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Supplementary Information for

Klotho controls the brain/immune system interface in the choroid plexus

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Supporting Information

Figure S1 | Klotho protein and *Klotho* mRNA levels in the CP and hippocampus. (*A*–*E*) Relative klotho protein (*A*–*D*) and *Klotho* mRNA (*E*) levels in the CP or hippocampus were determined by western blot analysis and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), respectively. The genotypes of mice were WT (*A, C*–*E*) or as indicated (*B*). Ages were 2–4 months (*A*, *B*), 6 months (*C*) or as indicated (*D*, *E*). No s-KL was detected in the hippocampus of WT mice (*C*). The antibody used for detection also recognizes the KL1 domain of p-KL, which makes up much of the s-KL isoform and is proteolytically released from p-KL. We therefore used klotho-overexpressing (OE) mice that had received a stereotaxic hippocampal injection of lentivirus encoding klotho amino acids 1–980 (roughly corresponding to p-KL) 3 months earlier as a positive control (*C*). *n* = 3–4 mice per age. n.s., not significant. Values in bar graphs are means ± s.e.m.

Figure S2 | Cre-mediated reduction of klotho does not affect the cytoarchitecture of the CP. (*A*) AAV5-CMV-Cre-GFP was injected stereotaxically into the left lateral ventricle of a 3-month-old WT mouse. Coronal brain sections were obtained one month later and stained with DAPI (blue) and an antibody against Cre (pink). (*B*, *C*) Sagittal CP sections stained with hematoxylin and eosin (*B*) or co-labeled for klotho (green) and aquaporin-1 (AQP-1, red) and counterstained with Hoechst 33342 dye (blue) (*C*) obtained from 7–8-month-old WT or *Klotho*$^{flox/flox}$ (Flox) mice 5–6 months after Cre injection. (*D*) Coronal CP sections stained for cytokeratin (green), the endothelial cell marker CD31 (red), and the tight junction marker claudin-1 (gray) obtained from 19–21-month-old WT or *Klotho*$^{flox/flox}$ mice 9 months after Cre injection. Scale bars: 250 μm (*A*: hemibrain), 100 μm (*A*: hippocampus, cortex, and thalamus), 50 μm (*A*: Lat. Ventricle, *B, D*), 25 μm (*C*).

Figure S3 | CP-specific reduction of klotho after ICV injection of AAV5-CMV-Cre-GFP. AAV5-CMV-Cre-GFP (Cre) was injected stereotaxically into the left lateral ventricles of 3-month-old WT and *Klotho*$^{flox/flox}$ (Flox) mice. Levels of klotho and Cre recombinase in the CP of the lateral (Lat.) and 4th ventricles (*A, C*) and in the kidney (*B, D*) were determined by western blot analysis one month later. Uninjected (Uninj) mice served as an additional negative control. (*A, B*) Representative western blots. #, non-specific band above Cre-specific band. (*C, D*) Quantitation of relative protein levels normalized to GAPDH levels and expressed relative to mean levels in WT controls that were (C) or were not (D) injected with Cre. n = 3 mice per group. ****P<0.0001 by two-way ANOVA and Holm-Sidak test. V, ventricle. Values are means ± s.e.m.

Figure S4 | Aging and klotho reduction increase the expression of immune regulators and mediators in the CP. (*A*) Immunostained brain sections illustrating levels of ICAM1 (green) and IRF7 (red) in the CP of 2- vs. 26-month-old WT mice. Scale bar: 100 μm. (*B*) Levels of the indicated mRNAs were measured in the CP of 20–

24-month-old WT and *Klotho*$^{flox/flox}$ (Flox) mice (n = 4–8 per group) 11 months after Cre injection. *P<0.05, **P<0.01 by unpaired, two-tailed t test. Values are means ± s.e.m.

Figure S5 | Expression of immune regulators and mediators is also increased in the CP of *kl/kl* mice but not in the hippocampus of *kl/kl* mice or of *Klotho*$^{flox/flox}$ mice with selective depletion of klotho in the CP. (*A*, *B*) ICAM1 expression in the CP of uninjected 2-month-old WT and *kl/kl* mice (n = 9–10 per group) was determined in coronal sections co-labeled for ICAM1 (red) and cytokeratin (gray). Scale bars: 100 μm. (*C*) Levels of the indicated mRNAs in the CP of uninjected, 2-month-old WT and *kl/kl* mice (n = 9–12 per group). (*D*) Hippocampal levels of the indicated mRNAs in 20–24-month-old WT and *Klotho*$^{flox/flox}$ (Flox) mice (n = 8 per group) measured 11 months after Cre injection. (*E*) Hippocampal levels of the indicated mRNAs in uninjected, 2-month-old WT and *kl/kl* mice (n = 12 per group). *P<0.05 or as indicated (unpaired, two-tailed t test). n.s., not significant. Values in bar graphs are means ± s.e.m.

