

1 **Choroid plexus NKCC1 mediates cerebrospinal fluid clearance during mouse**  
2 **early postnatal development**

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9 **SUPPLEMENTAL INFORMATION**

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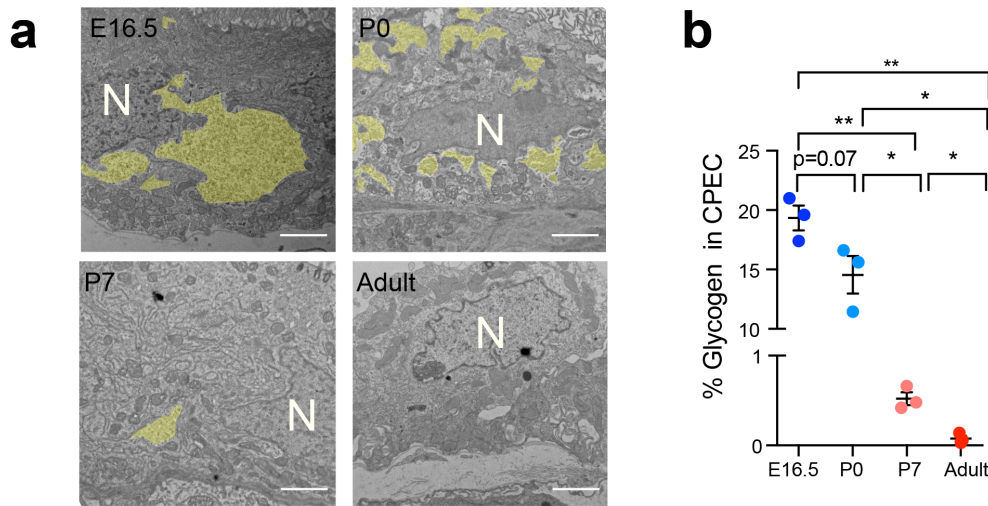
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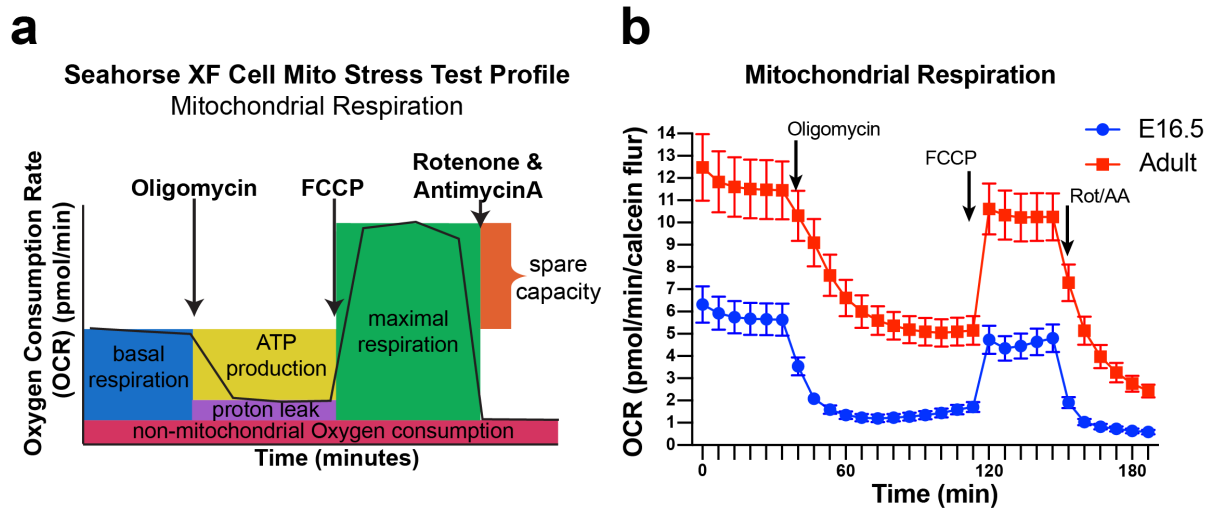
29 \*Correspondence should be addressed to: [maria.lehtinen@childrens.harvard.edu](mailto:maria.lehtinen@childrens.harvard.edu)

