PNAS

Supporting Information for

Inhibition of connexin hemichannels alleviates neuroinflammation and hyperexcitability in temporal lobe epilepsy

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This PDF file includes:

Figures S1 to S10

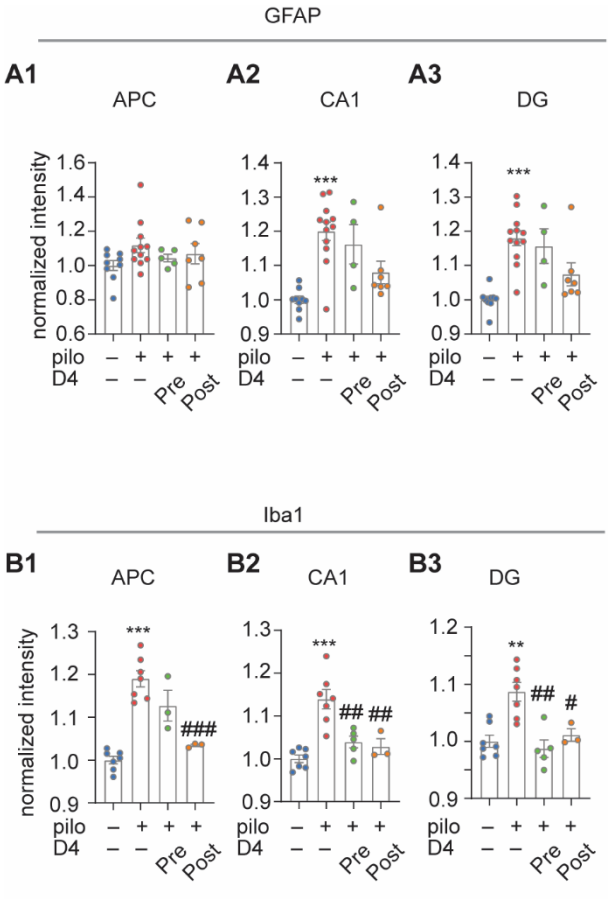


Fig. S1. D4 treatment decreased intensity of GFAP and Iba1 in TLE.

(A) Quantification of GFAP mean intensity in APC (ctrl: 1.0 ± 0.03, n = 9; pilo: 1.12 ± 0.04, n = 11; pilo + D4 (pre): 1.04 ± 0.02, n = 5; pilo + D4 (post): 1.07 ± 0.06, n = 7) **(A1)**, CA1 (ctrl: 1.0 ± 0.01, n = 9; pilo: 1.2 ± 0.03, n = 12, $p < 0.001$; pilo + D4 (pre): 1.2 ± 0.06, n = 4, p = 1.000; pilo + D4 (post): 1.1 ± 0.03, n = 7) **(A2)**, and DG (ctrl: 1.0 ± 0.01, n = 9; pilo: 1.2 ± 0.02, n = 12, $p <0.001$; pilo + D4 (pre): 1.2 ± 0.05, n = 4, p = 1.000; pilo + D4 (post): 1.1 ± 0.03, n = 7) **(A3). (B)** Quantification of Iba1 mean intensity in APC (ctrl: 1.0 ± 0.01, n = 7; pilo: 1.2 ± 0.02, n = 7, $p < 0.001$; pilo + D4 (pre): 1.13 ± 0.04, n = 3; pilo + D4 (post): 1.03 ± 0.002, n = 3, $p < 0.001$) **(B1)**, CA1 (ctrl: 1.0 ± 0.01, n = 7; pilo: 1.1 ± 0.02, n = 7, $p < 0.001$; pilo + D4 (pre): 1.04 ± 0.01, n = 5, p = 0.003; pilo + D4 (post): 1.03 ± 0.02, n = 3, p = 0.005) **(B2)**, and DG (ctrl: 1.0 ± 0.01, n = 7; pilo: 1.1 ± 0.02, n = 7, p = 0.001; pilo + D4 (pre): 0.99 ± 0.02, n = 5, p = 0.001; pilo + D4 (post): 1.01 ± 0.01, n = 3, p = 0.025) **(B3)**. ***$p < .001$, **$p < .01$: significant increase compared with ctrl; ###$p < .001$, ##$p < 0.01$, #$p < 0.05$: significant decrease compared with pilo. One-way ANOVA followed by Tukey's multiple comparisons post hoc test was used to compare normalized Iba1 intensity in APC, CA1 and DG. Independent-Samples Kruskal-Wallis test (significance values were adjusted by the Bonferroni correction) was used to compare normalized GFAP intensity in APC, CA1 and DG.

