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## Lipopolysaccharide-induced sepsis impairs M2R-GIRK signaling in the mouse sinoatrial node

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1

2 **Title:** Lipopolysaccharide-induced sepsis impairs M2R-GIRK  
3 signaling in mouse sinoatrial node

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## 20 **Author contributions**

21 S Scheruebel, K Zorn-Pauly, and B Pelzmann conceptualized and supervised the project. N  
22 Shrestha, P Mesirca, CN Koyani, G Wölkart, V Di Biase, E Torre, P Lang, A Gorischek, ME  
23 Mangoni, B Pelzmann, and S Scheruebel designed and performed experiments. N Shrestha and  
24 K Zorn-Pauly performed data analysis and statistics. N Shrestha wrote the original manuscript  
25 draft and all authors contributed to data interpretation and manuscript review and editing.

## 26 **Competing Interest Statement**

27 None declared.

## 28 **Classification**

29 **Major:** Biological Sciences

30 **Minor:** Physiology

## 31 **Keywords**

32 Carbachol, Ca<sup>2+</sup> transient, Heart rate variability,  $I_{KACH}$ , Langendorff's heart

33 **This PDF file includes:**

34 Main Text  
35 Figures 1 to 5

36 **Abstract**

37 Sepsis has emerged as a global health burden associated with multiple organ dysfunction and  
38 20% mortality rate in patients. Numerous clinical studies over the past two decades have  
39 correlated the disease severity and mortality in septic patients with impaired heart rate variability  
40 (HRV), as a consequence of impaired chronotropic response of sinoatrial node (SAN) pacemaker  
41 activity to vagal/parasympathetic stimulation. However, the molecular mechanism(s) downstream  
42 to parasympathetic inputs have not been investigated yet in sepsis, particularly in the SAN.  
43 Based on electrocardiography, fluorescence  $Ca^{2+}$  imaging, electrophysiology, and protein assays  
44 from organ to sub-cellular level, we report that impaired M2R-GIRK signaling in a  
45 lipopolysaccharide-induced proxy septic mouse model plays a critical role in SAN pacemaking  
46 and HRV. The parasympathetic responses to a muscarinic agonist, namely  $I_{KACH}$  activation in  
47 SAN cells, reduction in  $Ca^{2+}$  mobilization of SAN tissues, lowering of heart rate and increase in  
48 HRV, were profoundly attenuated upon lipopolysaccharide-induced sepsis. These functional  
49 alterations manifested as a direct consequence of reduced expression of key ion-channel  
50 components (GIRK1, GIRK4, and M2R) in the mouse SAN tissues and cells, which was further  
51 evident in the human right atrial appendages of septic patients and likely not mediated by the  
52 common pro-inflammatory cytokines elevated in sepsis.

53 **Significance statement**

54 Altered heart rate variability (HRV), arising from impaired chronotropic response of sinoatrial node  
55 (SAN) pacemaker activity to vagal input, has been proposed as an early diagnostic marker in  
56 sepsis. However, the underlying pathophysiological changes in the SAN have not been  
57 investigated yet. We discovered the downstream M2R-GIRK signaling in a lipopolysaccharide-  
58 induced proxy septic model is impaired, consequent to reduced expression of GIRK isoforms,  
59 leading to compromised chronotropic regulation of SAN pacemaking and HRV by  $I_{KACH}$ .  
60 Intriguingly, downregulation of GIRK channels was not directly mediated by the common pro-  
61 inflammatory cytokines elevated in sepsis. Our findings provide insights into parasympathetic  
62 regulation of SAN and clinical monitoring of altered HRV in sepsis, which potentially help to  
63 reduce risk of death in patients.