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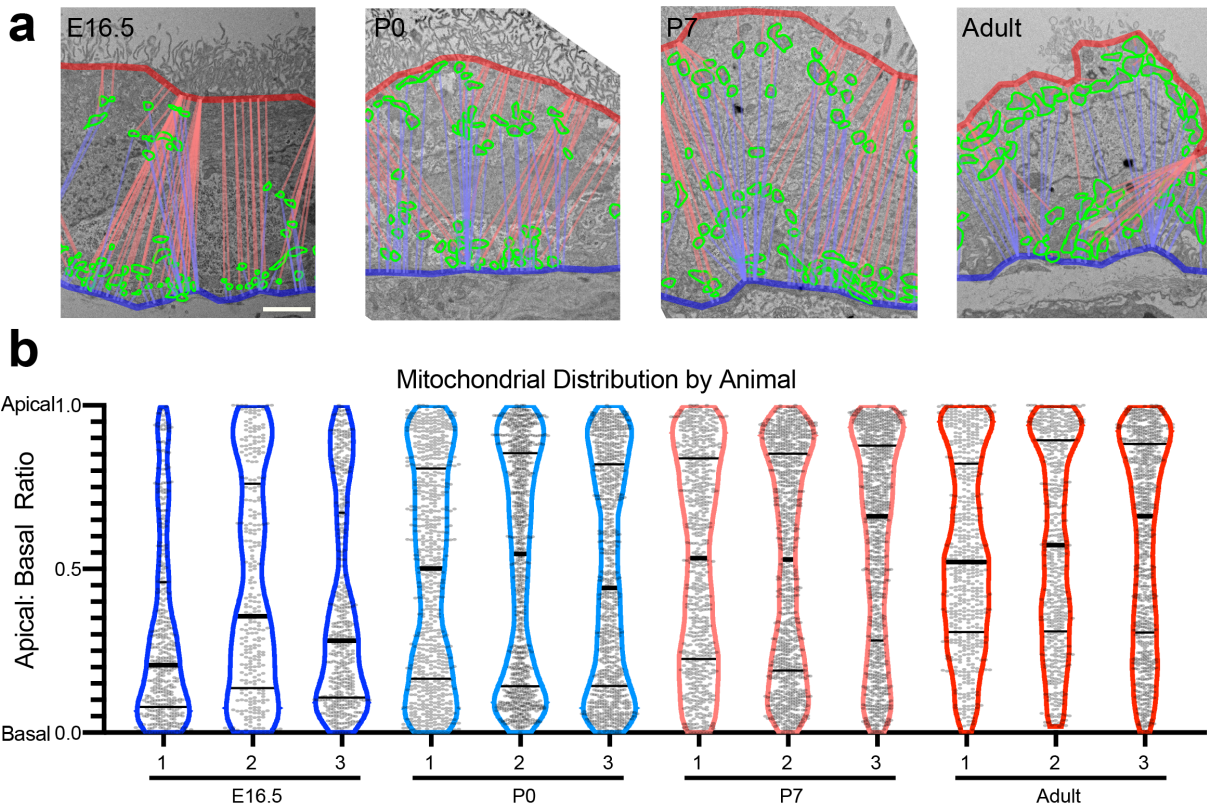
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 32 **Supplementary Fig. 1. Glycogen load in ChP epithelial cells.** **a** Representative transmission  
 33 electron micrographs of E16.5, P0, P7, and adult LV ChP. Glycogen granules are highlighted in  
 34 yellow. Scale bar = 2  $\mu$ m. N = Nucleus. **b** Proportion of TEM fields of view that are filled with  
 35 glycogen granules, N=3 animals from 2 independent experiments (same animals as those used in  
 36 Fig. 1d-f), 10-15 fields of view (FOV) per animal (See source data for exact numbers of FOV),  
 37 distinct cells were captured in each FOV., Blue to red color change represents age change, with  
 38 blue being the youngest (embryonic) and red the oldest (adult). E16 vs. P7: \*\*  $p = 0.0030$ ; E16  
 39 vs. adult: \*\*  $p = 0.0029$ ; P0 vs. P7: \*  $p = 0.0124$ ; P0 vs. adult \*  $p = 0.0117$ ; P7 vs. adult \*  $p =$   
 40  $0.0136$ ; Welch's two-tailed unpaired t-test. CEPC, choroid plexus epithelial cell. Source data are  
 41 provided as a Source Data file.