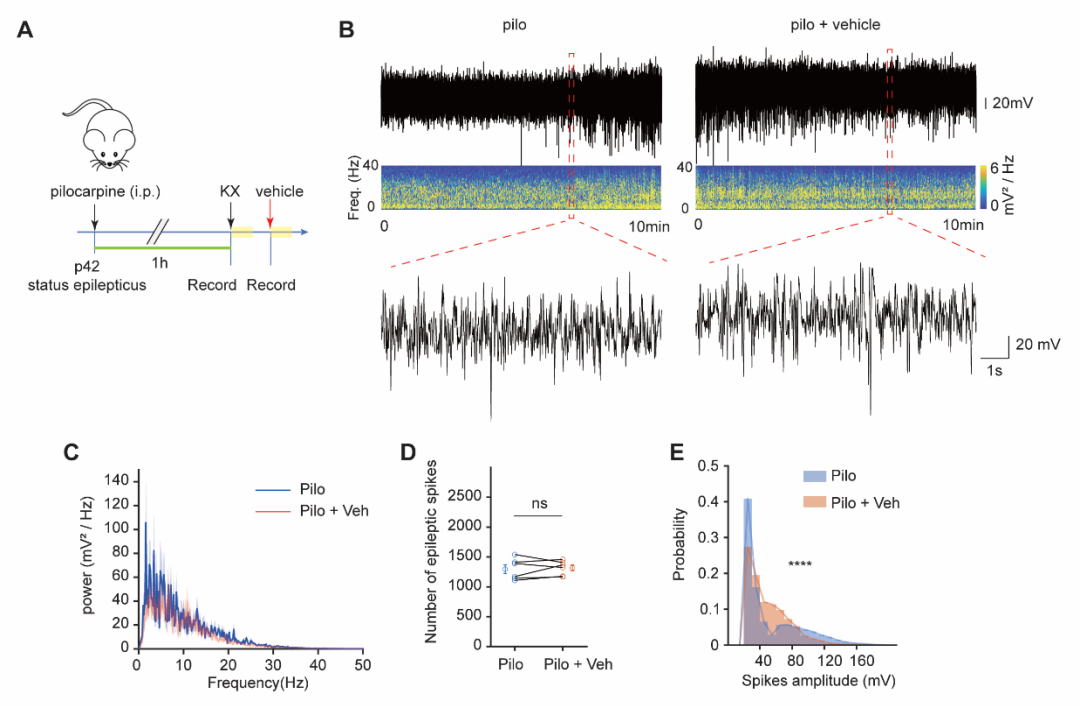


Fig. S2. Vehicle has no effects on pilocarpine-induced acute seizure.

(A) scheme of experiment for vehicle treatment in pilocarpine-induced acute epileptic mice. The yellow blocks represent the recording period. **(B)** Representative raw LFP and LFP spectrogram during pilocarpine-induced status epilepticus pre and post vehicle administration. Below are enlarged raw LFP segments corresponding to the red dotted boxes. **(C)** Power spectral analysis of pilocarpine and pilocarpine with the vehicle group. Thick dark lines indicated mean and shaded light-colored areas indicated SEM. **(D)** The effect of vehicle on the number of epileptic spikes in the ten-minute recording. Paired t-test. **(E)** The effect of vehicle on the distribution of the amplitude of epileptic spikes. Vehicle did not decrease the amplitude of epileptic spike but instead increased it ($P < 10^{-17}$) and the two distributions differed in shape (Two-sample Kolmogorov-Smirnov test, ****p<0.0001).

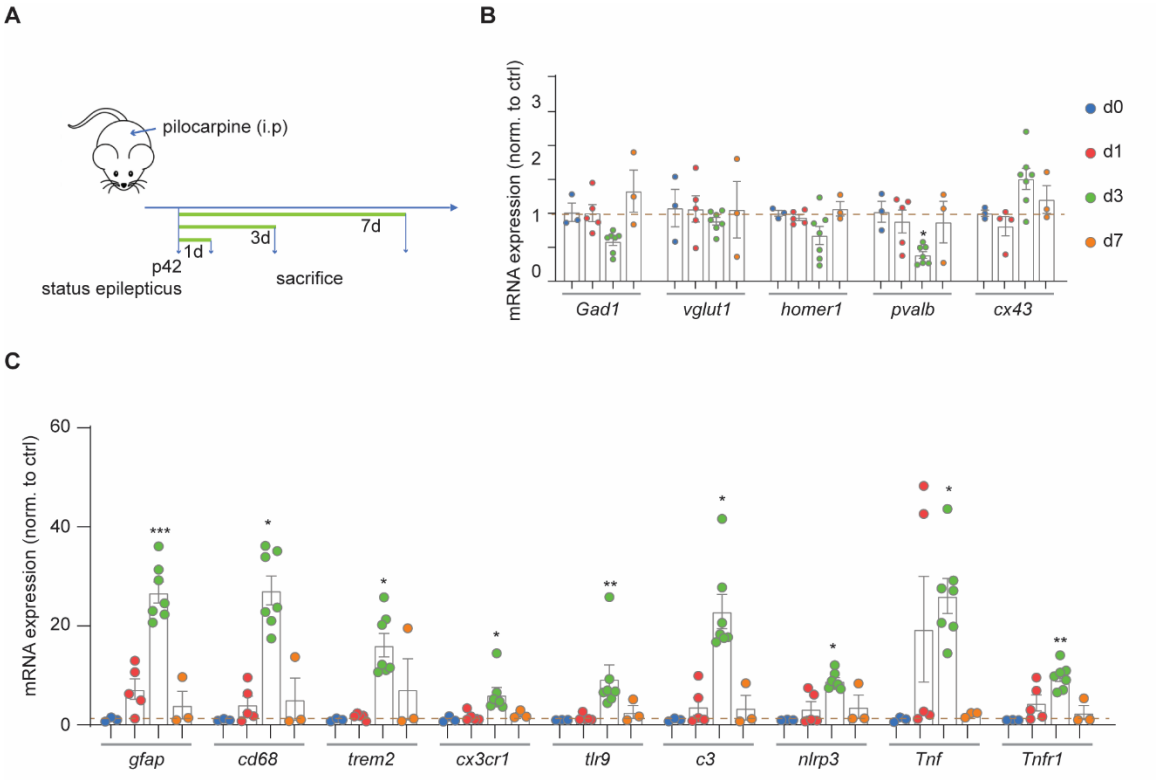


Fig. S3. Time course expression analysis of neuroinflammation and synapse genes in hippocampus after pilocarpine injection.