Figure S6 | Klotho reduction increases the expression of macrophage but not T-cell-related gene products in the CP. (*A*–*C*) CP levels of the indicated mRNAs in 19–21-month-old WT and *Klotho*$^{flox/flox}$ (Flox) mice (n = 4–8 per group) measured 11 months after Cre injection. (*D*, *E*) In the same mice, we also determined the number of cells in the CP that expressed the T-cell markers CD3 (*D*) or CD4 (*E*). *P<0.05, **P<0.01 by unpaired, two-tailed t test. n.s., not significant. Values are means ± s.e.m.

Figure S7 | Macrophage markers and complement expression are not increased in the CP of 2-month-old *kl/kl* mice. (*A*–*F*) Levels of the indicated mRNAs (*A*–*E*) and number of LY6C-positive cells (*F*) in the CP of 2-month-old WT and *kl/kl* mice (n = 9–12 per group). n.s., not significant. Values are means ± SEM.

Figure S8 | Klotho reduction increases *Txnip* expression in the CP but not in the hippocampus or kidney. (*A*–*D*) Hippocampal levels of *Txnip* mRNA (*A*, *B*) and renal levels of TXNIP (*C*) and klotho (*D*) protein in 20–24-month-old WT and *Klotho*$^{flox/flox}$ (Flox) mice measured 11 months after Cre injection (*A*) and in uninjected 2-month-old WT and *kl/kl* mice (*B*–*D*). n = 8–9 mice per group. ***P<0.001 by unpaired, two-tailed t test. n.s., not significant. Values are means ± s.e.m.

Figure S9 | LPS-ATP treatment does not increase IL-1β production in primary macrophages from *Nlrp3*$^{-/-}$ or *Casp1/4*$^{-/-}$ mice. At DIV 7-10, primary bone marrow-derived macrophages from WT, *Nlrp3*$^{-/-}$, or *Casp1/4*$^{-/-}$ mice were treated with vehicle (Veh) or LPS (100 EU/ml, equivalent to 1 μg/ml) for 20 h and then with vehicle or ATP (1 mM) for 1 h. IL-1β levels in the culture medium were determined by ELISA. Note log-10 scale. n = 3 independent experiments, each of which included 3 wells per treatment. ****P<0.0001 by unpaired, two-tailed t test. Values are means ± s.e.m.

Figure S1

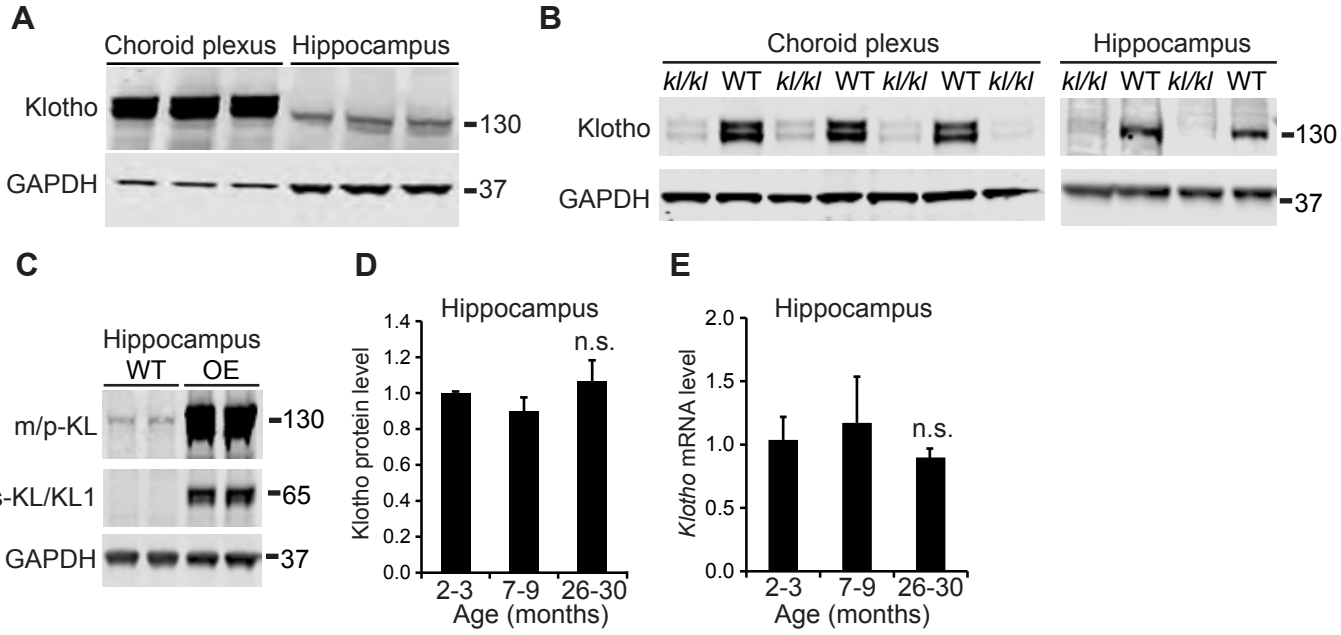
A
Choroid plexus
Hippocampus
Klotho
GAPDH
130
37
B
Choroid plexus
Hippocampus
kl/kl WT kl/kl WT kl/kl WT kl/kl
kl/kl WT kl/kl WT
Klotho
GAPDH
130
37
C
Hippocampus
WT OE
m/p-KL
s-KL/KL1
GAPDH
130
65
37
D
Hippocampus
n.s.
Klotho protein level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2-3 7-9 26-30
Age (months)
E
Hippocampus
n.s.
Klotho mRNA level
2.0
1.5
1.0
0.5
0.0
2-3 7-9 26-30
Age (months)