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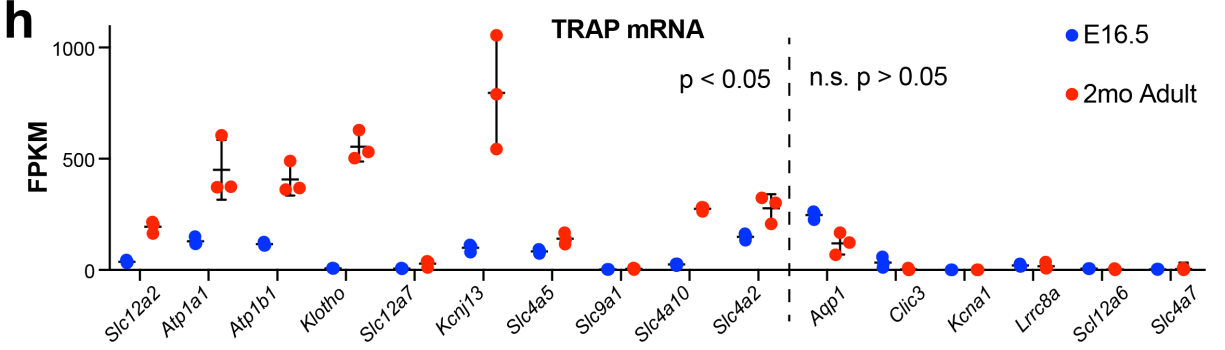
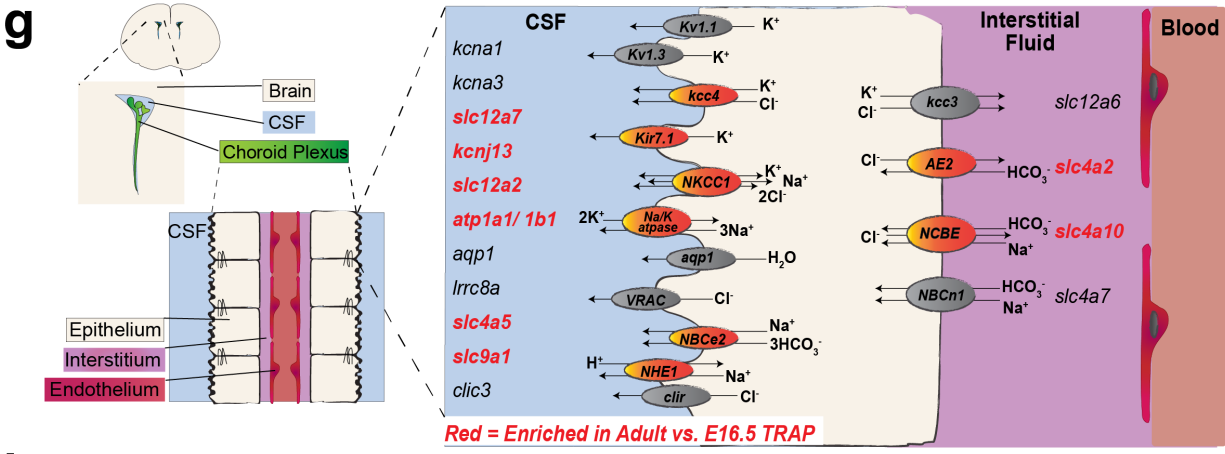
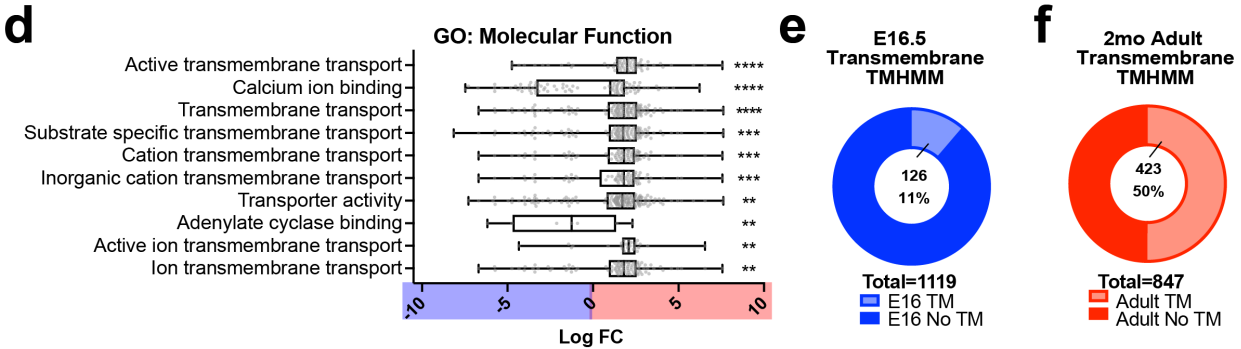
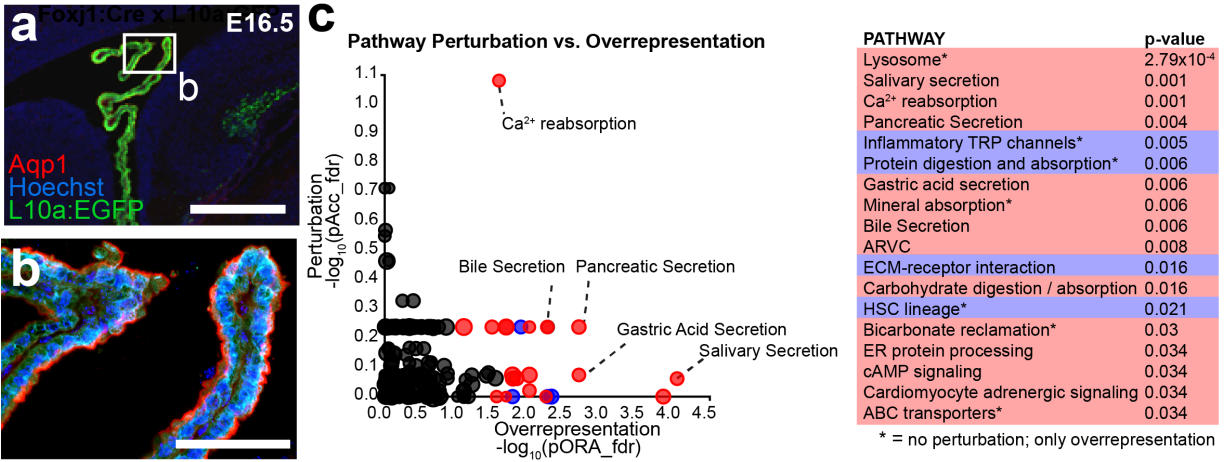


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 44 **Supplementary Fig. 2. Seahorse XF Cell mito stress test profile and representative curves. a**  
 45 Schematic of the Agilent Cell Mito Stress Test showing the experimental design to quantify  
 46 mitochondria basal respiration and ATP production. **b** Representative experiment of ChP in Cell  
 47 Mito Stress Test; N = 12 E16.5 animals and N = 4 adult animals. Red: adult; blue: E16.5. Source  
 48 data are provided as a Source Data file.

