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Supplemental Information

TMEM163 Regulates

ATP-Gated P2X Receptor and Behavior

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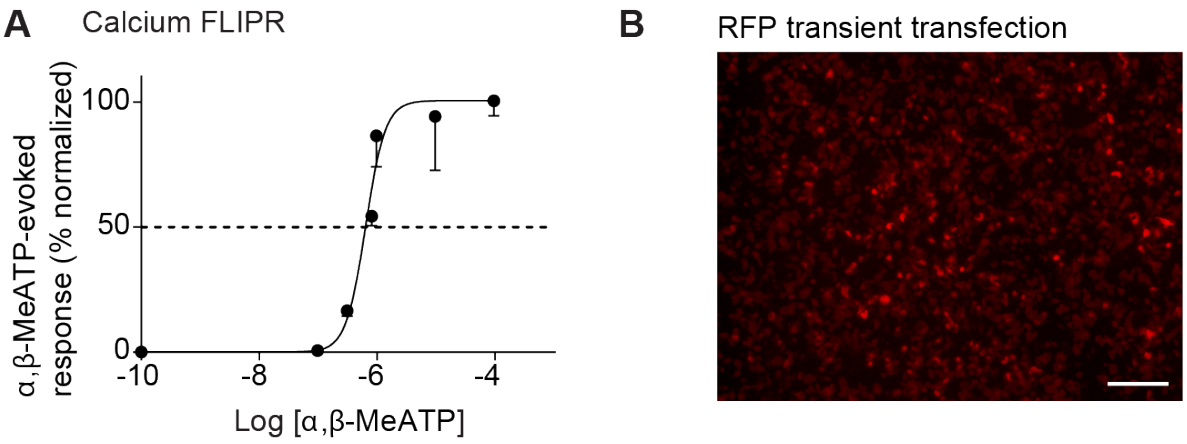

Figure S1. Conditions for genome-wide ORF-based FLIPR screening. Related to Figure 1.
(A) Dose–response curve of α,β-MeATP in the P2X3R/P2X2R-stably-expressing HEK cells used for the screening (Figure 1) was measured with the calcium FLIPR. The EC_{50} and Hill coefficient of α,β-MeATP were estimated as 656.5 ± 88.7 nM and 2.6 ± 0.8, respectively (n = 4).
(B) The transfection efficiency was estimated as 80–95% from the numbers of RFP-positive and total cells. Scale bar = 100 μm.
Data are mean ± s.e.m.

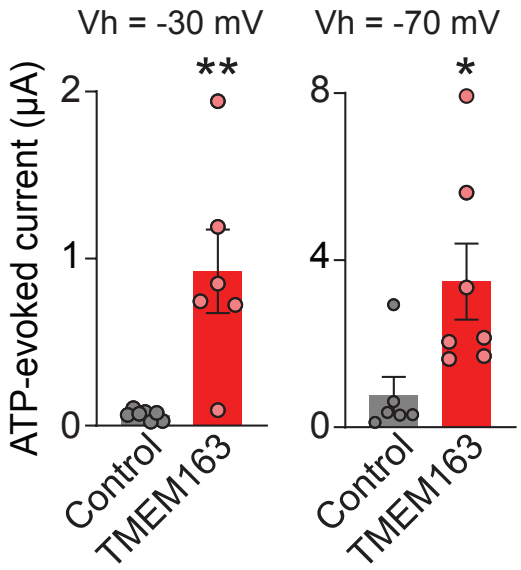

Figure S2. TMEM163 enhanced ATP-evoked P2X3R current at both -30 and -70 mV. Related to Figure 2.
300 nM ATP-evoked currents were measured with TEVC recording from oocytes injected with 25 pg P2X3R cRNA and 2 ng of TMEM163 or Neto2 (Control) cRNA at a holding potential of -30 and -70 mV. TMEM163 enhanced the ATP-evoked currents of P2X3R at both holding potentials (n = 6-7). Data are mean ± s.e.m. Mann-Whitney U-test. *$p < 0.05$, **$p < 0.01$.