Figure S2

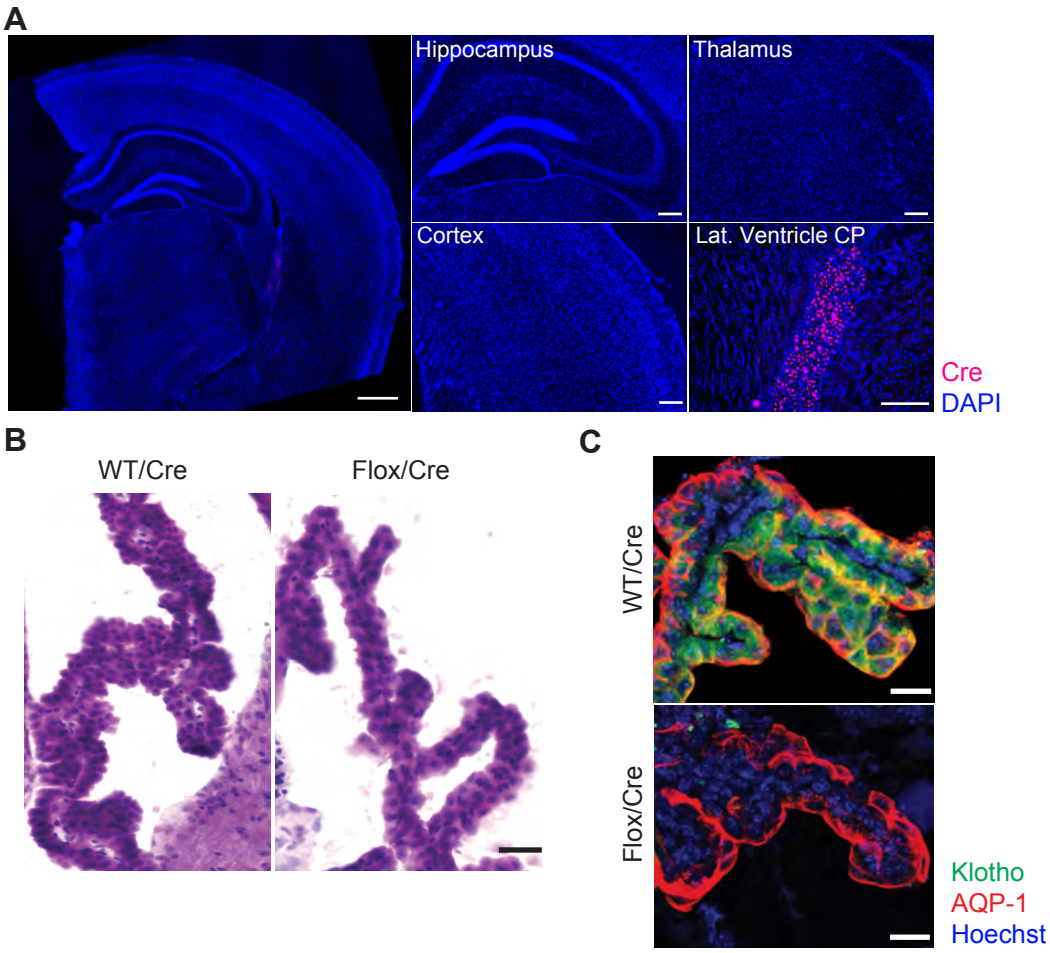
A
Hippocampus
Thalamus
Cortex
Lat. Ventricle CP
Cre
DAPI
B
WT/Cre
Flox/Cre
C
WT/Cre
Flox/Cre
Klotho
AQP-1
Hoechst
D
WT/Cre
Flox/Cre
Cytokeratin
CD31
Claudin-1
Merge

