Supplemental information

Title

A long noncoding RNA contributes to neuropathic pain by silencing Kcna2 in primary afferent neurons

Authors

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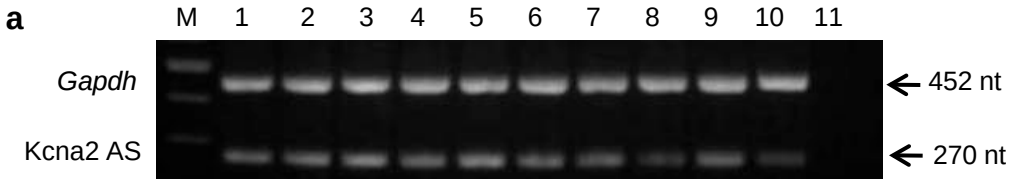
Supplemental Fig. 1 Expression of Kcna2 antisense (AS) RNA in tissues and its whole sequence in rats

a



b

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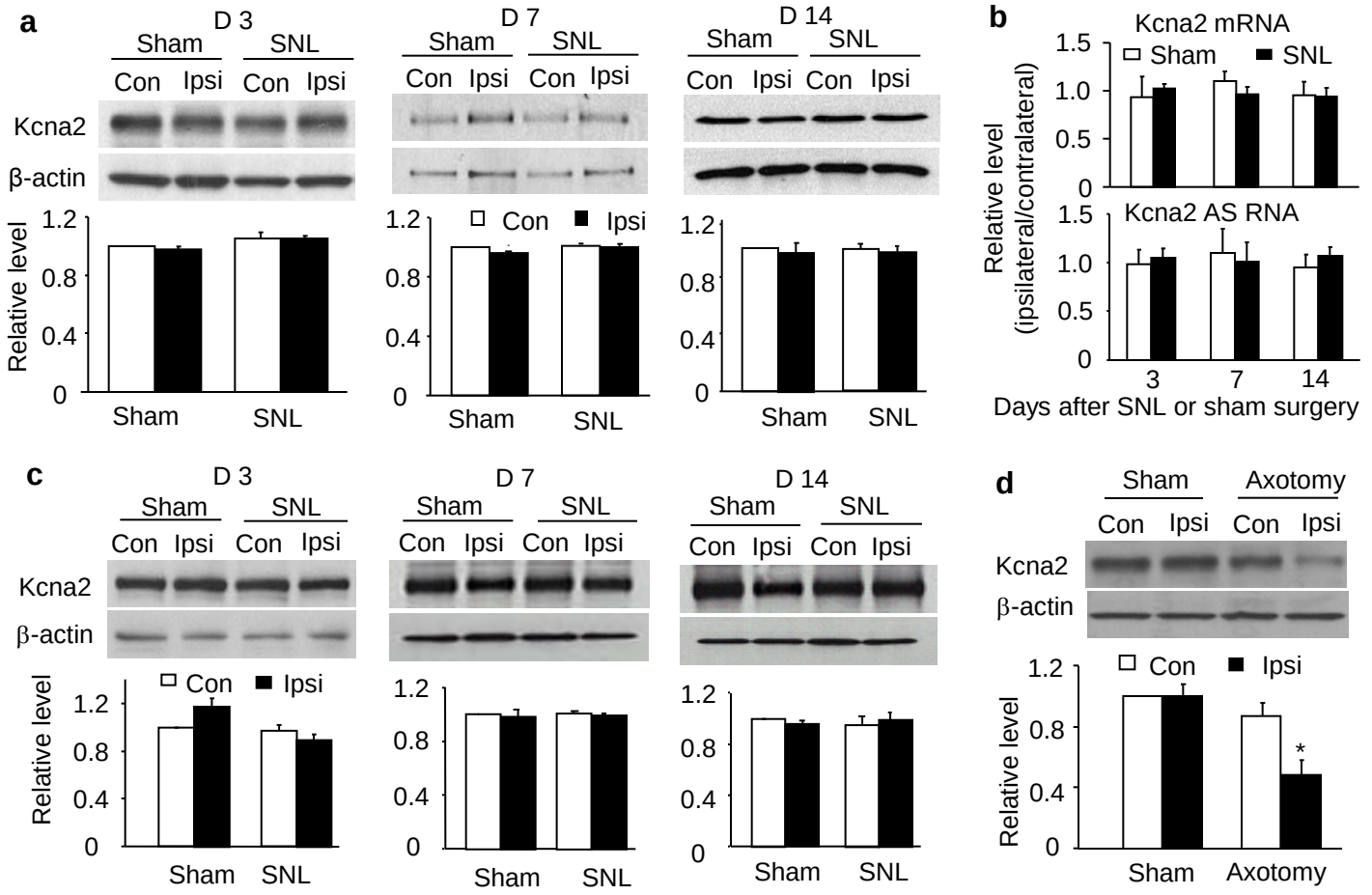
ATCCCAGGCCCGAGCGGGATCCAGCCCCGAGGGAGATATGTTGCTATTATTATGATGCTGCCAGACAGAATCTGCAGACCCCAGTTCCCCCCACTC
CAGTCTTCTGTTTCTGCGTCCCTTT –3’