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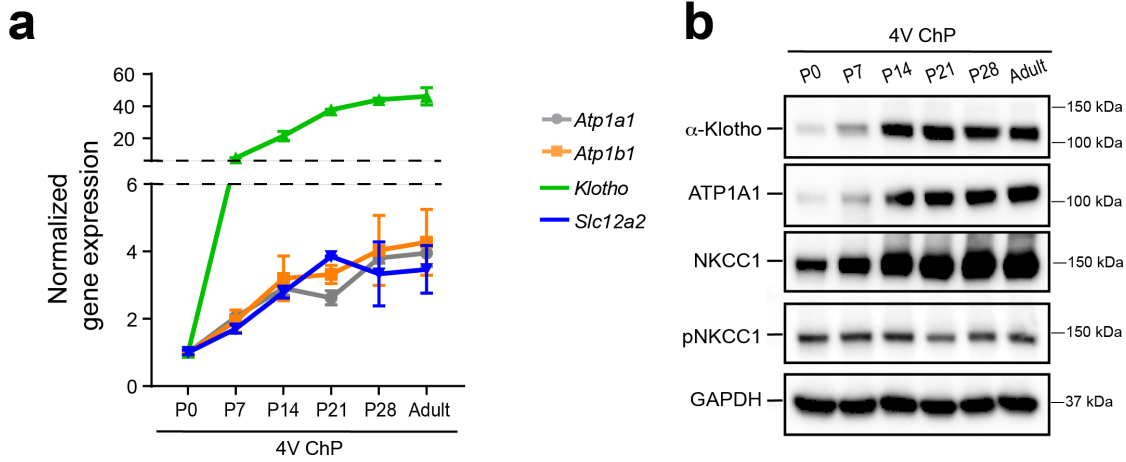


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 51 **Supplementary Fig. 3. Representative images and quantification of ChP epithelial**  
 52 **mitochondria distribution analysis.** **a** Representative transmission electron micrographs of  
 53 E16.5, P0, P7, and adult LV ChP. Mitochondria (green circle), apical membrane (red line), and  
 54 basal membrane (blue line) are labeled. Scale bar = 2 μm. Images are representative of 2  
 55 independent experiments with a total of 3 biologically independent animals at each age. **b**  
 56 Mitochondrial distribution plots from each animal (same as described in panel a). Apical: basal  
 57 ratio: 1 is touching the apical surface and 0 is touching the basal surface. Solid thick line  
 58 indicates median and thinner line indicates upper/lower quartiles. Blue to red color change  
 59 represents age change, with blue being the youngest (embryonic) and red the oldest (adult).  
 60 Source data are provided as a Source Data file.



63 **Supplementary Fig. 4. Supportive analysis of TRAP sequencing. a-b** Rpl10a-conjugated  
64 EGFP expression in ChP epithelial cells after *Foxj1*-Cre recombination in TRAP-BAC mice.  
65 Aqp1 marked ChP epithelial apical membrane. Scale bars = 500  $\mu\text{m}$  (a) and 100  $\mu\text{m}$  (b).  
66 Representative of 2 experiments, each with 2 biologically independent replicates. **c** Perturbation  
67 vs. overrepresentation analysis via iPathway (Advaita) reveals enriched pathways at E16.5 (blue)  
68 and Adult (red) (the same red vs. blue color scheme is used for the rest of this figure). \* indicates  
69 pathways that are only overrepresented, but not predicted to be additionally perturbed at the  
70 network level. **d** Top 10 significantly enriched GO terms for “molecular function”. Plotted with  
71 center bars as median, bounds of boxes for quartiles, and whiskers for maximum and minimum  
72 values. The  $\log_{10}$  fold change (LogFC) is plotted for each expressed gene for the network.  
73 Positive values (red) indicate Adult enrichment and negative values (blue) indicate E16.5  
74 enrichment.  $p$  values are corrected for multiple measures using Bonferroni correction. See  
75 Supplementary Data S1 for exact  $p$  - values; \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ . **e-f**  
76 Proportion of enriched genes in E16.5 (blue) and Adult (red) ChP with predicted transmembrane  
77 domains using TMHMM. **g** Schematic of ChP localization within brain ventricles, relative  
78 position to blood and CSF, and transporters. Red highlights: significantly enriched in Adult vs.  
79 E16.5 TRAP (adjusted  $p < 0.05$ ). **h** FPKM values from TRAP of transcripts associated with ChP  
80 transport.