Figure S3

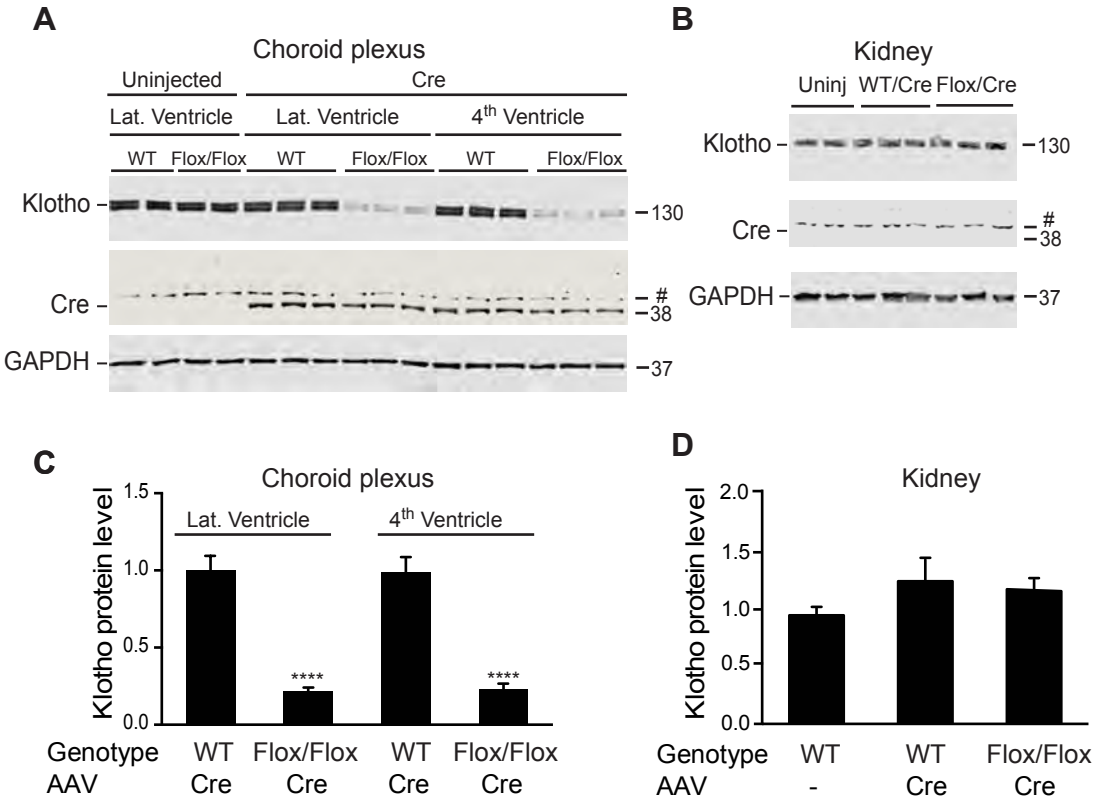
A
Choroid plexus
Uninjected
Cre
Lat. Ventricle
Lat. Ventricle
4th Ventricle
WT
Flox/Flox
WT
Flox/Flox
WT
Flox/Flox
Klotho
130
Cre
#
38
GAPDH
37
B
Kidney
Uninj
WT/Cre
Flox/Cre
Klotho
130
Cre
#
38
GAPDH
37
C
Choroid plexus
Klotho protein level
1.5
1.0
0.5
0.0
Lat. Ventricle
4th Ventricle
****
****
Genotype
WT
Flox/Flox
WT
Flox/Flox
AAV
Cre
Cre
Cre
Cre
D
Kidney
Klotho protein level
2.0
1.5
1.0
0.5
0.0
Genotype
WT
WT
Flox/Flox
AAV
-
Cre
Cre

Figure S4

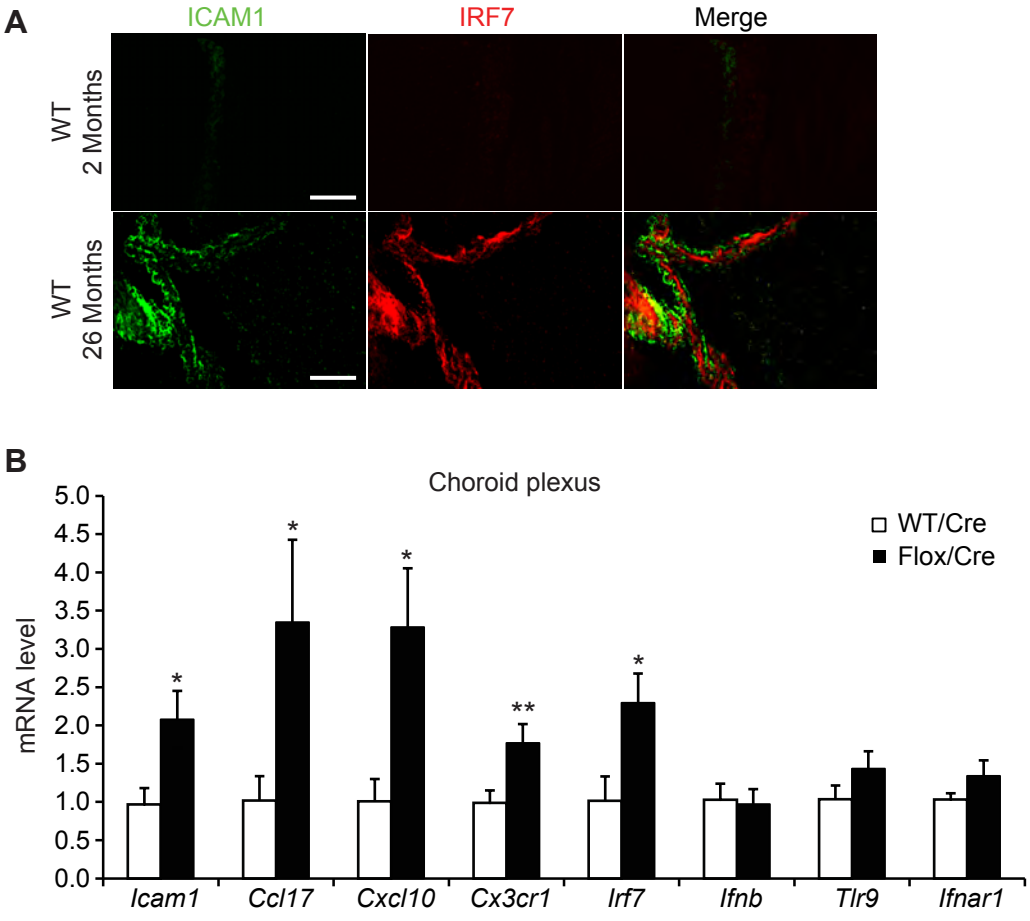
A
ICAM1
IRF7
Merge
WT
2 Months
WT
26 Months
B
Choroid plexus
WT/Cre
Flox/Cre
mRNA level
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
**
*
Icam1
Ccl17
Cxcl10
Cx3cr1
Irf7
Ifnb
Tlr9
Ifnar1

