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Supplementary Information for

GPCR-dependent biasing of GIRK channel signaling dynamics by RGS6 in mouse sinoatrial nodal cells

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Supplementary Information Text

Materials and Methods

***SAN cell culture*.** Adult male and female mice (8-10 wk) were anesthetized with intraperitoneal (i.p) ketamine (100 mg/Kg) and xylazine (10 mg/Kg). Hearts were excised into Tyrode's solution (in mM): 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.55 glucose, 5 HEPES (pH 7.4 with NaOH). SAN-containing tissue was excised into a modified Tyrode's solution containing (in mM): 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 0.2 $CaCl_2$, 50 taurine, 18.5 glucose, 5 HEPES, 0.1% BSA (pH 6.9 with NaOH) with elastase (0.3 mg/mL, Worthington Biochemical Corp., Lakewood, NJ) and collagenase II (0.21 mg/mL; Sigma Aldrich, St. Louis, MO). SAN tissue was digested at 37 °C for 30 min and then washed three times in a solution containing (in mM): 100 L-glutamic acid/potassium salt, 10 L-aspartic acid/potassium salt, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, 5 HEPES, 0.1% BSA (pH 7.2 with KOH). SAN tissue was then brought up to a volume of 500 μL of KB solution and triturated; 50 μL of the SAN cell containing solution was plated on each laminin-coated coverslips (25 μg/mL) and used within 8 h. In some experiments, SAN cells were treated with pertussis toxin (5 μg/mL; Tocris Bioscience, Bristol, UK) for 4-6 h prior to recordings.

***Electrophysiology*.** Whole-cell currents and voltages were measured with hardware (Axopatch-700B amplifier, Digidata 1440A) and software (pCLAMP v. 10.4) from Molecular Devices (Sunnyvale, CA). SAN cells were identified as thin, striated cells with capacitance values between 15–45 pF. Currents evoked by Ado (Sigma-Aldrich, St. Louis, MO), CCh (Sigma-Aldrich, St. Louis, MO), and ML297 (a generous gift from Dr. C. David Weaver and Dr. Corey Hopkins) were measured at a holding potential of −70 mV. Borosilicate patch pipettes were filled with (in mM): 140 K-gluconate, 2 $MgCl_2$, 1.1 EGTA, 5 HEPES, 2 Na_2ATP, 5 phosphocreatine, and 0.3 Na-GTP (pH 7.2 with KOH). Whole-cell access was achieved in a low-K^+ bath solution consisting of (in mM): 130 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose, 5 HEPES/NaOH (pH 7.4). A high-K^+ bath solution (+/−CCh, Ado, or ML297) consisting of (in mM): 120 NaCl, 25 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose, 5 HEPES/NaOH (pH 7.4) was applied via ValveLink 8.2 rapid perfusion system (AutoMate Scientific, Berkeley, CA). Current reversal with TTQ (Alomone Labs, Jerusalem, Israel) was measured in the presence of CCh or Ado. Activation and deactivation time constants were extracted from appropriate regions of stable current traces and were fit with a 1-term Boltzmann equation:

$$f(x) = \sum_{i=1}^{n} \frac{A_i}{1 + e^{-x/\tau_i}} + C$$

Only those experiments in which the access resistances were stable and low (<20 MΩ) were included in the final analysis.

***Quantitative RT-PCR*.** The following amplification program was used: 95 °C/20 s followed by 40 cycles of 95 °C/3 s, 60 °C/30 s. Intron-spanning primer pairs were as follows:

A_1R:	5'-CAGAGCTCCATCCTGGCTCT-3' (forward)
	5'-CGCTGAGTCACCACTGTCTTG-3' (reverse)
M_2R:	5′-GCCAGACTCCACCAGAT-3′ (forward)
	5′-CCATTGTTCGAGGAGTTAGTT-3′ (reverse)
GIRK1:	5′-AAACTCACTCTCATGTTCCG-3′ (forward)
	5′-TCCAGTTCAAGTTGGTCAAG-3′ (reverse)
GIRK4:	5′-GAGTTCGAAGTTGTGGTCATA-3′ (forward)
	5′-GCACCTCTGTATCCATGTAAG-3′ (reverse)
RGS6:	5′-CTGACATTGTACAGTGGCTTAT-3′ (forward)
	5′-GAGAACATGGTCTGAGATTGG-3′ (reverse)