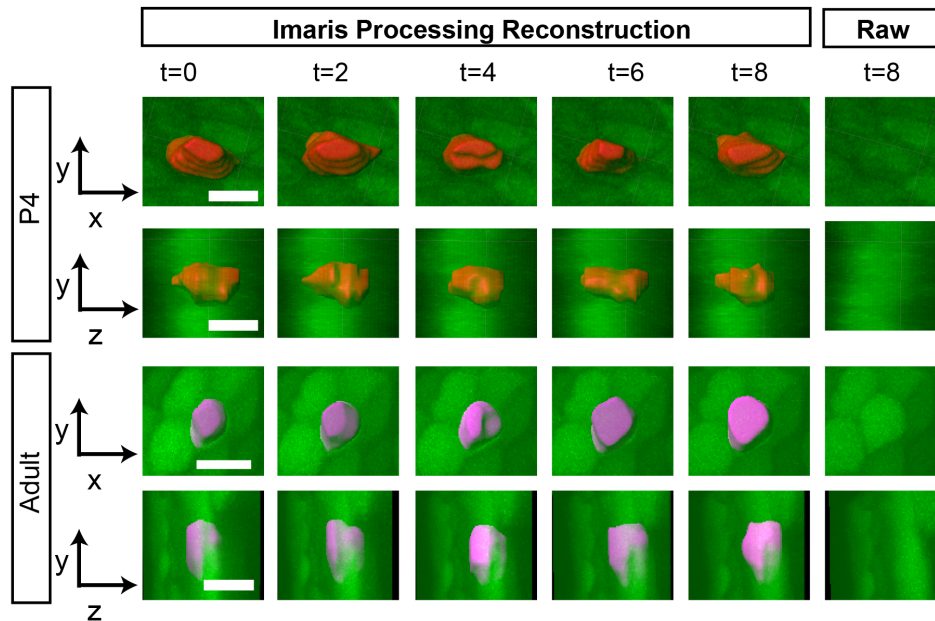
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83 **Supplementary Fig. 5. TRAP candidate validation in 4V ChP. a-b** RT-qPCR (N=4  
 84 biologically independent animals from two experiments for each timepoint, colors were chosen  
 85 to match with Fig. 2f) and immunoblotting of 4V ChP during postnatal development,  
 86 representative of 3 independent experiments, each with tissues from 1-2 animals pooled (2  
 87 animals for ages under P14, 1 animal for ages of P14 and older) for each timepoint Source data  
 88 are provided as a Source Data file.