Figure S5

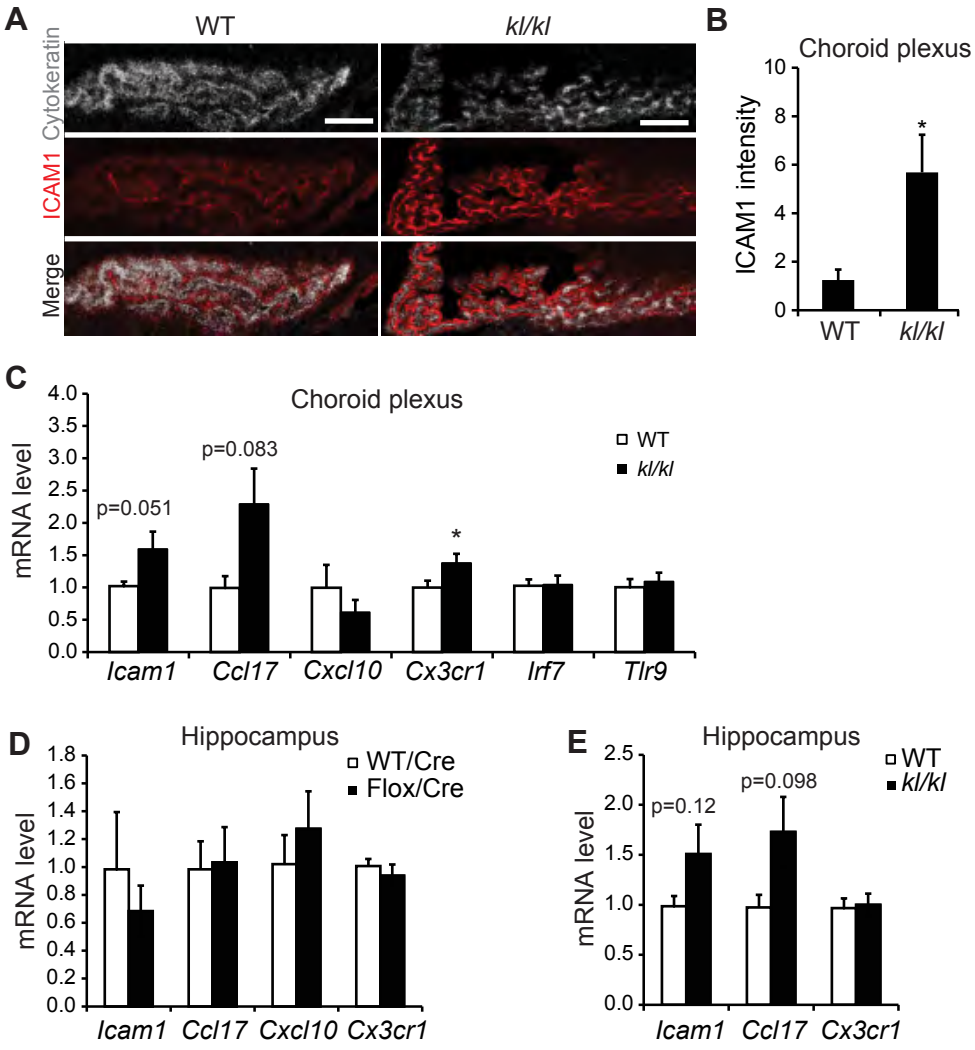
A
WT
kl/kl
Cytokeratin
ICAM1
Merge
B
Choroid plexus
ICAM1 intensity
10
8
6
4
2
0
*
WT
kl/kl
C
Choroid plexus
mRNA level
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
p=0.051
p=0.083
*
WT
kl/kl
Icam1
Ccl17
Cxcl10
Cx3cr1
Irf7
Tlr9
D
Hippocampus
mRNA level
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT/Cre
Flox/Cre
Icam1
Ccl17
Cxcl10
Cx3cr1
E
Hippocampus
mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
p=0.12
p=0.098
WT
kl/kl
Icam1
Ccl17
Cx3cr1