The specificity of all reactions was assessed with a melting curve at the end of the program. Samples were tested in triplicate, and the average of replicates was used in the final data analysis. GAPDH (Mm_Gapd_2_SG QuantiTect primers; Qiagen) was used as an internal control in each sample.

cDNA constructs. A_1 adenosine receptor (AY136746), M_2 muscarinic acetylcholine receptor (AF498916), $G\alpha_{oB}$ (AH002708), $G\alpha_z$ (J03260), $G\alpha_{11}$ (AF493900), $G\alpha_{14}$ (NM_004297), $G\alpha_{15}$ (AF493904), $G\alpha_s$ long isoform ($G\alpha$sL) (NM_000516), $G\alpha_{olf}$ (AF493893), $G\alpha_{12}$ (NM_007353), $G\alpha_{13}$ (NM_006572), RGS6 (NM_004296), and $G\beta$5S (NM_006578) in pcDNA3.1(+) were purchased from cDNA Resource Center (www.cDNA.org). Flag-tagged dopamine D2 receptors (NM_000795) containing the hemagglutinin signal sequence (KTIIALSYIFCLVFA) at the N-terminus was a gift from Dr. Abraham Kovoor. The pCMV5 plasmids encoding rat $G\alpha_{oA}$, rat $G\alpha_{i1}$, rat $G\alpha_{i2}$, rat $G\alpha_{i3}$, human $G\alpha_q$, and bovine $G\alpha_s$ short isoform ($G\alpha$sS) were gifts from Dr. Hiroshi Itoh. Venus 156-239-$G\beta$1 (amino acids 156-239 of Venus fused to a GGSGGG linker at the N terminus of $G\beta$1 without the first methionine (NM_002074)) and Venus 1-155-$G\gamma$2 (amino acids 1-155 of Venus fused to a GGSGGG linker at the N terminus of $G\gamma$2 (NM_053064)) were gifts from Dr. Nevin A. Lambert (6). Flag-tagged Ric-8A (NM_053194) in pcDNA3.1 was a gift from Dr. Jean-Pierre Montmayeur (7). Flag-tagged Ric-8B (NM_183172 with one missense mutation (A1586G)) in pcDNA3.1 was a gift from Dr. Bettina Malnic (8). The masGRK3ct-Nluc-HA constructs were constructed by introducing HA tag at the C-terminus of masGRK3ct-Nluc reported previously (9). PTX-S1 constructs were reported previously (10). GenBank accession numbers for each sequence are given in parentheses.

Fast kinetic BRET assay. The GPCR (M_2R (6), A_1R (1), and dopamine D2 receptor (D_2R) (1)), $G\alpha$ ($G\alpha_{oA}$ (2), $G\alpha_{oB}$ (1), $G\alpha_{i1}$ (1), $G\alpha_{i2}$ (2), $G\alpha_{i3}$ (1.5), $G\alpha_z$ (1.5), $G\alpha$q (2), $G\alpha_{11}$ (2), $G\alpha_{14}$ (4), $G\alpha_{15}$ (2), $G\alpha_s$ short (6), $G\alpha_s$ long (4), $G\alpha_{olf}$ (6), $G\alpha_{12}$ (3), or $G\alpha_{13}$ (4)), Venus 156-239-$G\beta$1 (1), Venus 1-155-$G\gamma$2 (1), and BRET donor (masGRK3ct-Nluc-HA (1)) were transfected with RGS6 (1) and $G\beta$5s (1) constructs; the number in parentheses indicates the ratio of transfected DNA (ratio 1 = 0.21 μg). $G\alpha_{14/15}$ and $G\alpha_{olf}$ were transfected with Ric-8A (1) and Ric-8B (1), respectively. A construct carrying catalytic subunit of pertussis toxin (PTX-S1) were transfected with $G\alpha_z$, $G\alpha_s$ subfamily, $G\alpha_q$ subfamily, and $G\alpha_{12/13}$ subfamily to inhibit the possible coupling of endogenous $G_{i/o}$ to GPCRs. An empty vector (pcDNA3.1(+)) was used to normalize the amount of transfected DNA. After transfection (16-24 h), HEK293T/17 cells were washed once with BRET buffer (Dulbecco's Phosphate-Buffered Saline containing 0.5 mM $MgCl_2$ and 0.1% glucose) and detached by gentle pipetting over the monolayer. Cells were harvested with centrifugation at 500xg for 5 min and resuspended in BRET buffer. Approximately 50,000 to 100,000 cells per well were distributed in 96-well flat-bottomed white microplates (Greiner Bio-One, Kremsmünster, Austria). The Nluc substrate furimazine was purchased from Promega (Madison, WI) and used according to manufacturer specifications. BRET measurements were made using a microplate reader (POLARstar Omega; BMG Labtech, Offenburg, Germany) equipped with two emission photomultiplier tubes, allowing for detection of two emissions simultaneously with a highest possible resolution of 20 ms per data point. All measurements were performed at room temperature. The BRET signal was determined by calculating the ratio of the light emitted by the Venus-$G\beta1\gamma2$ (535 nm with a 30 nm band path width) over the light emitted by the masGRK3ct-Nluc-HA (475 nm with a 30 nm band path width). The average baseline value (basal BRET ratio) recorded prior to agonist stimulation was subtracted from the experimental BRET signal values and obtained ΔBRET ratio. The time constants (tau) of the activation and deactivation phases were obtained by fitting a single exponential curve to the traces with Clampfit 10.3. k_{GAP} rate constants were determined by subtracting the basal deactivation rate from the deactivation rate measured in the presence of exogenous RGS protein. Obtained k_{GAP} rate constants were used to quantify GAP activity.