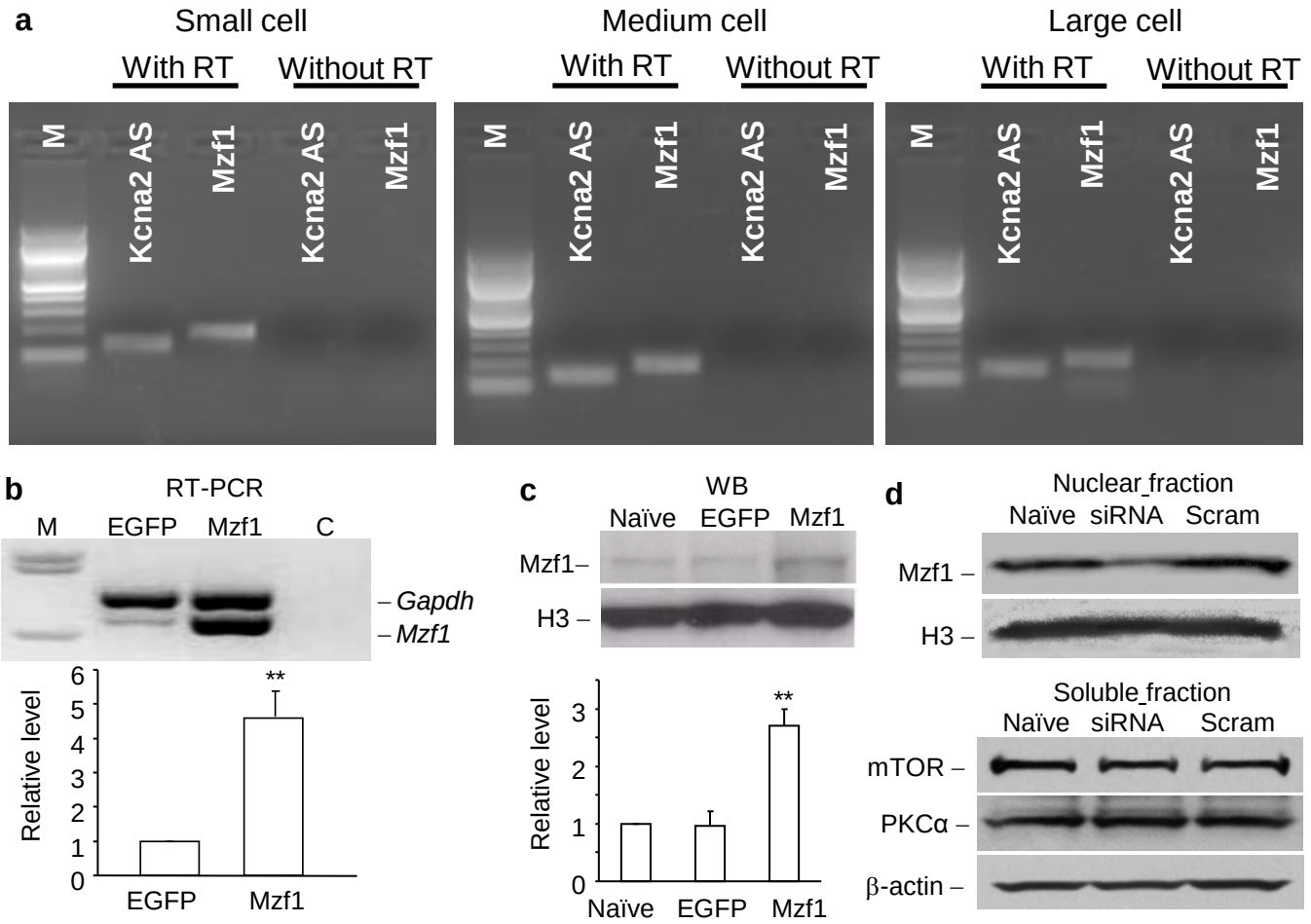
Supplemental data Fig. 1: (a) Reverse transcription-PCR analysis showing the expression of Kcna2 antisense (AS) RNA in different tissues from normal rats. Lane 1: dorsal root ganglion. Lane 2: spinal cord. Lane 3: brainstem. Lane 4: hippocampus. Lane 5: cerebellum. Lane 6: cortex. Lane 7: heart. Lane 8: liver. Lane 9: lung. Lane 10: kidney. Lane 11: no-template control. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as an internal control. n = 3 repeated experiments. M: a DNA ladder marker. (b) The full-length rat Kcna2 AS cDNA sequence (2.52 kb). It contains a unique sequence at each end (black letters) and complementary sequence in the middle. The pink letters indicate sequences that are complementary to the 3′ UTR and part of the 5′ UTR of Kcna2 cDNA, and the blue letters indicate the sequence complementary to the coding sequence of Kcna2 cDNA. Translation analysis with DNAMAN software shows more than 30 stop codons distributed throughout the sequence of Kcna2 AS cDNA.

Supplemental Fig. 2 Expression of Kcna2 mRNA, Kcna2 protein, or Kcna2 AS RNA in L_4 DRG, L_5 DRG, or L_5 dorsal horn after peripheral nerve injury



