowed to move freely for 10 min. Total distance, time spent in open vs closed arm and the frequency of open and closed arm visit was calculated. Animal movement was continuously recorded by a video camera placed above the apparatus. Between each test, the whole apparatus was cleaned with alcohol and water.

Inverted mesh

The test is a modified Kondziela's inverted screen test as described by Deacon.⁷⁰ Briefly, a mouse is placed on a 40 cm square wire mesh (12 mm mesh of 1 mm diameter wire). The edge of the screen is sealed with tape to prevent the mouse from escaping. Once the mouse holds the wire mesh, the screen is inverted 50 cm above the padded surface and kept in that position for 60 s. The latency to fall was recorded and scores were assigned accordingly as follows: score 1: 1–10 s, 2: 11–25 s, 3: 26–60 s, 4: > 60 s.

Hind limb clasp

A mouse is lifted by its tail and held in that position for 10 s while its hind limbs are observed and scored. If the hind limbs are consistently splayed outward, away from the abdomen, it is assigned a score of 0. If one hind limb is retracted toward the abdomen for more than 50% of the time suspended, it receives a score of 1. If both hind limbs are partially retracted toward the abdomen for more than 50% of the time suspended, it receives a score of 2. If its hind limbs are entirely retracted and touching the abdomen for more than 50% of the time suspended, it receives a score of 3.⁶⁶

Ledge test

A mouse was lifted from the cage and placed on the cage's ledge, whereby mice typically walk along the ledge and attempt to descend back into the cage. Mouse behavior is scored as described previously.⁶⁶ Score 0: the mouse walks along the ledge and lowers itself into the cage. 1: the mouse loses its footing while walking along the ledge. 2: the mouse does not effectively use its hind legs or lands on its head rather than its paws when descending into the cage. 3: the mouse falls off the ledge, loses its balance during walking, or refuses to engage in locomotion. In some cases, a gentle nudge was presented to encourage initiation of locomotion.

Rotarod

Rotarod test was performed using a Panlab Rotarod LE8205 (Harvard Apparatus, rod diameter 30 mm, drum diameter 250 mm, fall height 20 cm, lane width 50 mm). Mice were trained in the rotarod for 3 days before the final test day. On day 1, all mice were habituated on the stationary rod. On day 2, mice are habituated on the rotating rod at 4 rpm/min. On day 3, mice were trained on the rod, where the rod started to rotate after 30 s for 9.5 min with increasing acceleration of 4–40 rpm at 8.3 s increment. The latency to fall from the rod was recorded. On day 4, two tests in the accelerating mode were performed with a 45-min interval. The mean latency to fall is computed as the data point.

Cranial window preparation

Mice were anesthetized with 3–4% isoflurane for induction before being secured in a stereotaxic frame. During the surgical procedure, anesthesia was maintained at 1–1.5% isoflurane, and body temperature was regulated at 37°C using a heating pad. For initial analgesia, buprenorphine (0.05 mg/kg) was administered subcutaneously, while lidocaine (0.2 mg/kg) was injected locally at the

incision site for local pain relief. The skull was then exposed, and a headplate was affixed to the skull using dental cement (Super Bond C&B, Sun Medical, Shiga, Japan). A 3-mm diameter craniotomy was performed over the cerebellar vermis, after which a sterile 3-mm diameter coverslip was positioned and sealed with dental cement.

In vivo imaging of PCs

Two-photon imaging was performed by using the Bergamo microscope (Thorlabs) and a Chameleon Ultra 2 laser (Coherent) with XLPlan N objective lense (x25 NA = 1.05; Olympus) and the primary dichroic mirror FF705-Di01-25x36 (Chroma). The emission light was split using a secondary dichroic mirror (FF562-Di03, Semrock) along with band-pass filters (FF03-525/50, Semrock). The animals were acclimated to the experimental setup for a period of three days prior to the first imaging session. Image size was 802x 802 μm (512x512 pixels).

Histology image acquisition

Epifluorescence images of coronal or sagittal sections were acquired using an inverted fluorescence microscope (Nikon ECLIPSE Ni-E, Mono-Camera Nikon DS-Fi3) controlled by proprietary software (NIS-Elements Imaging AR 4.60.00). Confocal images were acquired with a Nikon Eclipse Ti2 microscope (NIS-Elements AR 4.50.00) with a Plan Apo x60/1.40 numerical aperture (NA) oil-immersion objective at a Z step of 1 μm . Image J (NIH) was used to measure the fluorescent intensity of ROIs. For the computation of area fraction of glycogen signal (image analysis), images were acquired with the 60 $\times$ objective lens with 2–6 $\times$ digital zoom depending on the cell type.

QUANTIFICATION AND STATISTICAL ANALYSIS

To compute the modulation depth of cerebellar stripes, image clips encompassing the molecular layer were extracted after rotating the coronal cerebellar IV glycogen immunostaining image as appropriate. For each image clip, the horizontal intensity profile was computed by averaging across the vertical dimension. Thereafter, a 50- μm averaging filter was applied. The modulation depth of possible cerebellar stripes was computed as the Michelson contrast: $(H-L)/(H+L)$, where H and L represent the medians of the 10% highest and lowest intensity values, respectively.

To compute the area fraction of glycogen signal, i.e., the proportion of glycogen-immunoreactive area in a ROI, fluorescent images of a cell type marker were binarized with a threshold of 30% of the maximum fluorescence intensity.⁹ The supra-threshold area was set as the ROI. Each frame of the image stack (10 frames, totaling to 5–10 μm in depth from the surface of a specimen) was analyzed separately. The number of glycogen puncta and area fraction of glycogen were determined from binarized images of glycogen immunofluorescence with a threshold of 30% of the maximum fluorescence intensity in each ROI.

Manders' overlap coefficients were calculated by quantifying colocalization between IV glycogen and cell-type markers (calbindin, GFAP) in the molecular and Purkinje cell layers using the Coloc2 plugin in Fiji.⁶⁵ After ROIs were drawn per layer, background was subtracted for each channel, and thresholded Manders' coefficients (tM1 and tM2) were obtained. Values were averaged across ROIs and are reported as mean $\pm$ SEM.

Electron microscopy images were analyzed with Fiji. Comparison of glycogen particles in BG processes from WT and PC *Gys1* KO mice was performed by randomly selecting ROIs (1 μm^2) in the molecular layer.

For comparisons of two sample means, unpaired *t*-test was used. For multiple group comparisons, one-way ANOVA followed by Tukey's post hoc test was used. Statistical tests were performed using Graphpad Prism.