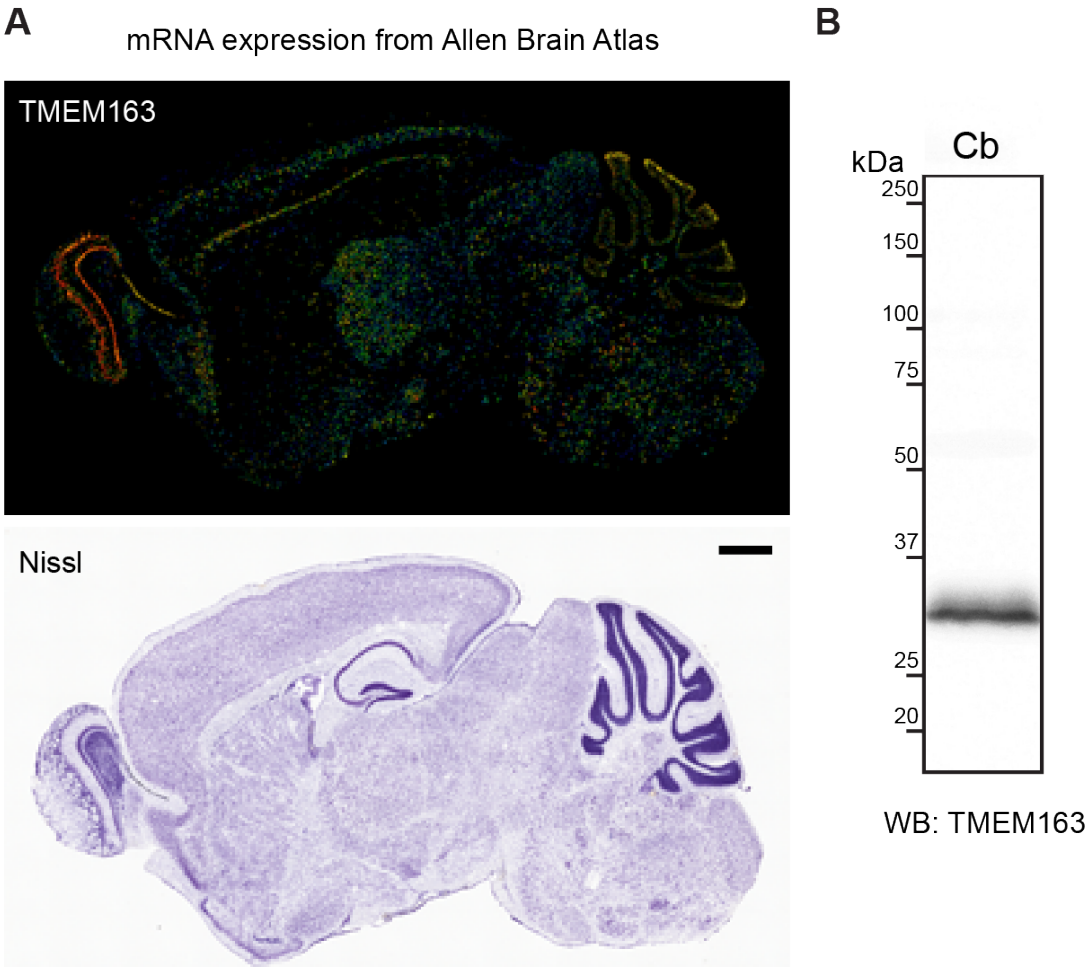

Figure S3. TMEM163 expression in the brain. Related to Figure 3.
(A) TMEM163 mRNA distribution in mouse brain (image generated from Allen Brain Atlas website http://mouse.brain-map.org/). Scale bar = 1 mm.
(B) The antibody against TMEM163 was generated against recombinant N-terminal cytoplasmic domain (for details, see Experimental Procedure). The anti-TMEM163 antibody recognized a strong band at the expected molecular weight of 31 kDa in mouse cerebellar lysate.