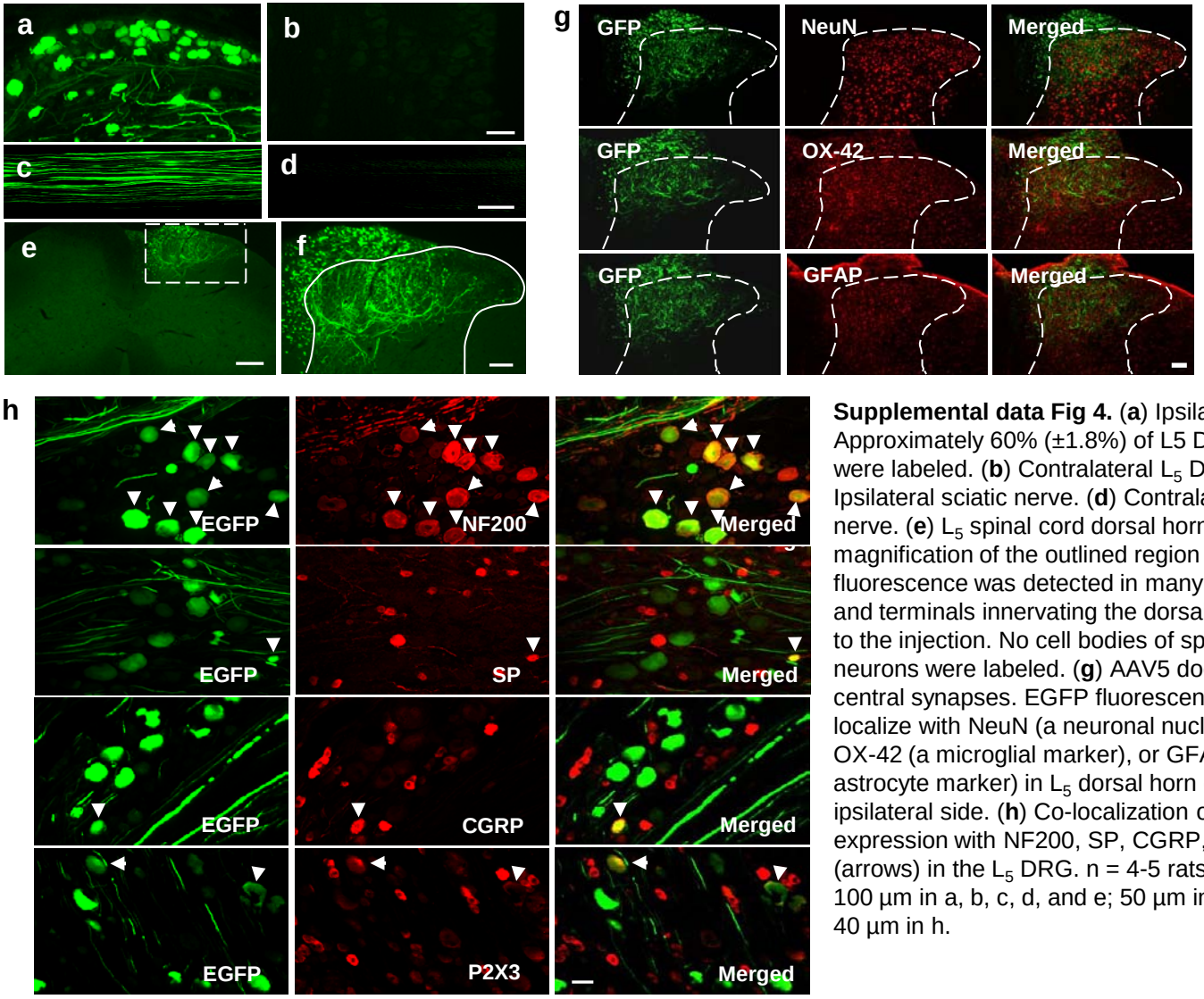
Supplemental data Fig. 2: (**a**) Western blot analysis revealed the expression of Kcna2 protein in the ipsilateral (Ipsi) and contralateral (Con) L_4 DRGs on days 3, 7, and 14 after L_5 SNL or sham surgery. n = 12 rats/group/time point. F = 0.51 for day 3, 0.71 for day 7, and 0.59 for day 14. (**b**) Quantitative real-time RT-PCR analysis showing the levels of Kcna2 mRNA and Kcna2 AS RNA in the ipsilateral and contralateral L_5 dorsal horns on days 3, 7, and 14 after L_5 SNL or sham surgery. n = 12 rats/group. F = 0.82 for mRNA and 0.79 for AS RNA. (**c**) Western blot analysis showing the expression of Kcna2 protein in the ipsilateral and contralateral L_5 dorsal horns on days 3, 7, and 14 after L_5 SNL or sham surgery. n = 12 rats/group/time point. F = 0.58 for day 3, 0.47 for day 7, 0.69 for day 14. (**d**) Western blot analysis shows a significant reduction in expression of Kcna2 protein in the ipsilateral $L_{4/5}$ DRGs on day 7 after sciatic nerve axotomy. n = 12 rats/group. F = 9.68. *P < 0.05 *vs* the contralateral side from the sham group.

Supplemental Fig.3 Mzf1 expression in Kcna2 AS RNA-containing DRG neurons and Mzf1 knockdown by its siRNA in HEK293T cells.



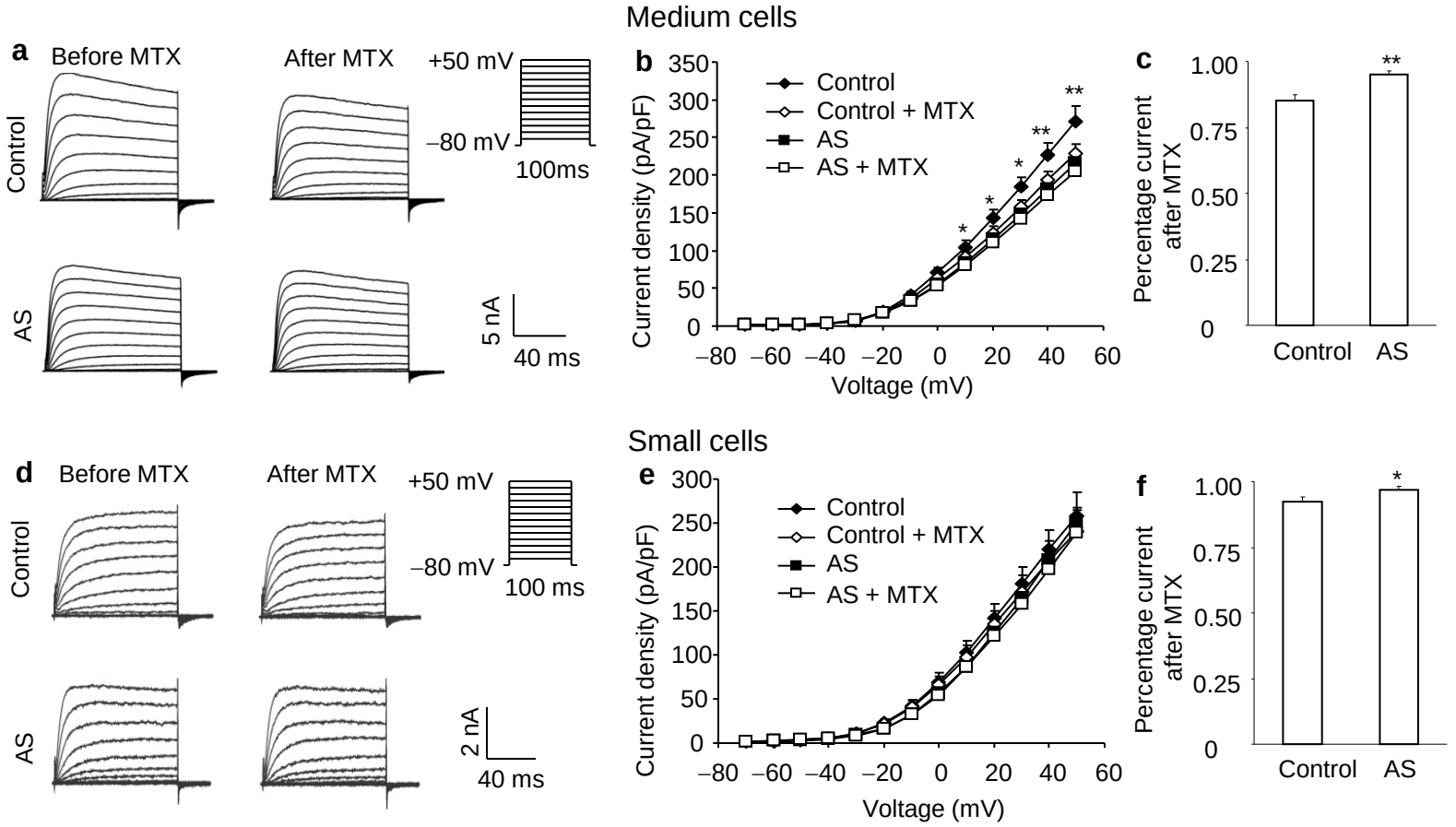
Supplemental data Fig. 3: (**a**) Co-expression of Kcna2 AS RNA with Mzf1 mRNA in small, medium, and large individual DRG neurons. Without RT primers, neither Kcna2 AS nor Mzf1 PCR products were detected in the DNase I-treated samples. n = 5 repeats/cell. M: 100-bp ladder. (**b**) Expression of Mzf1 mRNA in HEK293T cells transfected with control EGFP vector or the full-length Mzf1 vector. Gapdh was used as a loading control. n = 3 repeated experiments/treatment. t = -9.25. ***P* < 0.01 *vs* the EGFP group. M: DNA ladder. C: no-template control. (**c**) Expression of Mzf1 protein in naive HEK293T cell and HEK293T cells transfected with EGFP vector or the full-length Mzf1 vector. H3 was used as a loading control. n = 3 repeated experiments/treatment. F = 20.87. ***P* < 0.01 *vs* naïve group. (**c**) Mzf1 siRNA [but not scramble Mzf1 siRNA (Scram)] significantly knocks down Mzf1 protein expression but does not affect expression of nuclear protein H3 or cytosolic proteins mTOR, PKCα, and β-actin in HEK-293T cells.