64 **Introduction**

65 Sepsis refers to a life-threatening, dysregulated host response to infection, manifesting into  
66 multiple organ dysfunction and ultimately death, unless diagnosed and treated promptly (1).  
67 Sepsis is a major health problem that accounted for 11 million deaths out of 49 million reported  
68 cases worldwide in 2017 and nearly 40% of fatal outcome were related to cardiovascular

69 complications (2). The precise etiology and mechanism(s) underlying cardiac dysfunction in  
70 sepsis are complex and not entirely understood, which hinders timely diagnosis and therapeutic  
71 intervention in patients. Various mediators such as pro-inflammatory cytokines, nitric oxide, and  
72 reactive oxygen species have been linked to cardiac dysfunction (3, 4).

73 A number of clinical studies over the past two decades have correlated the disease  
74 severity and mortality in septic patients with altered heart rate variability (HRV), as a  
75 consequence of dampened cardiac response to vagal/parasympathetic stimulation (5-8). HRV  
76 refers to beat-to-beat variability (BBV) between successive RR intervals on an electrocardiogram  
77 (ECG). This variability predominantly arises from adaptive autonomic nervous system  
78 modulations of a specialized group of cardiac cells in sinoatrial node (SAN) that constitute the  
79 natural pacemaker of heart. Besides, a certain degree of BBV is intrinsic to the SAN cells due to  
80 fluctuations in the spontaneous pacemaking machinery (9, 10). Previous animal studies on  
81 isolated SAN cells and tissues showed that spontaneous diastolic depolarization and action  
82 potential firing in SAN cells is generated by a functional interplay between two tightly linked  
83 intracellular clocks, namely the membrane clock and the calcium clock. The membrane clock  
84 involves periodic opening of transmembrane ion channels: hyperpolarization-activated cyclic  
85 nucleotide-gated (HCN) channels encoding the funny current ( $I_f$ ), voltage-gated  $Ca^{2+}$  channels (L-  
86 type  $Ca_v1.3$  and T-type  $Ca_v3.1$ ),  $Ca^{2+}$ -activated  $K^+$  channels, and transient receptor potential  
87 channels (11). The calcium clock involves local  $Ca^{2+}$  release through ryanodine receptors from  
88 the sarcoplasmic reticulum (SR), which can be either spontaneous (12) or activated by  $Ca_v1.3$   
89 channels (13, 14). Local  $Ca^{2+}$  release is coupled to diastolic depolarization via activation of the  
90 cardiac  $Na^+/Ca^{2+}$  exchanger (NCX1) (10, 15-17). Sympathetic stimulation of  $\beta$ -adrenergic  
91 receptor on isolated SAN cells promotes action potential firing rate (18, 19) and reduces BBV (9)  
92 via coupled G-protein alpha stimulatory subunit ( $G\alpha_s$ )-mediated cyclic adenosine  
93 monophosphate-protein kinase A (cAMP-PKA) pathway, which is counterbalanced by the  
94 parasympathetic stimulation of muscarinic receptor subtype 2 (M2R)-inhibitory subunit  $G\alpha_i$   
95 leading to lower action potential firing rate and higher BBV (9, 10, 19-21). Moreover, upon M2R  
96 stimulation by agonists in isolated SAN cells, the coupled G-protein beta-gamma ( $G\beta\gamma$ ) subunit  
97 directly activates acetylcholine-activated inwardly-rectifying potassium current ( $I_{KACH}$ ). The cardiac  
98  $I_{KACH}$  is conducted by G protein-activated inwardly-rectifying potassium (GIRK) channels,  
99 consisting of GIRK1 and GIRK4 heteromers, which results in  $K^+$  efflux and membrane  
100 hyperpolarization with consequent reduction in action potential firing rate (19, 20, 22, 23).  $I_{KACH}$   
101 plays a major role in the generation of HRV in response to vagal stimulation and in recovering  
102 basal heart rate after sympathetic activation (22, 23).