(A) Timeline of experiment. Status epilepticus was induced in P42 wild-type mice by intraperitoneal injection of pilocarpine (200 – 300 mg/kg). 1, 3, or 7 days after status epileptics, mice were sacrificed for tissue extraction and further quantitative real-time PCR. **(B)** Relative mRNA expression of *Gad1*, *vglut1, homer1*, *pvalb* and *cx43* in four groups (ctrl; pilo d1; pilo d3; pilo d7) in hippocampus. Fold change is normalized to the control group. **(C)** Relative mRNA expression of *gfap, cd68, trem2, cx3cr1, tlr9, c3, nlrp3, Tnf* and *Tnfr1* in four groups (ctrl; pilo d1; pilo d3; pilo d7) in the hippocampus. Fold change is normalized to the control group. ***p < 0.001, **p < 0.01, *p < 0.05: significant increase/decrease compared with d0. One-way ANOVA followed by Tukey's multiple comparisons post hoc test was used to compare the expression of *Gad1*, *vglut1, homer1*, *pvalb, cx43,* and *gfap* among four groups; Independent-Samples Kruskal-Wallis test (significance values were adjusted by the Bonferroni correction) was used to compare the expression of c3, *cd68, trem2, cx3cr1, tlr9, c3, nlrp3, Tnf* and *Tnfr1* among four groups. d3: (*gfap*: 26.8 ± 2.1, n = 7, p < 0.001; *cd68*: 27.2 ± 2.9, n = 7, p = 0.023; *trem2*: 16.1 ± 2.3, n = 7, p= 0.028; *cx3cr1*: 6.1 ± 1.4, n = 7, p = 0.017; *tlr9*: 9.3 ± 2.8, n = 7, p = 0.006; *c3*: 22.9 ± 3.4, n = 7, p = 0.023; *nlrp3*: 8.9 ± 0.7, n = 7, p = 0.046; *Tnf*: 26.1 ± 3.5, n = 7, p = 0.027; *Tnfr1*: 9.7 ± 1.0, n = 7, p = 0.006; *Gad1*: 0.59 ± 0.06, n = 7, p = 0.178; *vglut1*: 0.89 ± 0.06, n = 7, p = 0.906; *homer1*: 0.68 ± 0.13, n = 7, p = 0.273; *pvalb*: 0.39 ± 0.05, n = 7, p = 0.049; *cx43:* 1.51 ± 0.16, n = 7)

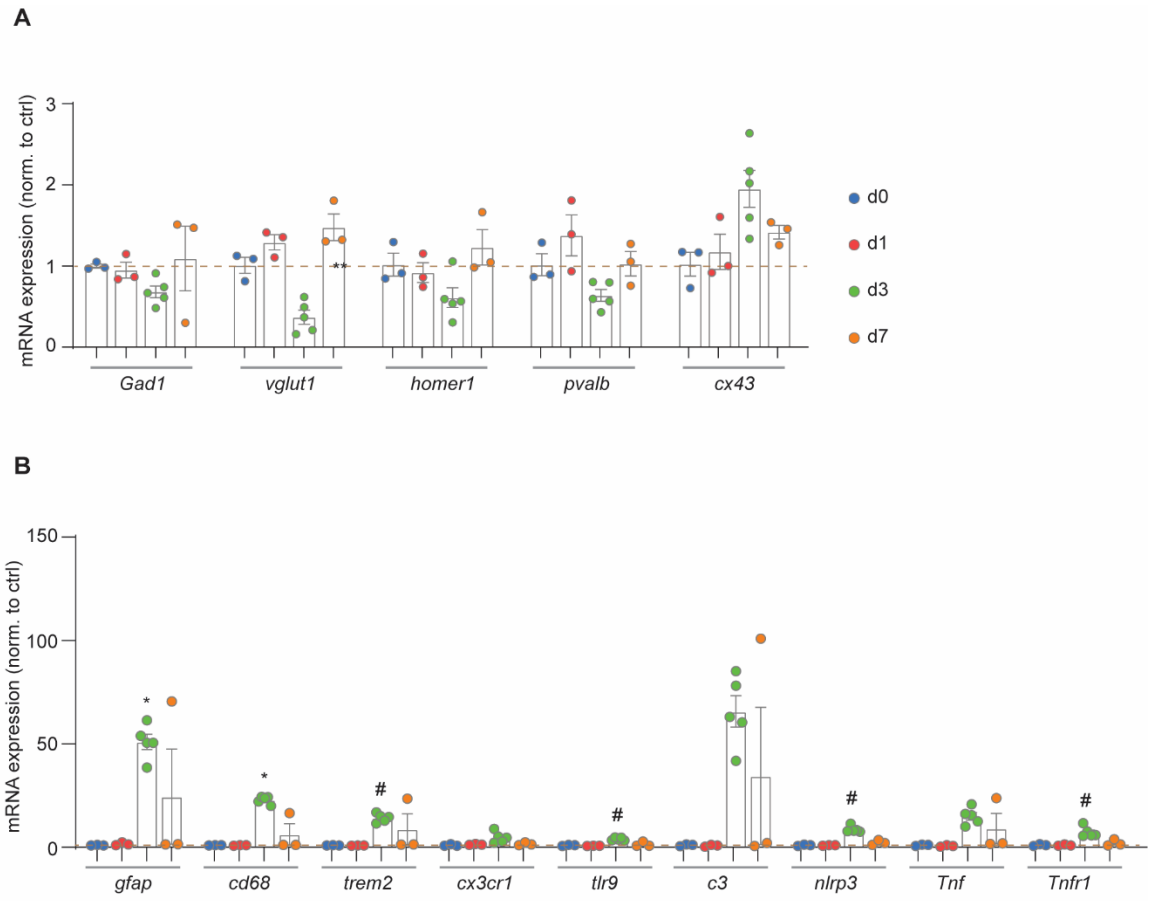


Fig. S4. Time course expression analysis of neuroinflammation and synapse genes in the APC after pilocarpine injection.