Supplemental Fig. 4 EGFP expression is limited in L_5 DRG and its fibers and terminals on the ipsilateral side 8 weeks after AAV5-EGFP injection into the left L_5 DRG.



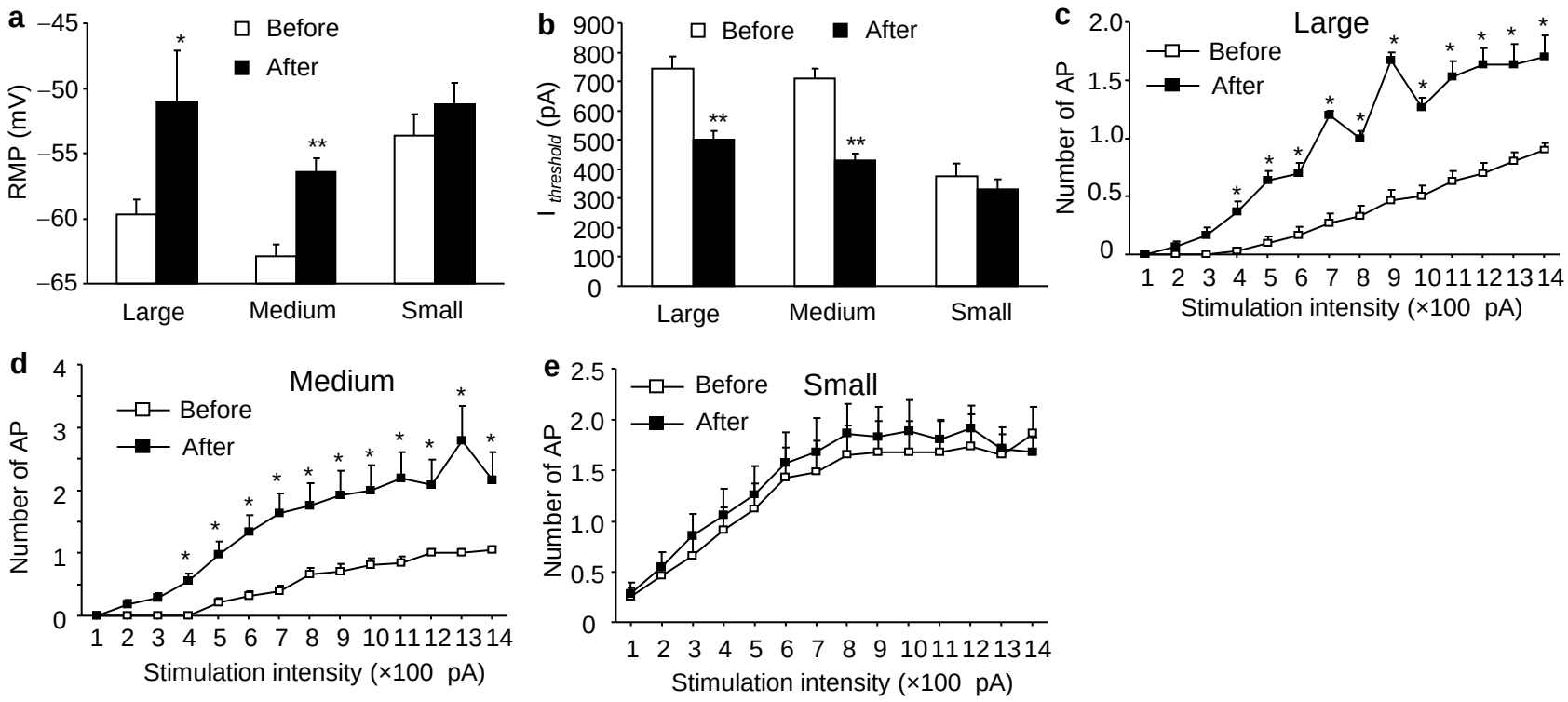
Supplemental data Fig 4. (**a**) Ipsilateral L_5 DRG. Approximately 60% (±1.8%) of L5 DRG neurons were labeled. (**b**) Contralateral L_5 DRG. (**c**) Ipsilateral sciatic nerve. (**d**) Contralateral sciatic nerve. (**e**) L_5 spinal cord dorsal horn. (**f**) High magnification of the outlined region from e. EGFP fluorescence was detected in many nerve fibers and terminals innervating the dorsal horn ipsilateral to the injection. No cell bodies of spinal cord neurons were labeled. (**g**) AAV5 does not cross central synapses. EGFP fluorescence did not co-localize with NeuN (a neuronal nuclear marker), OX-42 (a microglial marker), or GFAP (an astrocyte marker) in L_5 dorsal horn on the ipsilateral side. (**h**) Co-localization of EGFP expression with NF200, SP, CGRP, and P2X3 (arrows) in the L_5 DRG. n = 4-5 rats. Scale bars: 100 µm in a, b, c, d, and e; 50 µm in f; 200 µm in g; 40 µm in h.