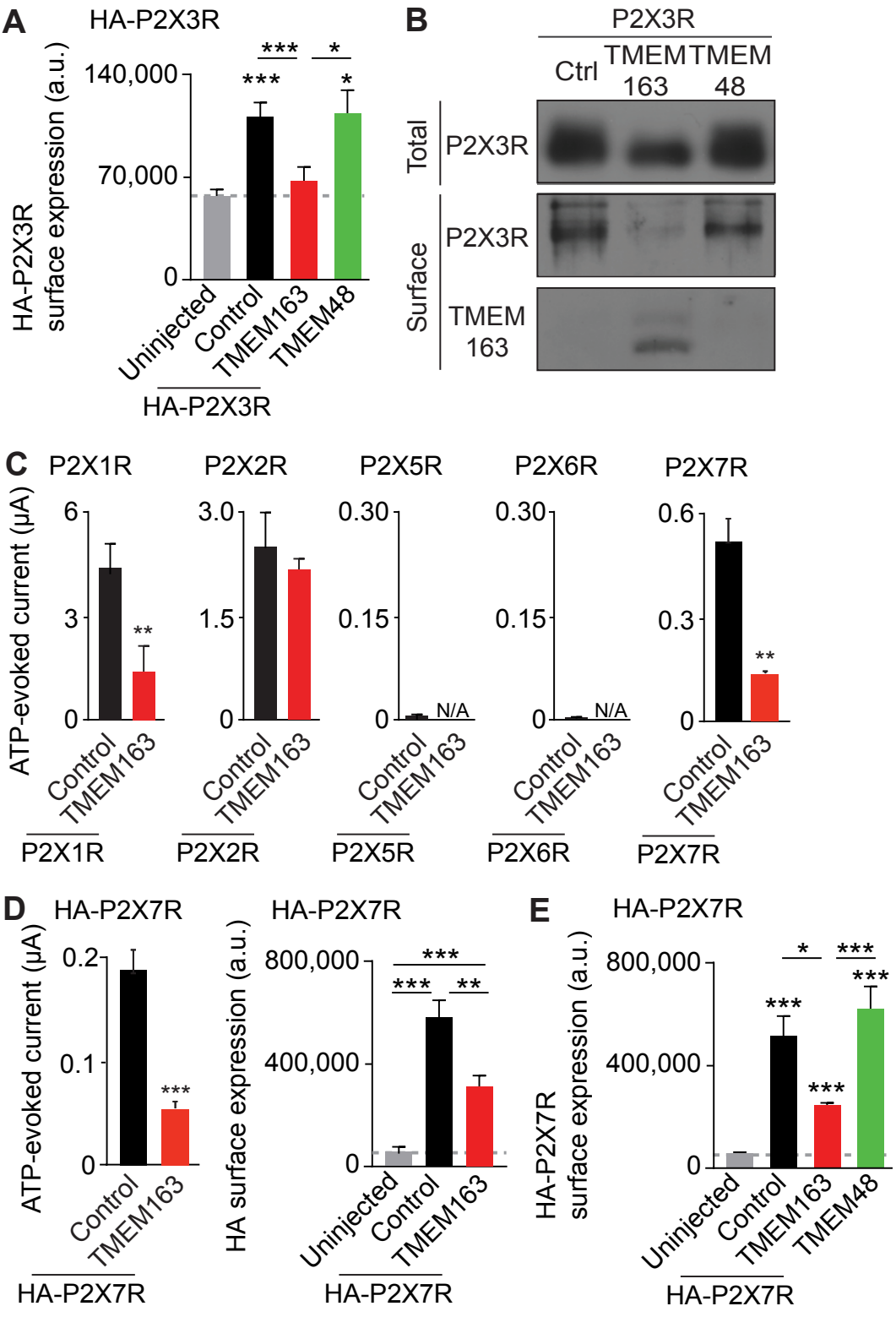
A
HA-P2X3R
HA-P2X3R surface expression (a.u.)
140,000
70,000
0
***
***
*
*
Uninjected
Control
TMEM163
TMEM48
HA-P2X3R
B
P2X3R
Ctrl
TMEM 163
TMEM 48
Total
P2X3R
Surface
P2X3R
TMEM 163
C
P2X1R
P2X2R
P2X5R
P2X6R
P2X7R
ATP-evoked current (μA)
6
3
0
3.0
1.5
0.30
0.15
0.6
0.3
**
N/A
Control
TMEM163
D
HA-P2X7R
ATP-evoked current (μA)
0.2
0.1
0
***
HA surface expression (a.u.)
800,000
400,000
***
**
Uninjected
E
HA-P2X7R
HA-P2X7R surface expression (a.u.)
800,000
400,000
*
***