Figure S6

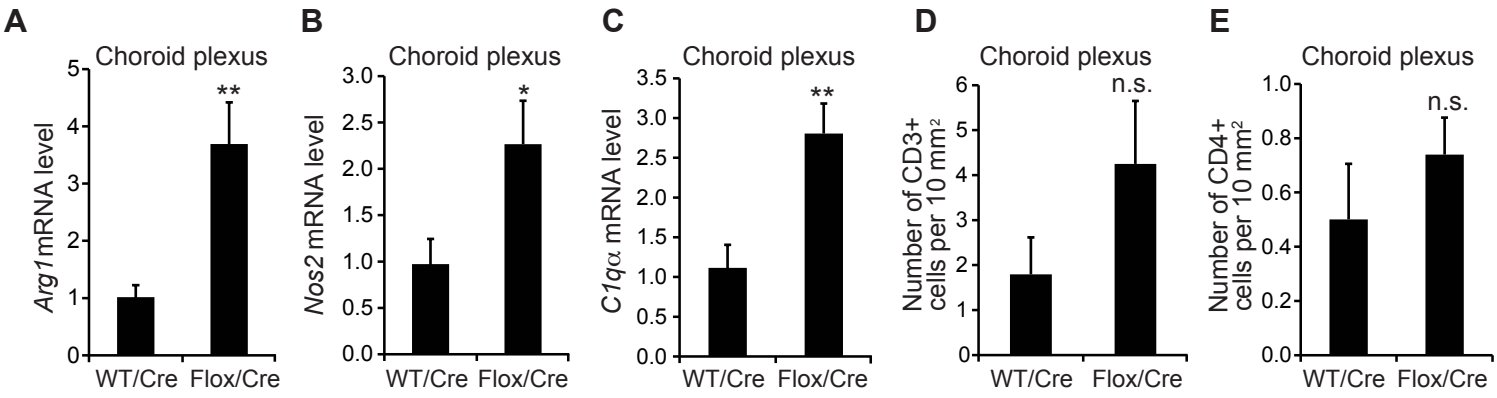
A
Choroid plexus
Arg1 mRNA level
**
WT/Cre
Flox/Cre
B
Choroid plexus
Nos2 mRNA level
*
WT/Cre
Flox/Cre
C
Choroid plexus
C1qα mRNA level
**
WT/Cre
Flox/Cre
D
Choroid plexus
Number of CD3+ cells per 10 mm²
n.s.
WT/Cre
Flox/Cre
E
Choroid plexus
Number of CD4+ cells per 10 mm²
n.s.
WT/Cre
Flox/Cre

Figure S7

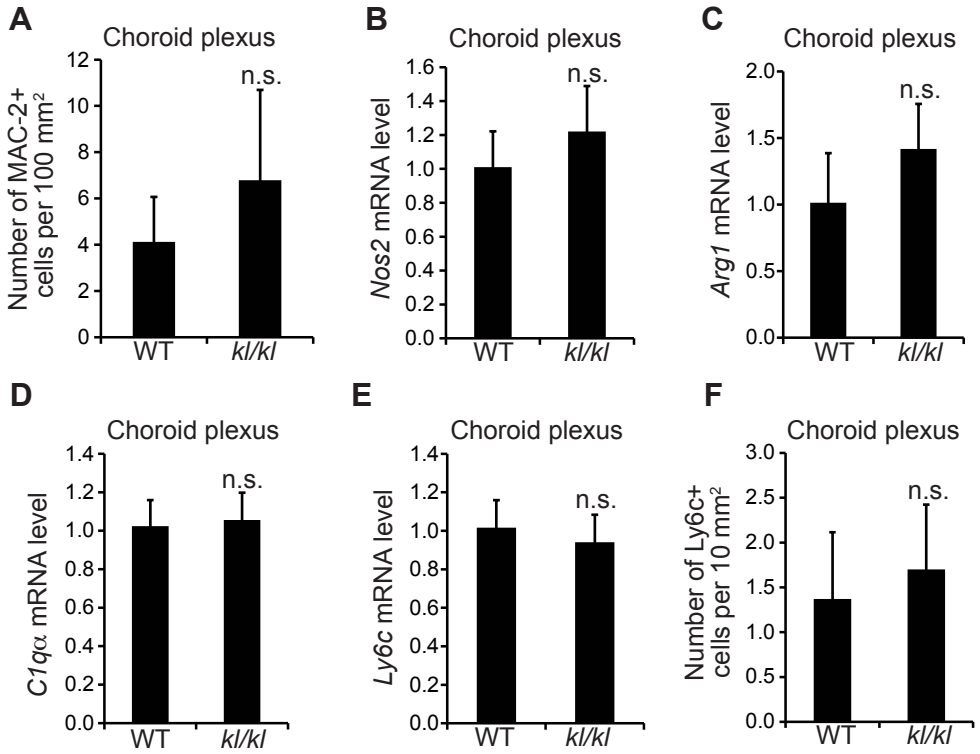
A
Choroid plexus
Number of MAC-2+ cells per 100 mm²
n.s.
WT
kl/kl
B
Choroid plexus
Nos2 mRNA level
n.s.
WT
kl/kl
C
Choroid plexus
Arg1 mRNA level
n.s.
WT
kl/kl
D
Choroid plexus
C1qα mRNA level
n.s.
WT
kl/kl
E
Choroid plexus
Ly6c mRNA level
n.s.
WT
kl/kl
F
Choroid plexus
Number of Ly6c+ cells per 10 mm²
n.s.
WT
kl/kl