Supplemental Fig. 5 Kcna2 AS RNA overexpression in DRG reduces total Kv current density in medium, but not small, DRG neurons 8–12 weeks post-viral injection.



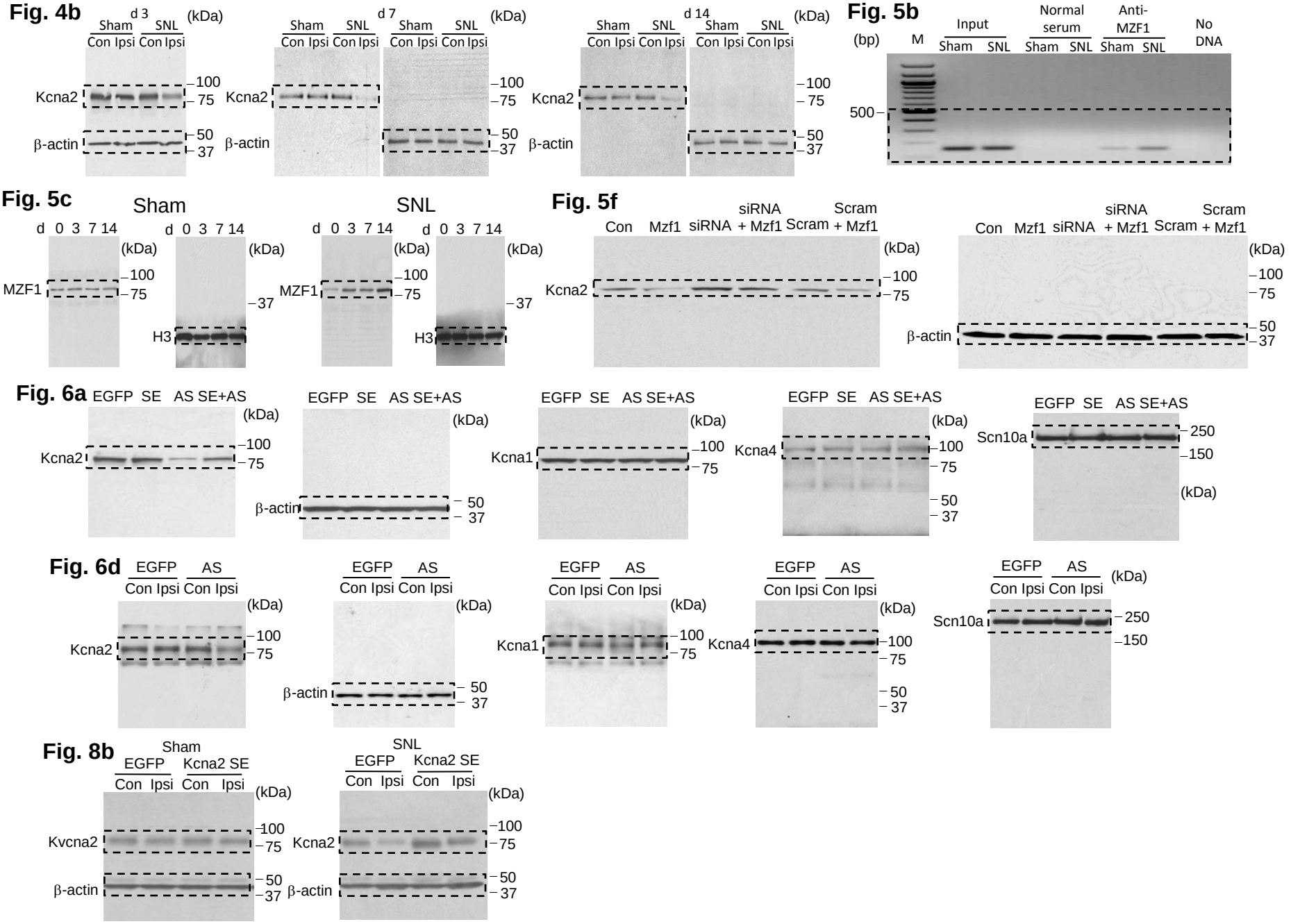
Supplemental data Fig. 5: (**a**) Representative traces of total Kv current in medium DRG neurons from control and AS–injected rats before or after bath perfusion of 100 nM MTX) (**b**) I-V curve for control (n = 17 cells, 7 rats) and AS-treated (n = 15 cells, 8 rats) medium DRG neurons before or after 100 nM MTX treatment. The current density was plotted against each voltage. F = 117.99, **P* < 0.05, ***P* < 0.01 *vs* the AS group. (**c**) At +50 mV, reduction in total Kv current after MTX treatment in medium DRG neurons was greater in the control group (n = 17 cells, 7 rats) than in the AS-treated group (n = 15 cells, 8 rats). t = -6.54, ***P* < 0.01 *vs* control. (**d**) Representative traces of total Kv current in small DRG neurons from control and AS-treated rats before or after bath perfusion of 100 nM MTX. (**e**) I-V curve for control and AS-treated small DRG neurons before or after 100 nM MTX treatment. The current density was plotted against each voltage. n = 11 cells/group. (**f**) At +50 mV, reduction in total Kv current after MTX treatment in small DRG neurons was greater in the control group (n = 11 cells, 7 rats) than in the AS-treated group (n = 11 cells, 8 rats). t = -2.83, **P* < 0.05 *vs* control.