Figure S4. TMEM163 modulation of ATP-evoked current and surface expression of P2XR subtypes. Related to Figure 4.

(A) Extracellularly HA tagged P2X3R cRNA (HA-P2X3R, 200 pg) was co-injected with 2ng cRNA of Neto2 (control), TMEM163 or TMEM48 into *Xenopus laevis* oocytes. Then, HA-P2X3R at the oocyte surface was quantified by the chemiluminescence assay with the anti-HA antibody and HRP-conjugated secondary antibody under non-permeabilized condition. The signal from uninjected oocytes indicates the background in this assay. A specific signal from HA-P2X3R was detected in control, and co-expression of TMEM163, but not TMEM48, reduced the surface expression of HA-P2X3Rs (n = 8 each).

(B) Surface and total expressions of P2X3R was biochemically measured in oocytes injected with cRNAs of 200 pg of P2X3R and 2 ng of Neto2 (Control), TMEM163 or TMEM48. Total and surface expression of P2X3R was reduced by co-expression of TMEM163, but not control and TMEM48.

(C) ATP-evoked currents were measured from oocytes co-injected with 2 ng of TMEM163 or Neto2 (Control) cRNA and the indicated amount of each P2XR subtype cRNA. TMEM163 co-expression reduced ATP-evoked current of P2X1R (33 μM ATP, 200 pg cRNA, Vh = -30 mV, n = 7) and P2X7R (100 μM ATP, 200 pg cRNA, Vh = -70 mV, n = 4-5), but not P2X2R (1 μM ATP, 50 pg cRNA, Vh = -70 mV, n = 9). 1 mM ATP-evoked currents were not detected in oocytes injected with 50 pg cRNA of human P2X5R (Vh = -70 mV, n = 9) or P2X6R (Vh = -70 mV, n = 6).

(D and E) Extracellularly HA tagged P2X7R cRNA (HA-P2X7R, 200 pg) was co-injected with 2ng of Neto2 (control) or TMEM163 cRNA into oocytes, followed by measurements of 100 μM ATP-evoked currents using TEVC recording (Vh = -70 mV) and HA signals at the oocyte surface using the chemiluminescence assay.

(D) TMEM163 co-expression reduced 100 μM ATP-evoked currents (n = 7 each) and the surface expression of HA-P2X7R (n = 8 each).

(E) Co-expression of TMEM163, but not TMEM48, reduced the surface expression of HA-P2X7R (n = 8 each)

Data are mean ± s.e.m. Mann-Whitney U-test (A, C, D, E). *p<0.05, **p < 0.01, ***p < 0.001.