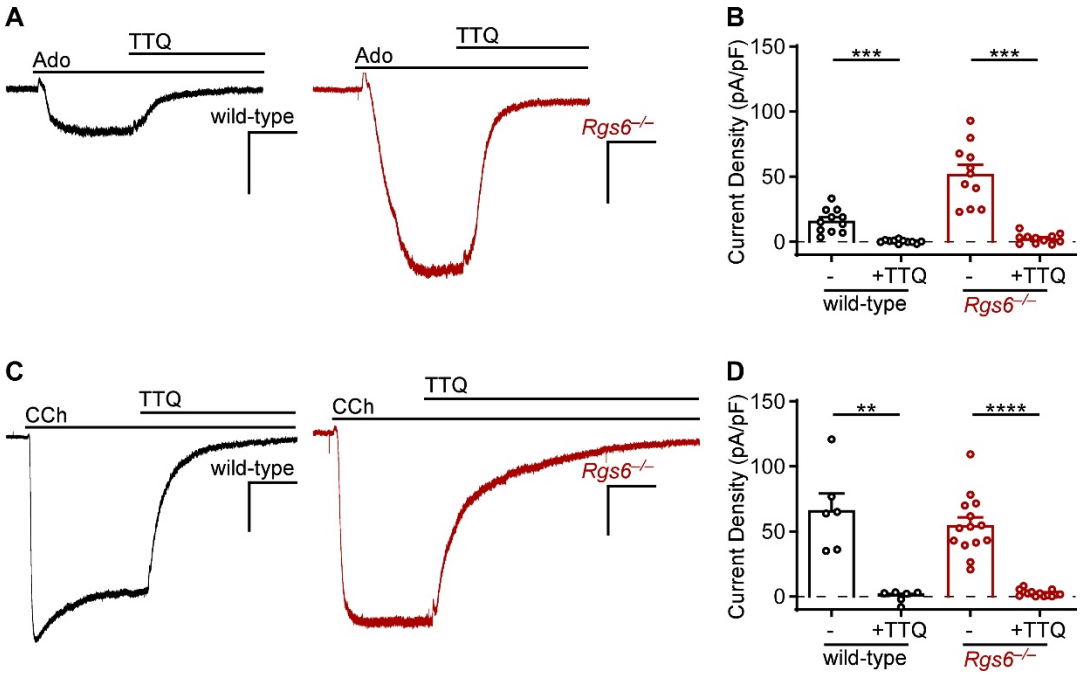

Fig S1. GIRK-dependence of Ado- and CCh-induced currents. (A) The GIRK channel blocker rTertiapin-Q (TTQ, 300 nM) reversed whole-cell currents evoked by Ado (10 μM) in SAN cells from wild-type and *Rgs6*$^{-/-}$ mice. **(B)** Peak current density of Ado-induced currents (-/+ TTQ) in SAN cells from wild-type (n=11 cells/3 mice) and *Rgs6*$^{-/-}$ (n=11 cells/3 mice) mice. There was a significant difference in the peak current density of Ado-induced currents before and after TTQ perfusion in SAN cells isolated from wild-type (t_{10}=5.6, ***p< 0.001; paired t-test) and *Rgs6*$^{-/-}$ (t_{10}=8.2, ****p<0.0001; paired t-test) mice. **(C)** TTQ (300 nM) reversed whole-cell currents evoked by CCh (10 μM) in SAN cells from wild-type and *Rgs6*$^{-/-}$ mice. **(D)** Peak current density of CCh-induced currents (-/+ TTQ) in SAN cells from wild-type (n=6 cells/3 mice) and *Rgs6*$^{-/-}$ (n=14 cells/4 mice) mice. There was a significant difference in the peak current density of CCh-induced currents before and after TTQ perfusion in SAN cells from wild-type (t_5=5.3, **p<0.01; paired t-test) and *Rgs6*$^{-/-}$ (t_{13}=8.7, ****p<0.0001; paired t-test) mice.

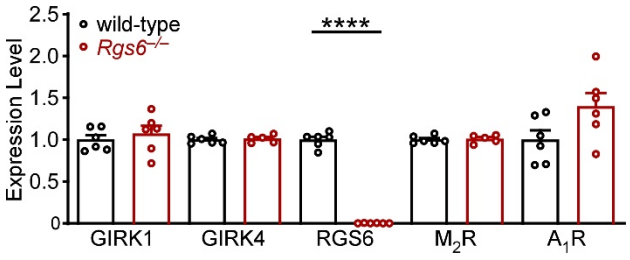