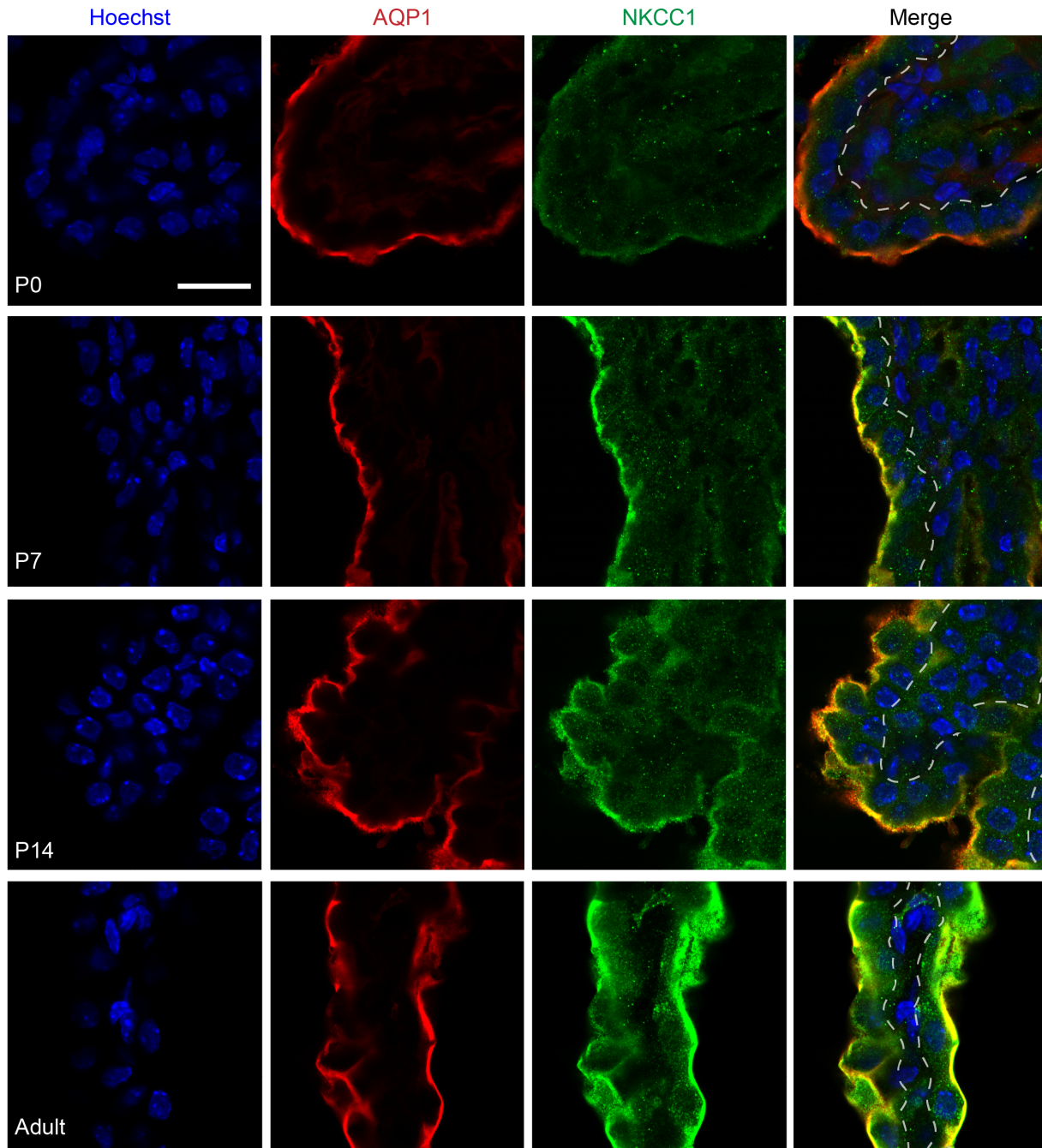
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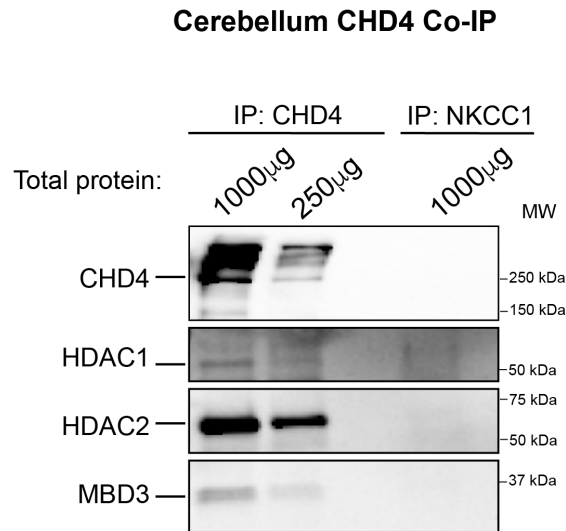
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91 **Supplementary Fig. 6.** Workflow of IMARIS demonstrating the cell volume quantification  
 92 process. At each time point, the reconstructed 3D cell mask is highlighted (P4 in red and adult in  
 93 pink) and views from x-y plane and y-z plane are displayed. Raw images from a single plane at  
 94 the last time point are shown on the right end. Scale bar = 10  $\mu\text{m}$  (P4) and 50  $\mu\text{m}$  (Adult). A  
 95 total of 3 independent experiments were conducted, each contained 2-3 biological replicates,  
 96 only tissues with good quality (i.e. tissues without tears or appearing stretched/crumpled due to  
 97 mounting, and with good calcein signal indicating viability) were included in quantification,  
 98 resulting in N=4 for each age.



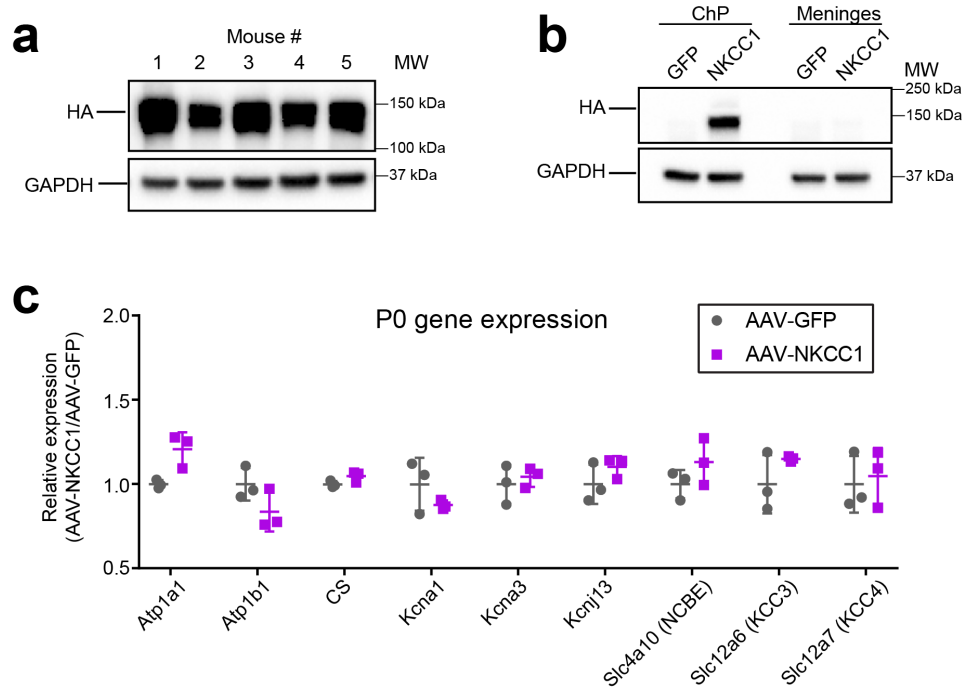


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 100 **Supplementary Fig. 7.** Localization of NKCC1 (green) at the apical membrane of ChP  
 101 epithelial cells across development (P0, P7, P14, and Adult). AQP1 (red) marks the apical  
 102 membrane; the white dashed line marks the basal membrane. Scale bar = 20  $\mu$ m. N = 4  
 103 biological replicates were included for each age, from 2 independent experiments.