Supplemental Fig. 6 Inhibition of Kcna2 function increases neuronal excitability in rat large and medium DRG neurons.

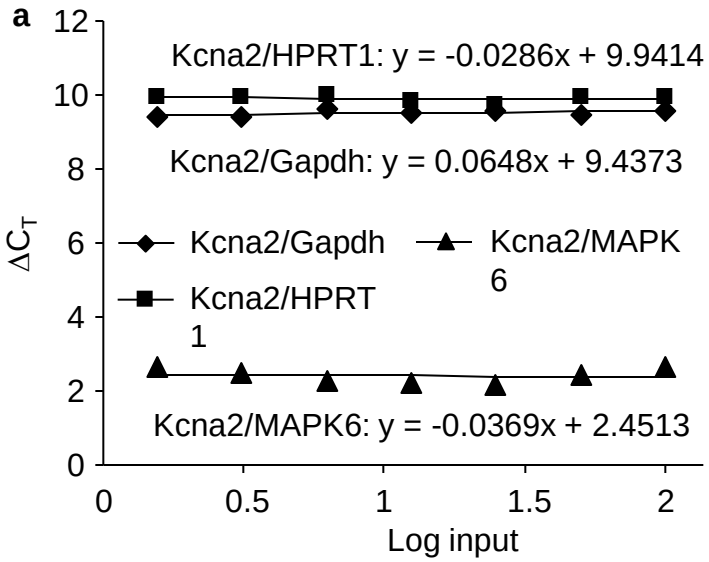


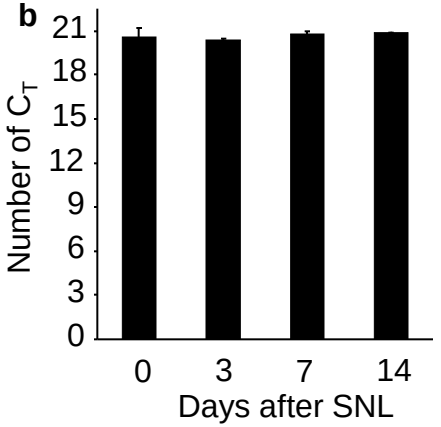
Supplemental data Fig. 6: DRG neurons were recorded before and 3–5 min after bath perfusion of 100 nM maurotoxin (MTX). n=30 large cells, 38 medium cells, and 35 small cells from 7 naïve rats. (**a**) Resting membrane potentials (RMP) before and after bath perfusion of MTX. t = -2.17 for large cells, -4.59 for medium cells, and -1.03 for small cells. **P* < 0.05, ***P* < 0.01 *vs* the corresponding cells before MTX treatment. (**b**) Current threshold for pulses ($I_{threshold}$) before and after bath perfusion of MTX. t = 4.60 for large cells, 7.25 for medium cells, and 0.77 for small cells. ***P* < 0.01 *vs* the corresponding cells before MTX treatment. (**c, d, e**) Numbers of evoked action potentials (APs) produced in large (F **=** 15.45), medium (F = 17.18), and small (F = 0.78) DRG neurons before or after bath perfusion of MTX. **P* < 0.05 *vs* the same stimulation intensity before MTX treatment.

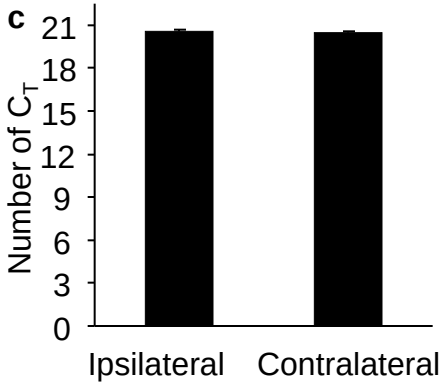
Supplementary Fig. 7 Full-length pictures of the blots presented in the main figures

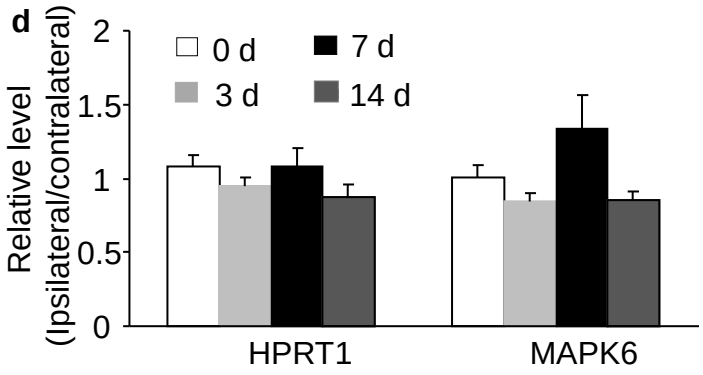


Supplemental Fig. 8 SNL does not alter expression of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) mRNA in the injured DRG.