Fig. S2. Impact of *Rgs6* ablation on the expression of GPCR-GIRK signaling elements. mRNA levels of GIRK1 (t_{10}=0.7, p=0.52), GIRK4 (t_9=0.4, p=0.72), RGS6 (t_{10}=27.4, ****p<0.0001), M_2R (t_9=0.3, p=0.76), and A_1R (t_{10}=2.0, p=0.07) in atrial/SAN tissue samples from wild-type (black) and *Rgs6*$^{-/-}$ (red) mice, compared using unpaired student's *t*-tests. mRNA levels of each target were normalized to the level of GAPDH mRNA measured in each sample, and the mRNA levels for each target in *Rgs6*$^{-/-}$ samples were normalized to the level present in wild-type samples (n=5–6 samples per tissue and target).

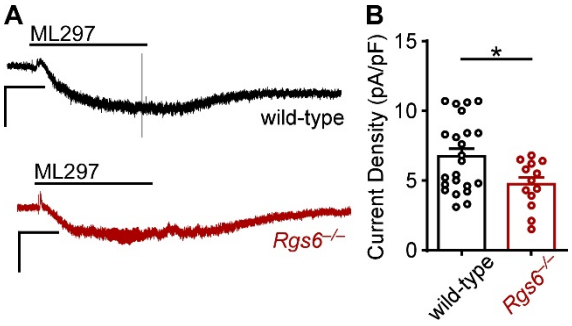

Fig. S3. ML297-induced currents in wild-type and *Rgs6*$^{-/-}$ SAN cells. (A) Representative whole-cell currents evoked by ML297 (10 μM) in SAN cells from wild-type (top) and *Rgs6*$^{-/-}$ (bottom) mice. Scale bars: 5 s/200 pA. **(B)** There was a modest but significant decrease in the peak ML297-induced current density in SAN cells from *Rgs6*$^{-/-}$ (n=13 cells/4 mice) as comparison to wild-type (n=23 cells/4 mice) mice (t_{34}=2.5, **p*<0.05; unpaired *t*-test).

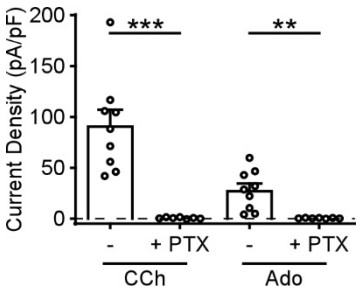

Fig. S4. Impact of pertussis toxin (PTX) on CCh- and Ado-induced currents. Summary of CCh- and Ado-induced current densities from control (-; n=9 cells/4 mice) or PTX-treated (+ PTX; n=7 cells/3 mice) wild-type SAN cells. PTX treatment (5 μg/mL; 4-6 h) abolished CCh-induced (t_{14}=5.1, ***p<0.001; unpaired t-test) and Ado-induced (t_{14}=3.7, **p<0.01; unpaired t-test) currents.

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