Figure S8

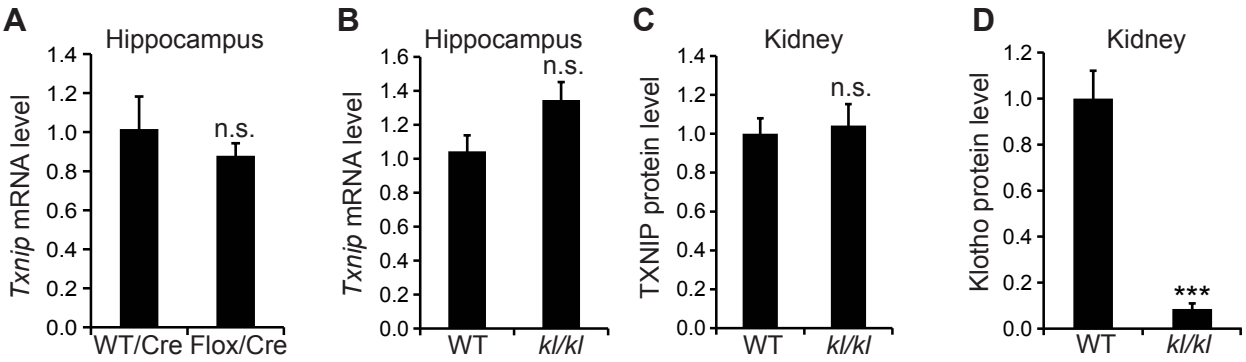
A
Hippocampus
Txnip mRNA level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
n.s.
WT/Cre
Flox/Cre
B
Hippocampus
Txnip mRNA level
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
n.s.
WT
kl/kl
C
Kidney
TXNIP protein level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
n.s.
WT
kl/kl
D
Kidney
Klotho protein level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
WT
kl/kl