(A) Relative mRNA expression of *Gad1*, *vglut1, homer1*, *pvalb* and *cx43* in four groups (ctrl; pilo d1; pilo d3; pilo d7) in APC. Foldchange is normalized to the control group. **(B)** Relative mRNA expression of *gfap, cd68, trem2, cx3cr1, tlr9, c3, nlrp3, Tnf,* and *Tnfr1* in four groups (ctrl; pilo d1; pilo d3; pilo d7) in APC. Foldchange is normalized to the control group. **p < .01, *p < .05: significant increase/decrease compared with d0; #p < 0.05: significant increase compared with d1. One-way ANOVA followed by Tukey's multiple comparisons post hoc test was used to compare the expression of *Gad1*, *vglut1, homer1*, *pvalb,* and *cx3cr1* among four groups; Independent-Samples Kruskal-Wallis test (significance values were adjusted by the Bonferroni correction) was used to compare the expression of *cx43, gfap, cd68, trem2, tlr9, c3, nlrp3, Tnf* and *Tnfr1* among four groups. d3: (*gfap*: 51.0 ± 3.7, n = 5, p = 0.027; *cd68*: 23.0 ± 0.8, n = 5, p = 0.019; *trem2*: 14.3 ± 0.9, n = 5, p = 0.297; *cx3cr1*: 4.8 ± 1.2, n = 5, p = 0.055; *tlr9*: 4.0 ± 0.3, n = 5, p = 0.229; *nlrp3*: 8.6 ± 0.7, n = 5, p = 0.053; *Tnf*: 15.1 ± 1.8, n = 5, p = 0.053; *Tnfr1*: 7.3 ± 1.1, n = 5, p = 0.098; *Gad1*: 0.68 ± 0.07, n = 5, p = 0.579; *vglut1*: 0.37 ± 0.09, n = 5, p = 0.007; *homer1*: 0.61 ± 0.12, n = 5, p = 0.258; *pvalb*: 0.64 ± 0.07, n = 5, p = 0.281; *cx43:* 1.95 ± 0.23, n = 5, p = 0.190)

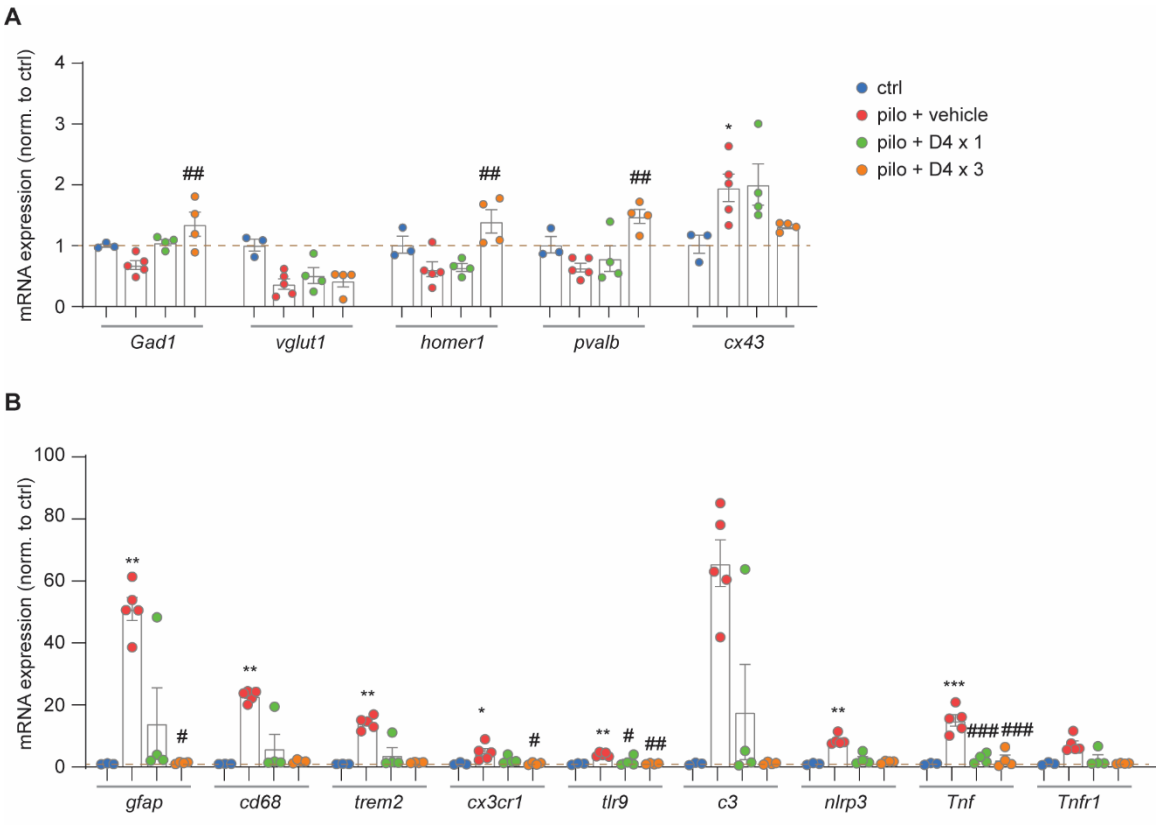


Fig. S5. Post-treatment of D4 rescues the altered mRNA levels of neuroinflammatory and synaptic proteins induced by SE in the anterior piriform cortex.

(A) Relative mRNA expression of *Gad1*, *vglut1, homer1*, *pvalb* and *cx43* in four groups: ctrl; pilo; pilo + D4 x 1 (+5h); pilo + D4 x 3 (+5h, +1d, +2d) in APC. Fold change is normalized to control group. **(B)** Relative mRNA expression of *gfap, cd68, trem2, cx3cr1, tlr9, c3, nlrp3, Tnf* and *Tnfr1* in four groups: ctrl; pilo; pilo + D4 x 1 (+5h); pilo + D4 x 3 (+5h, +1d, +2d) in APC. Fold change is normalized to control group. ***p < 0.001, **p < 0.01, *p < 0.05: significant increase/decrease compared with ctrl; $^{###}$p < 0.001, $^{##}$p < 0.01, $^{#}$p < 0.05: significant increase/decrease compared with pilo. One-way ANOVA followed by Tukey's multiple comparisons post hoc test was used to compare expression of *Gad1*, *homer1*, *pvalb, Tnf, cx3cr1* and *tlr9* among four groups; Independent-Samples Kruskal-Wallis test (significance values were adjusted by the Bonferroni correction) was used to compare expression of *vglut1, cx43, gfap, cd68, trem2, nlrp3, c3* and *Tnfr1* among four groups. pilo + D4 x 1 (+5h): (*Gad1*: 1.05 ± 0. 50, n = 4, p = 0.123; *homer1*: 0.64 ± 0.06, n = 4, p = 0.998; *pvalb*: 0.79 ± 0.21, n = 4, p = 0.851; *Tnf*: 2.7 ± 0.8, n = 4, p < 0.001; *tlr9*: 1.8 ± 0.8, n = 4, p = 0.012). pilo + D4 x 3 (+5h, +1d, +2d: (*Gad1*: 1.35 ± 0.20, n = 4, p = 0.004; *homer1*: 1.40 ± 0.19, n = 4, p = 0.005; *pvalb*: 1.48 ± 0.12, n = 4, p = 0.003; *gfap*: 1.4 ± 0.2, n = 4, p = 0.045; *cd68*: 1.8 ± 0.3, n = 4, p = 0.251; *trem2*: 1.4 ± 0.1, n = 4, p = 0.139; *cx3cr1*: 1.1 ± 0.2, n = 4, p = 0.028; *tlr9*: 1.1 ± 0.1, n = 4, p = 0.002; *c3*: 1.2 ± 0.2, n = 4, p = 0.08; *nlrp3*: 1.7 ± 0.2, n = 4, p = 0.113; *Tnf*: 2.5 ± 1.4, n = 4, p < 0.001; *Tnfr1*: 1.1 ± 0.07, n = 4, p = 0.064)