Supplemental data Fig. 8: (**a**)The amplification reactions of Kcna2mRNA and three reference genes, Gapdh mRNA, hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA, and mitogen-activated protein kinase 6 (MAPK6) mRNA have similar PCR efficiency. The ΔC_T (C_T Kcan2 – C_T reference) values are plotted *vs* log nputs (RNAs, 1–100 ng). The absolute values of the three slopes of ΔC_T *vs* log inputs were less than 0.1, indicating that the efficiency of four mRNA amplifications are approximately equal. (**b**) Quantitative real-time RT-PCR showed no difference in expression of Gapdh mRNA in the injured L_5 DRG on days 0 (naïve), 3, 7, or 14 after SNL. n = 6 rats/time point. F = 2.17 (*P* = 0.19). (**c**) Quantitative real-time RT-PCR showed no difference in expression of Gapdh mRNA in the ipsilateral and contralateral L_5 DRGs on day 7 after SNL. n = 6 rats. t = -1.63 (*P* = 0.12). (**d**) Quantitative real-time RT-PCR showed no difference in expression of Gapdh mRNA in the injured L_5 DRG on days 0 (naïve), 3, 7, or 14 after SNL, as evidenced by no significant alteration in ratios of ipsilateral-side mRNA level to contralateral-side mRNA level after normalization to either HPRT1 or MAPK6. n = 6 rats/time point. F = 1.45 (*P* = 0.29) for HPRT1 and 3.45 (*P* = 0.071) for MAPK6.

Supplemental Table 1 Effect of Kcna2 AS RNA or MTX on membrane input resistance and other action potential parameters in the DRG

	Large neurons					Medium neurons					Small neurons				
	Control		AS		t/p value	Control		AS		t/p value	Control		AS		t/p value
n	39 cells, 12 rats		43 cells, 14 rats			56 cells, 12 rats		72 cells, 14 rats			40 cells, 12 rats		44 cells, 14 rats		
R_{in}, MΩ	109.46	21.32	117.88	23.09	-0.26/0.79	233.49	71.77	297.56	34.45	-1.36/0.18	268.17	31.64	250.24	37.68	0.36/0.72
APA, mV	98.13	2.86	93.8	2.54	1.13/0.26	100.07	1.67	101.88	1.04	-0.97/0.33	93.63	2.32	98.88	2.58	-1.5/0.14
APT, mV	-21.80	0.98	-22.72	1.24	0.85/0.58	-17.07	0.64	-18.22	0.62	1.22/0.23	-18.1	1.67	-17.9	0.9	-0.14/0.9
APO, mV	40.32	2.3	39.45	2.02	0.29/0.77	37.78	1.79	40.19	1.17	-1.42/0.16	36.63	1.83	41.11	2.37	-1.5/0.14
AHPA, mV	-5.86	1.83	-9.44	1.32	1.63/0.11	-7.37	0.97	-9.12	1.16	1.04/0.3	-5.35	1.32	-9.09	1.84	0.36/0.72
	Large neurons					Medium neurons					Small neurons				
	Before MTX		After MTX		t/p value	Before MTX		After MTX		t/p value	Before MTX		After MTX		t/p value
n	30 cells, 7 rats		30 cells, 7 rats			38 cells, 7 rats		38 cells, 7 rats			35 cells, 7 rats		35 cells, 7 rats		
R_{in}, MΩ	117.52	16.21	121.81	17.47	-0.18/0.86	223.13	32.5	223.58	32.58	-0.01/0.99	256.5	36.81	262.49	36.81	-0.12/0.91
APA, mV	96.68	1.09	100.54	1.84	-1.81/0.08	102.34	2.9	100.41	1.29	0.61/0.55	99.82	1.18	98.51	1.54	0.68/0.5
APT, mV	-19.40	1.47	-20.67	1.17	0.68/0.5	-18.48	0.95	-19.22	1.11	0.5/0.62	-20.78	0.99	-21.33	0.89	0.41/0.69
APO, mV	36.90	1.09	35.13	2.83	0.59/0.56	36.43	1.67	38.43	1.34	-0.99/0.32	43.79	2.03	45.07	1.3	-0.53/0.6
AHPA, mV	-5.25	1.71	-8.09	1.04	1.4/0.17	-7.64	0.96	-9.48	0.81	1.47/0.15	-7.69	1.71	-7.6	1.69	0.04/0.97

Values are mean SEM. R_{in}: membrane input resistance. APA: action potential amplitude. APT: action potential threshold. APO: action potential overshoot. AHPA: afterhyperpolarization amplitude.

Supplemental table 2 All primers and probes used

Names	Sequences
RT-PCR	
Rat-Kcna2 AS-RT	5'-CGTCACACCTCCTGAGGACAG-3'
Rat-Kcna2 AS-F	5'-CTGCCCCCAGACTGGTAGTA-3'
Rat-Kcna2 AS-R	5'-CCCAGTGGATGAGGCTGCTG-3'
Mouse-Kcna2 AS-RT	5'-TCTCCTCCTCAAGTCGTGGTGC-3'
Mouse-Kcna2 AS-F	5'-TTCCAGACAGAAGCTGACGA-3'
Mouse-Kcna2 AS-R	5'-ACCTGTGAACGTGCCCTTAG-3'
Human/monkey-Kcna2 AS-RT	5'-ACTCACCATTATTTCTAGCTCG-3'
Human/monkey-Kcna2 AS-F	5'-TCTAAGGGCACATTCACAGGTC-3'
Human/monkey-Kcna2 AS-R	5'-TGTTGGTGCATCTCAGATTCCT-3'
Rat-Gapdh-RT	5'-GAGGGTGCAGCGAACTTTATTGAT-3'
Mouse-Gapdh-RT	5'-GAGGGTGCAGCGAACTTTATTGAT-3'
Human/Monkey-Gapdh-RT	5'-GAGCACAGGGTACTTTATTGAT -3'
Gapdh-F	5'-ACCACAGTCCATGCCATCAC-3'
Gapdh-R	5'-TCCACCACCCTGTTGCTGTA-3'
Rat-Mzf1-RT	5'-TCACCGCAGTGGTAGGGCTTTTCG-3'
Rat-Mzf1-F	5'-AGGTGAAAGAGGAGTCAGAGGTTA-3'
Rat-Mzf1-R	5'-GATCCTCGTCCGTGGGGTCCTGTT-3'
Cloning	
Kcna2 AS RT	5'-CGTCACACCTCCTGAGGACAG-3'
Kcna2 AS PCR-F	5'-CCTGTAAGTCGGTGATAAA-3'
Kcna2 AS PCR-R	5'-TACCTCCTTCCTTCCAAA-3'
Kcna2 AS N-PCR-F	5'-GGCTCCGGAGGATGACACGGAGAATGG-3'
Kcna2 AS N-PCR-R	5'-ATAGTTTAGCGGCCGCCTCGAGGACCTGCCCTCCTGCT-3'
Kcna2 SE RT	5′-GGGTGACTCTCATCTTTGGA-3′
Kcna2 SE PCR-F	5′-CAGGAACATGGAGGCTCGGGTAC-3′
Kcna2 SE PCR-R	5′-CACTGACTACAATGCAGGCTATT-3′
Kcna2 SE N-PCR-F	5'-GGCTCCGGATCGAGGACCTGCCCTCCTGCT-3'
Kcna2 SE N-PCR-R	5'-ATAAGAATGCGGCCGCCAGCAGCCTCATCCACTGGG-3'
Mzf1 RT	5′-CTGCGTAAGCCAGAAGTATTGT-3′
Mzf1 PCR-F	5′-GACAGACACTGGCCTCAAAGAC-3′
Mzf1 PCR-R	5′-GGTTGGGGAAGCATTATCTATT-3′
Mzf1 N-PCR-F	5‘-TGTCTAGAGCCACCATGAAACCCATTGTGCCAGACTC-3'
Mzf1 N-PCR-R	5‘-TCAGTGCGGTACCGCTACTCAGTGCTGTGGATGCGTT-3'
EMSA	
Minizmal probe	ATGGTCCTCTCAATGCGAAAGTGGGGATGA CCTTTGGAGTCG
Mutant probe	ATGGTCCTCTCAATGCGAAAGTGGATGACC TTTGGAGTCG