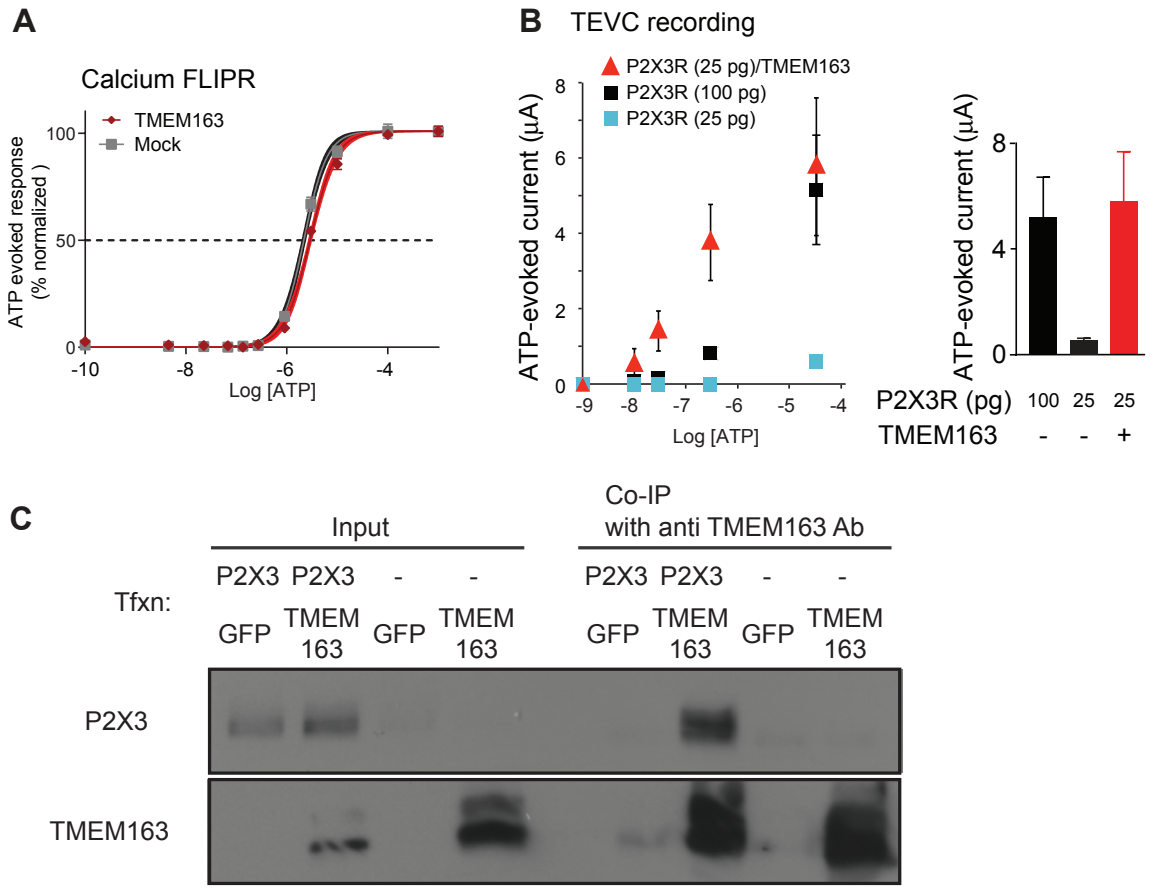

Figure S5. TMEM163 does not shift EC_{50} of ATP for endogenous P2 receptors. Related to Figure 5.

(A) Calcium FLIPR response at various concentrations of ATP was measured in the parent HEK cells transfected transiently with TMEM163 or pcDNA3 (Mock). Apyrase was pre-incubated for one hour before stimulation to reduce extracellular ATP. Transfected HEK cells responded to ATP (EC_{50} of 2.19 ± 0.8 μM for Mock and 2.91 ± 0.08 μM for TMEM163; Hill coefficient of 1.9 ± 0.1 for Mock and 1.71 ± 0.08 for TMEM163, n = 4) corresponding to ATP responses mostly from endogenous P2 receptors in HEK cells.

(B) ATP-evoked currents at various ATP concentrations were measured from oocytes injected with cRNAs (n = 6 each). 33 μM ATP-evoked currents were similar in the oocytes injected with 100 pg P2X3R cRNA and 25 pg P2X3 cRNA co-injected with 2 ng of TMEM163 cRNA (Bar graph, right). However, ATP-evoked currents from oocytes injected with 25 pg P2X3R cRNA were small at 33 μM and difficult to measure ATP-evoked currents at the lower ATP concentrations reliably.