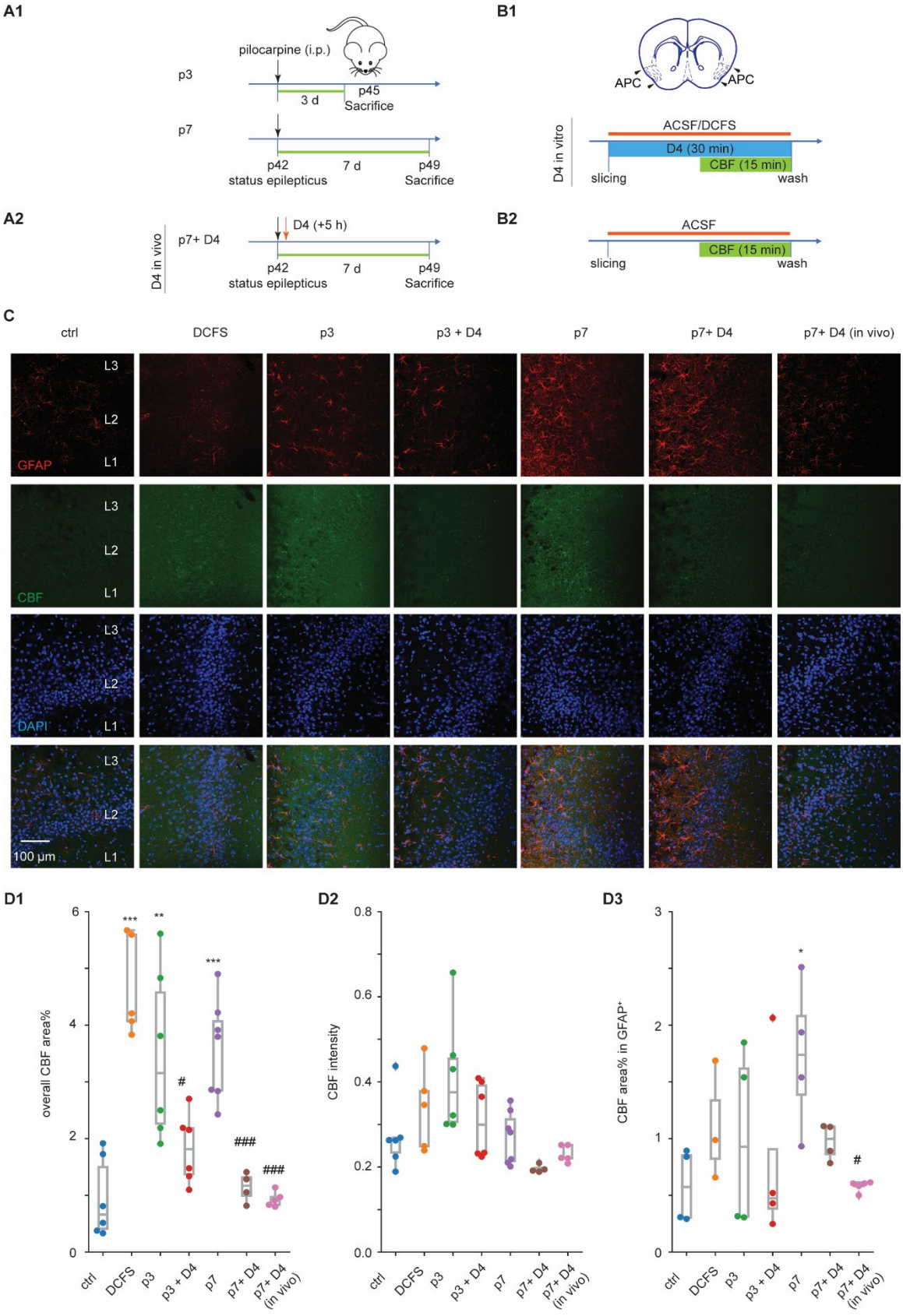

A1
pilocarpine (i.p.)
p3
3 d
p45
Sacrifice
p7
p42
status epilepticus
7 d
p49
Sacrifice
A2
D4 in vivo
p7+ D4
D4 (+5 h)
p42
status epilepticus
7 d
p49
Sacrifice
B1
APC
APC
D4 in vitro
ACSF/DCFS
D4 (30 min)
CBF (15 min)
slicing
wash
B2
ACSF
CBF (15 min)
slicing
wash
C
ctrl
DCFS
p3
p3 + D4
p7
p7+ D4
p7+ D4 (in vivo)
L3
L2
L1
GFAP
CBF
DAPI
100 µm
D1
overall CBF area%
D2
CBF intensity
D3
CBF area% in GFAP+
ctrl
DCFS
p3
p3 + D4
p7
p7+ D4
p7+ D4 (in vivo)

Fig. S6. D4 in vitro or in vivo inhibits astrocytic hemichannel activity induced by pilocarpine treatment in anterior piriform cortex acute slices.