Figure S9

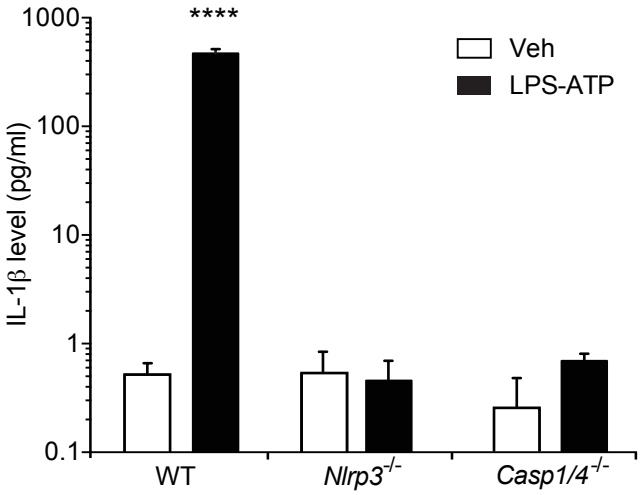

Table S1. mRNA and protein changes in the CP caused by klotho reduction

Gene Product	Analysis	*Klotho*$^{flox/flox}$ vs. *Klotho*$^{+/+}$ mice after i.c.v. injection of Cre/GFP or GFP	*kl/kl* vs. WT mice
Arg1	mRNA	↑ (3.6-fold, *P*=0.002, n=6)	↑ (1.4-fold, *P*=0.43, n=12)
C1qa	mRNA	↑ (2.5-fold, *P*=0.006,n=4-7)	No change (n=9-10)
Ccl17	mRNA	↑ (3.3-fold, *P*=0.03, n=5-7)	↑ (2.3-fold, *P*=0.08, n=10)
Cx3cr1	mRNA	↑ (1.8-fold, *P*=0.003, n=5-6)	↑ (1.4-fold, *P*=0.04, n=12)
Cxcl10	mRNA	↑ (3.3-fold, *P*=0.02, n=5-7)	No change (n=10-11)
Cyp27b1	mRNA	↑ (1.8-fold, *P*=0.02, n=8)	↑ (2.1-fold, *P*=0.046, n=12)
IBA-1	Protein (IHC)	No change (n=3-6)	No change (n=5-6)
ICAM-1	Protein (IHC)	↑ (2.1-fold, *P*=0.049, n=5-8)	↑ (5.1-fold, *P*=0.02, n=5-6)
Icam1	mRNA	↑ (2.1-fold, *P*=0.05, n=5-6)	↑ (1.6-fold, *P*=0.03, n=9-10)
Ifnar1	mRNA	No change (n=8)	Not analyzed
IL-6	mRNA	↑ (4.9-fold, *P* = 0.06, n=5-7)	↑ (2.9-fold, *P*=0.26, n=9-10)
Irf7	mRNA	↑ (2.3-fold, *P*=0.03, n=5-7)	No change (n=10)
Klotho	mRNA	↓ (0.5-fold, *P*=0.003, n=8)	↓ (0.2-fold, *P*<0.0001, n=10)
Ly6C	mRNA	↑ (4.4-fold, *P*=0.04, n=5-7)	No change (n=12)
LY6C	Protein (IHC)	↑ (2.0-fold, *P*=0.049, n=4-9)	No change (n=6-7)
MAC-2	Protein (IHC)	↑ (2.2-fold, *P*=0.037, n=7)	No change (n=6)
Nos2	mRNA	↑ (2.3-fold, *P*=0.02, n=8)	No change (n=12)
Tlr9	mRNA	↑ (1.4-old, *P*=0.06, n=8)	No change (n=10)
Txnip	mRNA	↑ (1.8-fold, *P*=0.04, n=8)	↑ (1.8-fold, *P*=0.0004, n=12)

i.c.v., intracerebroventricular. IHC, immunohistochemistry. n, number of mice per group. *P* values were generated by unpaired, two-tailed *t* tests.

Table S2. Antibodies used for immunohistochemistry or western blotting

Antibody	Company	Catalog No.	Species	Application	Dilution
AE2	Abcam	ab42687	Rabbit	IHC	1:500
AQP1	Santa Cruz Biotechnology	sc-20810	Rabbit	IHC	1:1000
CD3	Abcam	ab16669	Rabbit	IHC	1:500
CD4	BioLegend	100402	Rat	IHC	1:250
CD31	BD Biosciences	550274	Rat	IHC	1:250
Claudin-1	Cell signaling	13255	Rabbit	IHC	1:250
Cre recombinase	EMD Millipore	69050-3	Rabbit	WB, IHC	1:5000, 1:10000
Cytokeratin	Sigma	C5992	IgG1	IHC	1:400
GAPDH	Millipore	MAB374	Mouse	WB	1:2000
IBA-1	Abcam	ab5076	Goat	IHC	1:1000
ICAM1	Abcam	ab119871	Rat	IHC	1:500
IRF7	LSBio	LS-B2945	IgG1	IHC	1:500
Klotho	Abcam	ab154163	Rabbit	WB, IHC	1:1000, 1:500
Klotho	Cosmobio	KO603	Rat	IHC	1:100
LY6C	Abcam	ab15627	Rat	IHC	1:500
MAC-2 (Galectin-3)	Cedarlane	CL8942AP	Rat	IHC	1:500
MHCII	eBioscience	14-5321-81	Rat	IHC	1:200
TMEM119	Abcam	ab209064	Rabbit	IHC	1:500
Transthyretin	Sigma-Aldrich	SAB3500378	Chicken	IHC	1:500
TXNIP	Abcam	ab188865	Rabbit	WB	1:500

IHC, immunohistochemistry; WB, western blotting.

Table S3. Taqman qPCR primers from Life Technologies

Target Gene/Transcript	Taqman Catalog No.
Arg1	Mm00475988_m1
C1qa	Mm00432142_m1
Ccl17	Mm01244826_g1
Cx3cr1	Mm00438354_m1
Cxcl10	Mm00445235_m1
Cyp27b1	Mm01165918_g1
Icam1	Mm00516023_m1
Ifnar1	Mm00439544_m1
Ifnβ	Mm00439552_s1
IL-1β	Mm00434228_m1
Irf7	Mm00516793_g1
Klotho	Mm00502002_m1
Ly6c	Mm03009946_m1
Nlrp3	Mm00840904_m1
Nos2	Mm00440502_m1
Tlr9	Mm00446193_m1
Txnip	Mm01265659_g1

Table S4. Primers for SYBR Green qPCR quantification

Target Gene/Transcript	Forward (5'-3')	Reverse (3'-5')
Klotho: m-KL, mouse	GGACAATGGCTTTCCTCCTT	TGCACATCCCACAGATAGACA
Klotho: s-KL, mouse	GGACAATGGCTTTCCTCCTT	TCGGATGAGATCCTGACACA