103 Various animal models of sepsis point out the impact of the parasympathetic nervous  
104 system on cardiac dysfunction. In a lipopolysaccharide (LPS)-induced endotoxemic rat model, the  
105 observed narrowed BBV of the isolated SAN-atria region was linked to blunted or uncoupled

106 cardiac response to cholinergic stimulation (24). Other studies reported that electrical stimulation  
107 of the vagus nerve attenuated the myocardial inflammation and hemodynamic impairment in LPS-  
108 induced endotoxemia in vagotomized rats (25, 26). Another rat model of sepsis obtained by cecal  
109 ligation and puncture demonstrated altered biphasic redistribution and phosphorylation of M2R  
110 receptor in the isolated heart (27). However, the molecular mechanism(s) downstream to  
111 vagal/parasympathetic stimulation particularly in the SAN tissue have not been explored in detail  
112 in sepsis. Given the fact that GIRK1/4 channels are mainly expressed in SAN and atrial tissues,  
113 and are involved in determination of HRV (22, 23), we hypothesized that impaired vagal activity  
114 during sepsis might be related to pathological changes in the M2R-GIRK signaling in SAN tissue.  
115 Thus, the present study was designed to elucidate whether the responsiveness of SAN cells,  
116 tissue, and heart to parasympathetic stimulation is compromised under septic conditions due to  
117 modifications in the M2R-GIRK signaling, thereby potentially impairing HRV.

## 118 **Results**

### 119 **LPS attenuates parasympathetic modulation of heart rate and its variability**

120 We first induced sepsis in mice via intra-peritoneal (i.p.) injection of LPS (5 mg/kg), an endotoxin  
121 from gram-negative bacteria, and after 6 h, recorded ECGs from the isolated hearts under  
122 Langendorff's perfusion in relation to parasympathetic stimulation. We compared the beating rate  
123 and HRV measurements (SDNN, RMSSD, and pNN6, defined in the method section) calculated  
124 from ECGs on the hearts isolated from septic versus phosphate-buffered saline (PBS)-injected  
125 control mice. As illustrated in Fig. 1A, the beating rate of isolated hearts was unaffected by LPS  
126 injection in mice ( $414.22 \pm 10.23$  /min) compared to controls ( $399.58 \pm 13.11$  /min). Perfusion for  
127 10 min with  $0.1 \mu\text{M}$  carbachol (CCh), a muscarinic receptor agonist, significantly reduced the  
128 heart rate in controls by 39% ( $243.81 \pm 12.06$  /min). In contrast, CCh could marginally, albeit non-  
129 significant, reduce the beating rate in septic hearts ( $383.73 \pm 14.08$  /min, 7.4% reduction). LPS  
130 altered neither the SDNN (Fig. 1B,  $0.81 \pm 0.22$  ms vs.  $0.83 \pm 0.12$  ms) nor RMSSD (Fig. 1C,  $0.69$   
131  $\pm 0.21$  ms vs.  $0.46 \pm 0.09$  ms) compared to controls. However, upon CCh perfusion, hearts from  
132 control mice exhibited significantly higher SDNN (Fig. 1B,  $2.88 \pm 0.16$  ms, 3.5 fold) and RMSSD  
133 (Fig. 1C,  $2.46 \pm 0.39$  ms, 5.4 fold). SDNN of hearts from LPS-injected mice also increased  
134 significantly following CCh perfusion (Fig. 1B,  $1.85 \pm 0.31$  ms, 2.3 fold) but to a much lesser  
135 extent than in controls, as evident from the significant interaction effect in the mixed two-way  
136 ANOVA ( $p=0.032$ ). Meanwhile, the increase in RMSSD of hearts from LPS-injected mice did not  
137 reach significance (Fig. 1C,  $1.19 \pm 0.35$  ms, 1.7 fold) following CCh perfusion. SDNN and  
138 RMSSD data together reflect that LPS attenuated the increase in total autonomic variability as  
139 well as short-term variation between successive heart beats in response to CCh. Further,  
140 baseline pNN6 data were zero in most of the control and LPS-injected mice. So, the difference or  
141 increase in pNN6 data between baseline and CCh response were compared in Fig. 1D. Clearly,

142 the percentage increase in pNN6 following CCh perfusion was significantly higher in the hearts  
143 from control mice than those from LPS-injected mice (3.15% vs. 0.3%), which further reflects a  
144 lower response of heart rate to parasympathetic stimulation.