(A) Representative images showing GFAP (red) and CBF (green) uptake of acute APC slices from control mice (ctrl: slices incubated in ACSF; DCFS: slices incubated in DCFS) and pilocarpine injected mice (p3: 3 days after SE; p3 + D4: 3 days SE, slices treated with D4; p7: 7 days after SE; p7 + D4: 7 days SE, slices treated with D4; p7 + D4 (in vivo): 7 days after SE, D4 applied in vivo after pilocarpine injection). **(B)** Quantification of overall CBF area percentage (ctrl: 0.95 ± 0.29, n = 6; DCFS: 4.7 ± 0.4, n = 5, $p < 0.001$; p3: 3.5 ± 0.6, n = 6, $p = 0.002$; p3 + D4: 1.8 ± 0.3, n = 6, $p = 0.036$; p7: 3.57 ± 0.33, n = 7, $p < 0.001$; p7 + D4: 1.1 ± 0.1, n = 4, $p < 0.001$; p7 + D4 (in vivo): 0.9 ± 0.1, n = 5, $p < 0.001$) (B1), CBF mean intensity (ctrl: 0.27 ± 0.03, n = 6, DCFS: 0.34 ± 0.04, n = 5, $p = 0.055$) (B2) and CBF area percentage in $GFAP^+$ area (ctrl: 0.6 ± 0.16, n = 4; p7: 1.7 ± 0.3, n = 4, $p = 0.05$; p7 + D4 (in vivo): 0.6 ± 0.02, n = 5, $p = 0.039$) (B3). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$: significant increase compared with ctrl; ###$p < 0.001$, ##$p < .01$, #$p < .05$: significant decrease compared with corresponding pilocarpine group (p3 + D4 compared with p3; p7 + D4 compared with p7). Independent-Samples Kruskal-Wallis test (significance values were adjusted by the Bonferroni correction) was used to compare CBF mean intensity and CBF area percentage in $GFAP^+$ area among three groups (ctrl, p3 and p3 + D4). One-way ANOVA followed by Tukey's multiple comparisons post hoc test was used to compare overall CBF area percentage for the rest dataset among three groups (ctrl, p3 and p3 + D4; ctrl, p7 and p7 + D4; ctrl, p7 and p7 + D4 (in vivo)). A two-sided test-test was used to compare overall CBF area percentage, CBF means intensity, and CBF area percentage in $GFAP^+$ area between the ctrl and DCFS group.