(C) HEK cells were co-transfected (Tfxn) with P2X3R and TMEM163 or GFP as control. Two days later cells were solubilized with 1% CHAPS, followed by co-immunoprecipitation with anti-TMEM163 antibody. P2X3R was co-immunoprecipitated with TMEM163 only in the cell lysate expressing both TMEM163 and P2X3R. Input lanes contained 5% of the protein used for immunoprecipitation.

Data are mean ± s.e.m.

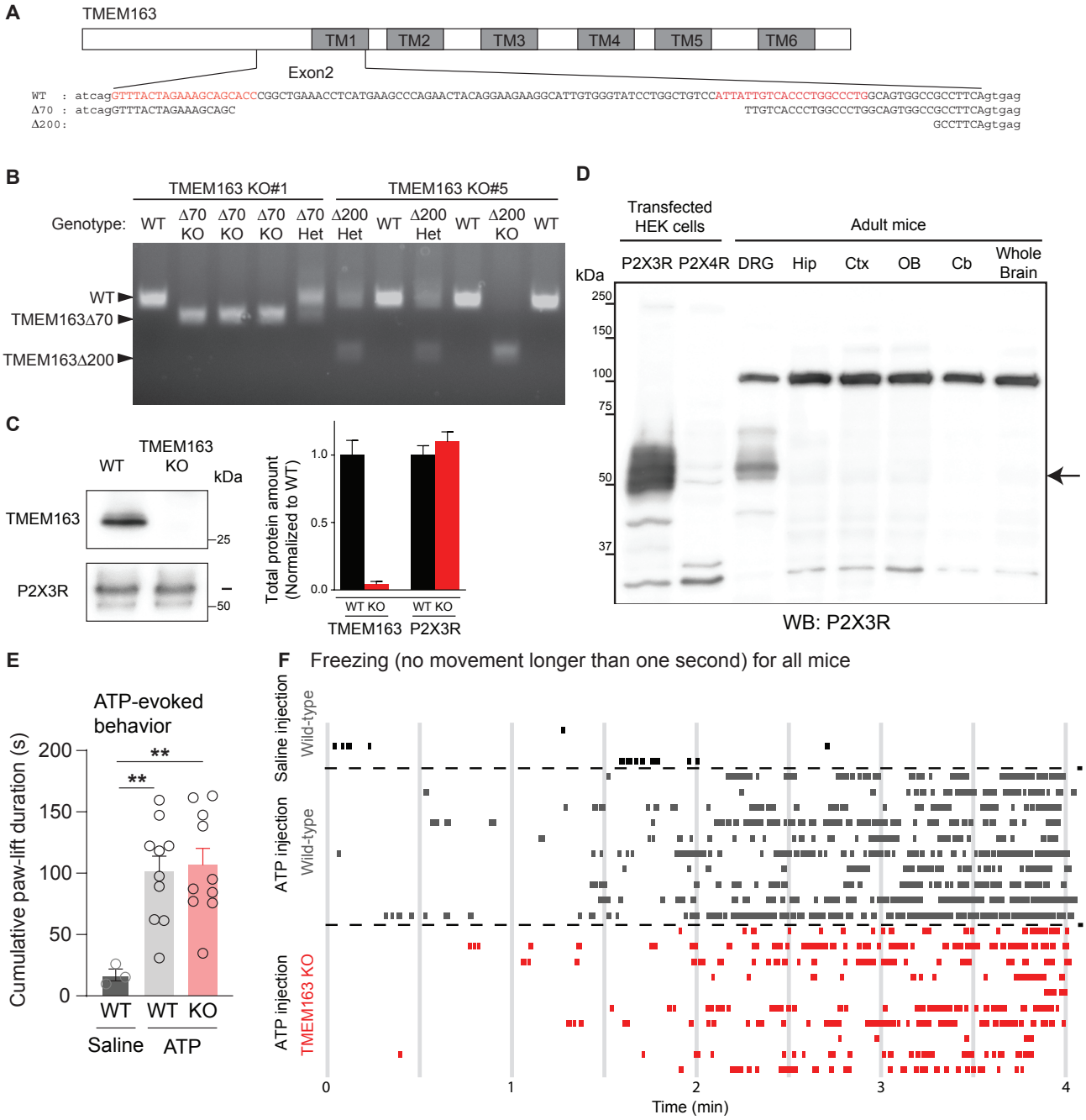
A
TMEM163
TM1
TM2
TM3
TM4
TM5
TM6
Exon2
WT : atcagGTTTACTAGAAAGCAGCACCCGGCTGAAACCTCATGAAGCCCAGAACTACAGGAAGAAGGCATTGTGGGTATCCTGGCTGTCCATTATTGTCACCCTGGCCCTGGCAGTGGCCGCCTTCAgtgag
Δ70 : atcagGTTTACTAGAAAGCAGC TTGTCACCCTGGCCCTGGCAGTGGCCGCCTTCAgtgag
Δ200: GCCTTCAgtgag
B
TMEM163 KO#1
TMEM163 KO#5
Genotype: WT Δ70 KO Δ70 KO Δ70 KO Δ70 Het Δ200 Het WT Δ200 Het WT Δ200 KO WT
WT
TMEM163Δ70
TMEM163Δ200
C
WT
TMEM163 KO
kDa
TMEM163
25
P2X3R
50
Total protein amount (Normalized to WT)
1.0
0.5
0.0
WT KO
WT KO
TMEM163
P2X3R
D
Transfected HEK cells
Adult mice
P2X3R P2X4R DRG Hip Ctx OB Cb Whole Brain
kDa
250
150
100
75
50
37
WB: P2X3R
E
ATP-evoked behavior
Cumulative paw-lift duration (s)
200
150
100
50
0
**
**
WT WT KO
Saline ATP
F
Freezing (no movement longer than one second) for all mice
Saline injection
Wild-type
ATP injection
Wild-type
ATP injection
TMEM163 KO
0
1
2
3
4
Time (min)