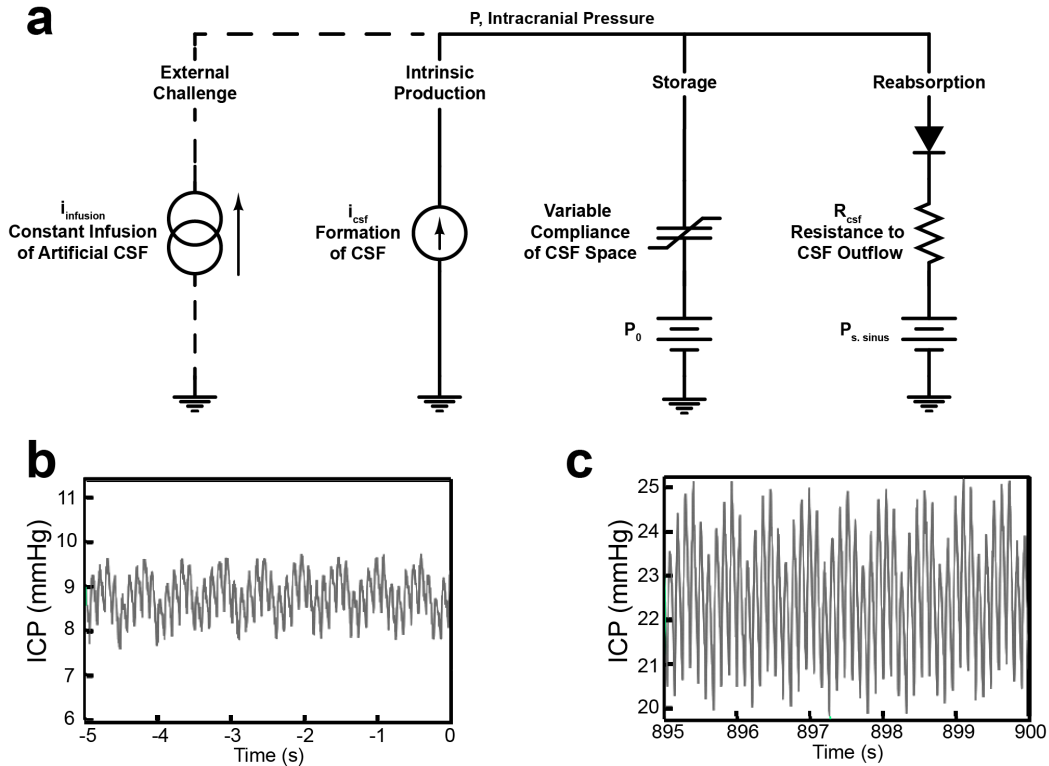


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 105 **Supplementary Fig. 8.** Co-IP immunoblots using adult mouse cerebellum to validate the Co-IP  
 106 protocol, representative of 3 independent experiments, each contained tissues collected from 1-2  
 107 mice. An antibody targeting CHD4 co-immunoprecipitated several other complex members  
 108 including HDAC1, HDAC2, and MBD3, from mouse cerebellar lysate, while a negative control  
 109 performed with a control antibody of the same host species (in this case anti-NKCC1 antibody)  
 110 failed to pull down NuRD-CHD4 complex members. Source data are provided as a Source Data  
 111 file.  
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 115 **Supplementary Fig. 9. Validation of AAV2/5-NKCC1 transduction efficiency and**  
 116 **specificity. a** Immunoblots of AAV2/5-NKCC1 transduced ChP showing successful but variable  
 117 transduction rate within one litter. N=5 biologically independent animals collected from two  
 118 experiments. **b** Immunoblot of AAV transduced ChP and meninges showing non-detectable  
 119 meningeal transduction by AAV2/5-NKCC1. Representative of 2 experiments, each with 2  
 120 biologically independent replicates. **c** RT-qPCR analysis of all other K<sup>+</sup> transporters and  
 121 channels in the ChP after *in utero* viral transduction, showing no significant changes; Grey:  
 122 AAV-GFP; purple: AAV-NKCC1.  $\alpha > 0.05$ , multiple t-Test corrected for multiple comparisons  
 123 using the Holm-Sidak method. N=3 biologically independent animals collected from 2  
 124 experiments. Source data are provided as a Source Data file.