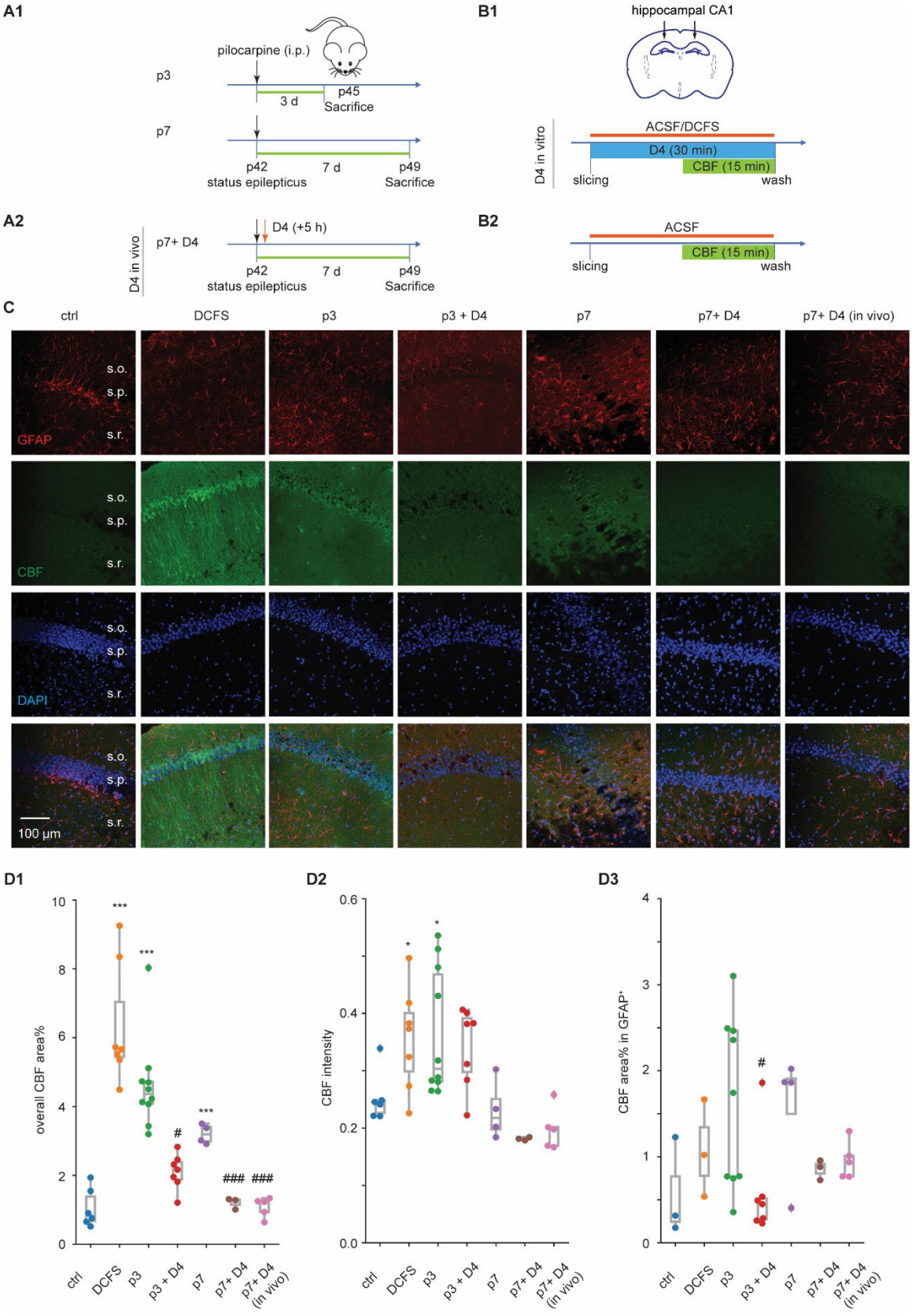

A1
pilocarpine (i.p.)
p3
3 d
p45
Sacrifice
p7
p42
status epilepticus
7 d
p49
Sacrifice
A2
D4 in vivo
p7+ D4
D4 (+5 h)
p42
status epilepticus
7 d
p49
Sacrifice
B1
hippocampal CA1
D4 in vitro
ACSF/DCFS
D4 (30 min)
CBF (15 min)
slicing
wash
B2
ACSF
CBF (15 min)
slicing
wash
C
ctrl
DCFS
p3
p3 + D4
p7
p7+ D4
p7+ D4 (in vivo)
s.o.
s.p.
s.r.
GFAP
CBF
DAPI
100 µm
D1
overall CBF area%
D2
CBF intensity
D3
CBF area% in GFAP+
ctrl
DCFS
p3
p3 + D4
p7
p7+ D4
p7+ D4 (in vivo)

Fig. S7. D4 in vitro or in vivo inhibits astrocytic hemichannel activity induced by pilocarpine treatment in the CA1 area in hippocampal acute slices.

(A) Representative images showing GFAP (red) and CBF (green) uptake of acute hippocampal slices from control mice (ctrl: slices incubated in ACSF; DCFS: slices incubated in DCFS) and pilocarpine injected mice (p3: 3 days after SE; p3 + D4: 3 days SE, slices treated with D4; p7: 7 days after SE; p7 + D4: 7 days SE, slices treated with D4; p7 + D4 (in vivo): 7 days after SE, D4 applied in vivo after pilocarpine injection). Images were taken from the CA1 zone. **(B)** Quantification of overall CBF area percentage (ctrl: 1.05 ± 0.23, n = 6; DCFS: 6.3 ± 0.7, n = 7, $p < 0.001$; p3: 4.61 ± 0.42, n = 10, $p < 0.001$; p3 + D4: 2.1 ± 0.2, n = 7, $p = 0.023$; p7: 3.2 ± 0.1, n = 4, $p < 0.001$; p7 + D4: 1.2 ± 0.1, n = 3, $p < 0.001$; p7 + D4 (in vivo): 1.1 ± 0.1, n = 5, $p < 0.001$) (B1), CBF mean intensity (ctrl: 0.25 ± 0.02, n = 6; DCFS: 0.36 ± 0.03, n = 7, $p = 0.035$; p3: 0.37 ± 0.04, n = 10, $p = 0.028$) (B2) and CBF area percentage in $GFAP^+$ area (p3 + D4: 0.6 ± 0.2, n = 7, $p = 0.049$) (B3). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$: significant increase compared with ctrl; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$: significant decrease compared with corresponding pilocarpine group (p3 + D4 compared with p3; p7 + D4 compared with p7). One-way ANOVA followed by Tukey's multiple comparisons post hoc test was used to compare overall CBF area percentage among three groups (ctrl, p7 and p7 + D4; ctrl, p7 and p7 + D4 (in vivo)). Independent-Samples Kruskal-Wallis test (significance values were adjusted by the Bonferroni correction) was used for the rest dataset among three groups (ctrl, p3 and p3 + D4; ctrl, p7 and p7 + D4). A two-sided test-test was used to compare overall CBF area percentage and CBF area percentage in $GFAP^+$ area between the ctrl and DCFS groups. A two-sided Mann-Whitney test was used to compare CBF mean intensity between ctrl and DCFS groups.

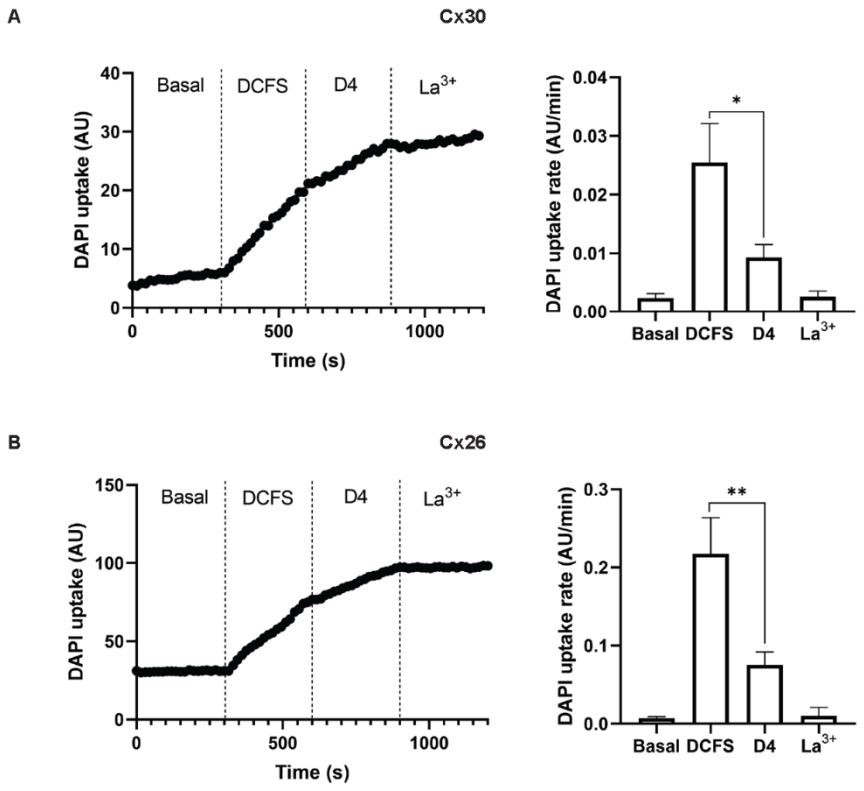


Fig. S8. D4 inhibits the activity of hemichannels formed by connexin26 or connexin30.