Names	Sequences
Real-time RT-PCR	
Kcan2 AS RNA RT	5'- CGTCACACCTCCTGAGGACAG-3'
Kcan2 AS RNA Probe	5'-/56-FAM/TGCTGTTGGAATAGGTGTGGAAGGT/BHQ_1/-3'
Kcan2 AS RNA PCR-F	5'-AGAAAGGGTCGGTGAAGGAGGT-3'
Kcan2 AS RNA PCR-R	5'-GTGTGGCTTCTCTTTGAATACC-3'
Kcna2 mRNA RT	5'-GGGTGACTCTCATCTTTGGA-3'
Kcna2 mRNA Probe	5'-/56-FAM/TGCTGTTGGAATAGGTGTGGAAGGT/BHQ_1/-3'
Kcna2 mRNA PCR-F	5'-GTGTGGCTTCTCTTTGAATACC-3'
Kcna2 mRNA PCR-R	5'-AGAAAGGGTCGGTGAAGGAGGT-3'
Kcna1 mRNA RT	5'-AAAGTATCTACAGAGTGGGACA-3'
Kcna1 mRNA Probe	5'-/56-FAM/AGTCATCTACACTTCTAACATCTTCACAGAC/BHQ_1/-3'
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Kcna1 mRNA PCR-R	5'-TCAAAAGAGAACCAGATGATACAC-3'
Kcna4 mRNA RT	5’-TGTTTTTATCTGTCTCGCTGTCA- 3’
Kcna4 mRNA Probe	5'-/56-FAM/CTTCTTCTTCCCTGGGGGACA /BHQ_1/-3'
Kcna4 mRNA PCR-F	5’-CCCATACCTACCTTCTAATTTGC -3’
Kcna4 mRNA PCR-R	5’-TGTTTTTATCTGTCTCGCTGTCA -3’
Gapdh RNA RT	5'-GAGGGTGCAGCGAACTTTATTGATG-3'
Gapdh RNA Probe	5’-/56-FAM/ATCAACGGGAAACCCATCACCATCTT/BHQ_1/-3’
Gapdh RNA PCR-F	5’-TCGGTGTGAACGGATTTGGC-3’
Gapdh RNA PCR-R	5’-CCTTCAGGTGAGCCCCAGC-3’
HPRT1 RNA RT	5’-AAGCTTGCTGGTGAAAAGGA-3’
HPRT1 RNA Probe	5'-/56-FAM/AAATTCCAGACAAGTTTGTTGTTGG/BHQ_1/-3'
HPRT1 RNA PCR-F	5’-AAGCTTGCTGGTGAAAAGGA-3’
HPRT1 RNA PCR-R	5’-CCGCTGTCTTTTAGGCTTTG-3’
MAPK6 RNA RT	5’-TAAAGCCATTGACATGTGGG-3’
MAPK6 RNA Probe	5’-/56-FAM/ACCCTCTTTGCAGGTGCACATGAAC/BHQ_1/-3’
MAPK6 RNA PCR-F	5’-TAAAGCCATTGACATGTGGG-3’
MAPK6 RNA PCR-R	5’-TCGTGCACAACAGGGATAGA-3’
RACE	
Extended primers	
Rat-Kcna2 AS-RT	5'-CGTCACACCTCCTGAGGACAG-3'
Rat-Kcna2 AS-F	5'-CCTGTAAGTCGGTGATAAA-3'
Rat-Kcan2 AS-R	5'-TACCTCCTTCCTTCCAAA-3'
5'-end primers	
SP1 for RT	5'-CAACTGTACCTTGGCTAACA-3'
5' Oligo Td-Anchor primer	Provided by 5’ RACE kit (03353621001)
SP2 for the first nested PCR	5'-CTGCATTGTAGTCAGTGTTC-3'
5' PCR anchor primer	Provided by 5' RACE kit (03353621001)
SP3 for the second nested PCR	5'-GCATGGAAAGCAATAGTCGT-3'
3'-end primers	
3' end RT/R primer	5'- GAGCATGCGGCCGCTAAGAACAGTG-3 '
Rat-Kcna2 AS-F	5'-AGGGAACTCAGGGTGCTGGTAA-3'
3' RNA adapter	5'-phosphate-UUCACUGUUCUUAGCGGCCGCAUGCUC-idT-3'

RT: Reverse-transcription. F: Forward. R: Reverse. N-PCR: Nested PCR. Underlined letters: the restriction enzyme recognition sites.