Figure S6. Generation of TMEM163 knockout mice. Related to Figure 7.
(A) Targeting sequence, genomic structure, and deleted regions are shown. Exon 2 of TMEM163 contains most of transmembrane domain 1 (TM1). This exon was targeted with a Cas9/CRISPR system with the four sgRNAs and two are highlighted in red. Their target allele was amplified from the genome of founder lines, and deletions of either 70 or 200 bp were confirmed by Sanger sequencing in a total of three founder lines.
(B) Example of genotyping PCR. The knockout (KO) lines #1 and #5 showed a deletion of 70 (Δ70) or 200 bp (Δ200), respectively. Wild type (WT). Het (Heterozygous).
(C) Total protein amounts of TMEM163 and P2X3R in dorsal root ganglion (DRG) from TMEM163 KO and WT littermate mice. TMEM163 protein expression was not detected in TMEM163 KO mice, and P2X3R protein expression (arrow) was unaltered (n = 4).
(D) The anti P2X3R antibody recognized strong bands around 60 kDa in HEK cells transfected with human P2X3R, but not P2X4R. Among various tissues of adult mice, the anti-P2X3R antibody detected the 60 kDa band only in the dorsal root ganglion (DRG), but not in the hippocampus (Hip), Cerebral cortex (Ctx), Olfactory bulb (OB), Cerebellum (Cb) and whole brain.
(E) Cumulative durations of hind-paw lifting induced by the intraplantar injection of 3 μmols ATP in saline and by saline alone. . Mean total durations were greater to ATP than to saline but unaltered between WT and TMEM163 KO mice (n = 10 for ATP-injected mice and 3 for saline-injected mice).
(F) Time of occurrence and duration of the ATP-evoked freezing behaviors of each mouse. Each black block in a row indicates the duration of freezing for a mouse for a given experimental condition. The mean cumulative duration of freezing durations for each condition is shown in Figure 7A.
Data are mean ± s.e.m. Student's t-test (C) and one-way ANOVA with Bonferroni's post-test (E); $^{**}p < 0.01$.