(**A**) Left, graph showing time-lapse measurements of fluorescence intensity of DAPI bound to nucleic acids of HeLa cells transfected with mCx30 under basal conditions, after exposure to DCFS followed by 1 μM D4 and finally 200 μM La^{3+}. (**B**) Left, same protocol as A but in HeLa cells transfected with mCx26. Graphs on the right represent the DAPI uptake rate of experiments as shown on the left. Each bar represents the mean value of the rate of DAPI uptake obtained from time-lapse curves. Mean ± SEM for 10 (A) and 11 (B) independent experiments measuring in 5-10 cells in each experiment. $^{**}P < 0.001$ and *P<0.05 using a one-way ANOVA test followed by Tukey's post hoc test.

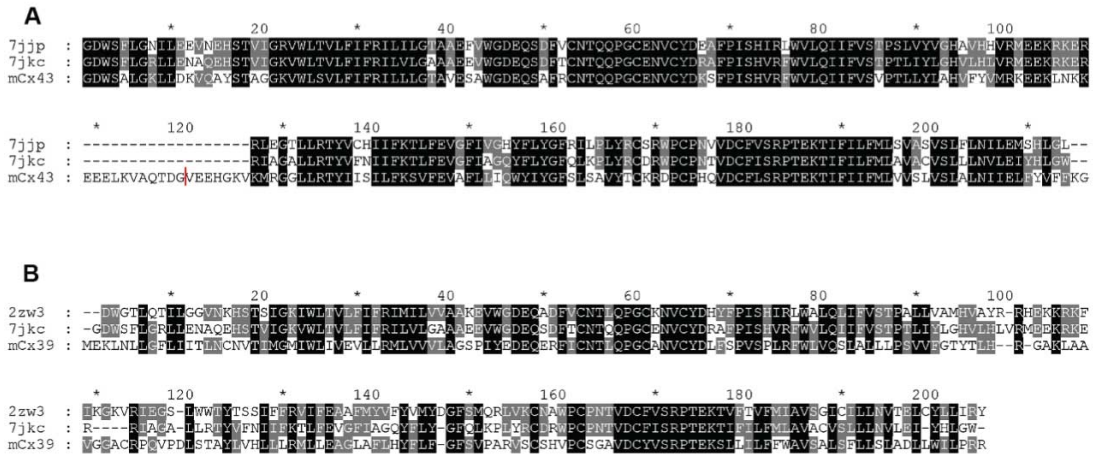
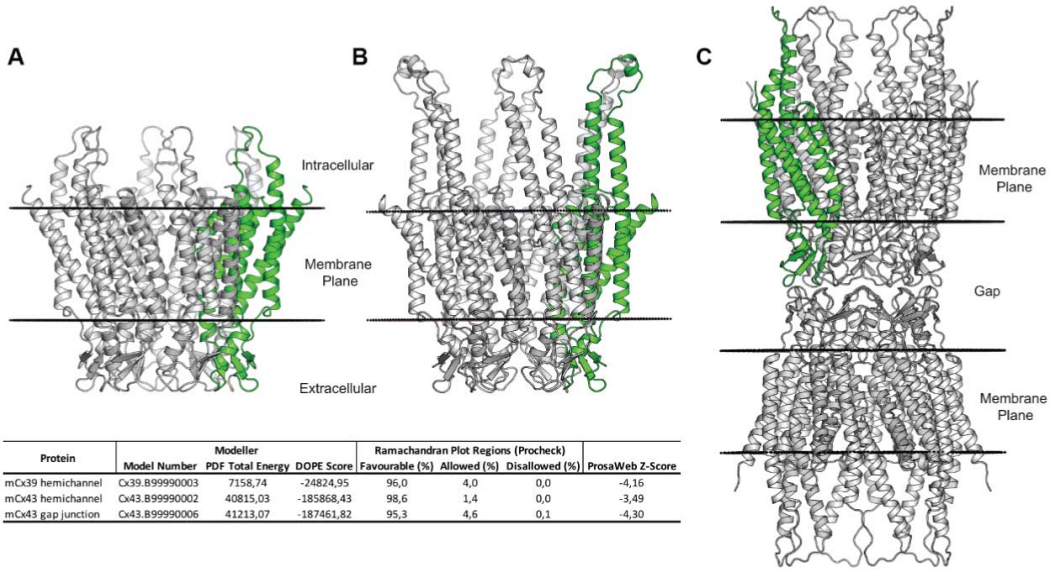


Fig. S9. Sequence alignment for mCx43 or mCx39.

Sequence alignment for mCx43 (**A**) or mCx39 (**B**) with their corresponding template structures indicated by their PDB id entry codes (2zw3, human Cx26 hemichannel; 7jjp and 7jkc, ovine connexin 46/50). The proteins were symmetrically modeled as connexons (hexamers) or gap junction channels (dodecamer).



Protein	Modeller			Ramachandran Plot Regions (Procheck)			
	Model Number	PDF Total Energy	DOPE Score	Favourable (%)	Allowed (%)	Disallowed (%)	ProsaWeb Z-Score
mCx39 hemichannel	Cx39.B99990003	7158,74	-24824,95	96,0	4,0	0,0	-4,16
mCx43 hemichannel	Cx43.B99990002	40815,03	-185868,43	98,6	1,4	0,0	-3,49
mCx43 gap junction	Cx43.B99990006	41213,07	-187461,82	95,3	4,6	0,1	-4,30

Fig. S10. Comparative models for mCx43 hemichannel (A), mCx39 hemichannel (B), or mCx43 gap junction channel (C).

A single protomer is shown in green color. Ramachandran plot and Prosa Z-score statistics were obtained using the SAVES and ProSA web servers respectively.