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**Supplementary Fig. 10. Mechanisms of constant CSF infusion test by Marmarou's model. a**

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Marmarou's model of CSF dynamics. In this model, the physiologic processes of the cycle of CSF turnover are represented by analogous electric circuit elements, with ICP expressed as a solution to the circuit model in terms of lumped parameters describing the net effect of the processes on the level of ICP without attributing them to specific microscopic pathways. At the most basic level, the model is a statement of conservation of mass, with the rate of CSF production balanced by the rate of CSF storage in intracranial and spinal compartments plus the rate of CSF reabsorption. **b-c** Higher magnification of the ICP data of infusion test showing the normal arterial and respiratory components of the ICP waveform at the beginning of the test (**b**). The increase in waveform amplitude with ICP is expected with increasing volume load (**c**).

138 **Supplementary Table 1.** CSF ion concentrations at developmental stages. *p*: statistical comparison to adult values. \* *p* < 0.05; \*\* *p* <  
 139 0.01; \*\*\* *p* < 0.001; \*\*\*\* *p* < 0.0001; ns = not significant.

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	K <sup>+</sup>			Na <sup>+</sup>			Cl <sup>-</sup>			Ca <sup>2+</sup>			Mg <sup>2+</sup>							
	mM	<i>p</i>	N	mM	<i>p</i>	N	mM	<i>p</i>	N	mM	<i>p</i>	N	mM	<i>p</i>	N					
<b>E14.5</b>	7.969 ± 1.90	****	<0.0001	8	141.1 ± 6.10	ns	0.1851	5	104.9 ± 5.28	**	0.0054	5	3.42 ± 0.47	*	0.0205	3	1.76 ± 0.07	****	<0.0001	4
<b>P0</b>	9.590 ± 3.50	**	0.0015	5	126.2 ± 11.84	ns	0.03605	4	104.0 ± 3.27	**	0.0078	4	3.70 ± 0.84	*	0.0456	3	1.07 ± 0.04	****	0.0002	4
<b>P4</b>	4.903 ± 0.47	**	0.0013	4	131.9 ± 4.52	ns	0.1	4	111.0 ± 2.00	*	0.035	4	2.63 ± 0.21	ns	0.1273	3	1.09 ± 0.01	****	<0.0001	4
<b>P7</b>	4.363 ± 0.92	*	0.0348	4	144.5 ± 6.65	ns	0.8313	4	125.0 ± 2.00	ns	0.7499	4	3.13 ± 0.39	*	0.0328	3	2.04 ± 0.19	**	0.001	4
<b>P14</b>	3.283 ± 0.18	ns	0.6672	4	137.3 ± 3.22	ns	0.2686	4	115.1 ± 6.00	ns	0.1124	4	2.51 ± 0.36	ns	0.3443	4	0.53 ± 0.05	****	<0.0001	4
<b>Adult</b>	3.142 ± 0.61	/	/	6	146.2 ± 14.40	/	/	5	127.1 ± 12.01	/	/	5	2.24 ± 0.28	/	/	3	0.89 ± 0.04	/	/	5

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142 **Supplementary Table 2.** Summary of publications reporting various values of ChP epithelium  
 143 intracellular  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$  concentrations. (N.D., not determined).

Publication	Species	Age	$[\text{Na}^+]_i$	$[\text{K}^+]_i$	$[\text{Cl}^-]_i$
Gregoriades, J. M. C., Madaris, A., Alvarez, F. J. & Alvarez-Leefmans, F. J. Genetic and pharmacological inactivation of apical $\text{Na}^+$ - $\text{K}^+$ - $2\text{Cl}^-$ cotransporter 1 in choroid plexus epithelial cells reveals the physiological function of the cotransporter. <i>American journal of physiology. Cell physiology</i> 316, (2019).	Mouse	ChP epithelial culture collected from P10-21 mice	$9.2 \pm 2.5\text{mM}$	ND	$60.7 \pm 12.3\text{mM}$
Steffensen, A. B. <i>et al.</i> Cotransporter-mediated water transport underlying cerebrospinal fluid formation. <i>Nat Commun</i> 9, (2018).	Mouse	8-12 weeks	$31 \pm 5\text{mM}$	$141 \pm 12\text{mM}$	$35 \pm 9\text{mM}$
-Keep, R. F., Xiang, J. & Betz, A. L. Potassium cotransport at the rat choroid plexus. <i>The American journal of physiology</i> 267, (1994).  -Zeuthen, T. The effects of chloride ions on electrodiffusion in the membrane of a leaky epithelium. Studies of intact tissue by microelectrodes. <i>Pflugers Archiv : European journal of physiology</i> 408(3), (1987).	Necturus maculosus	Mature	$30.0\text{mM}$	$119\text{mM}$	$50\text{mM}$
Johanson, C. E. & Murphy, V. A. Acetazolamide and insulin alter choroid plexus epithelial cell $[\text{Na}^+]_i$ , pH, and volume. <i>The American journal of physiology</i> 258, (1990).	Rat	Adult	$48 \pm 0.7\text{mmol/kg}$	$95 \pm 1.2\text{mmol/kg}$	$62 \pm 0.3\text{mmol/kg}$
Saito, Y. & Wright, E. M. Regulation of intracellular chloride in bullfrog choroid plexus. <i>Brain Res</i> 417, (1987).	Bullfrog	Mature	$10.5\text{mM}$	ND	$24\text{mM}$