#### 145 **LPS dampens CCh-mediated negative chronotropic effect on $Ca^{2+}$ dynamics in SAN tissue**

146 The heartbeat is generated by SAN pacemaker mechanism, which depends on the activity of the  
147 membrane and the calcium clocks. Cholinergic stimulation is known to modulate  $Ca^{2+}$   
148 homeostasis during SAN pacemaking and LPS impaired CCh effect on HR and HRV in isolated  
149 hearts (Fig. 1). So, we examined the effect of CCh on  $Ca^{2+}$  dynamics in SAN tissues isolated  
150 from control and LPS-administered mice. Fig. 2A illustrates pacemaker activity reflected by  
151 automatic intracellular  $Ca^{2+}$ -transients, expressed as change in fluorescence signal of Cal-520-  
152 indicator over time in the SAN tissue of control vs. LPS-injected mouse in response to CCh. CCh  
153 perfusion for 10 min significantly reduced the rate of spontaneous  $Ca^{2+}$ -transients in control SAN  
154 tissues by 25% (Fig. 2B,  $284.76 \pm 27.21$  /min to  $213.51 \pm 32.19$  /min), which correlates well to the  
155 reduced beating rate of isolated hearts as observed in Fig. 1A. Meanwhile, the rate of  $Ca^{2+}$ -  
156 transients following CCh perfusion was also significantly reduced in SAN tissues from LPS-  
157 injected mice by 9.4% (Fig. 2B,  $328.99 \pm 32.29$  /min to  $298.17 \pm 34.26$  /min). However, the  
158 magnitude of reduction in LPS-treated SAN tissues was significantly lower than in control tissues,  
159 evident from the significant interaction effect in the mixed two-way ANOVA ( $p=0.015$ ). CCh  
160 reduced the peak amplitude by 23.6% (Fig. 2C) and prolonged the relaxation time to 90% from  
161 peak ( $RT_{90}$ ) by 15.5% (Fig. 2D) in the control SAN tissues whereas these parameters were  
162 unaffected in the SAN tissues of LPS-injected mice. Under basal conditions, there was no  
163 difference in these parameters between SAN tissues of LPS-injected mice and control mice (Fig.  
164 2B-D), supporting the results obtained from isolated hearts (Fig. 1).

#### 165 **LPS inhibits CCh-mediated increase in steady-state current density and $I_{KACH}$ in isolated** 166 **SAN cells**

167 Since LPS blunted CCh-induced reduction in heart rate and frequency of spontaneous  $Ca^{2+}$   
168 transients (Fig. 1 and 2), we investigated the impact of CCh on membrane current in SAN cells  
169 isolated from control and LPS-injected mice. In whole-cell patch-clamp recordings, a ramp  
170 protocol from -100 mV to +40 mV was applied over 1.5 s to elicit quasi steady-state current  
171 before and after 0.1  $\mu$ M CCh perfusion (Fig. 3A and B). The major differences between control  
172 and LPS groups in response to CCh were noticeable at negative and positive voltages (Fig. 3C  
173 and S1A-E). Upon CCh perfusion, control SAN cells showed significant increase in the inward  
174 current density at -100 mV (Fig. 3C), -90 and -80 mV (Fig. S1A and B) as well as the outward  
175 current density at +20, +30, and +40 mV (Fig. S1C-E). In contrast, CCh perfusion failed to alter  
176 the current density in inward and outward direction in SAN cells from LPS-injected mice, except  
177 for reduction at -80 mV. In Fig. S1F, plotting the difference between CCh and basal current ( $I_{CCh}$ -

178  $I_{\text{basal}}$ , referred as CCh-induced) revealed that  $I_{\text{KACH}}$  mediated CCh effect at both negative and  
179 positive voltages, and showed inward rectifying properties in control SAN cells. In contrast, LPS  
180 suppressed the CCh-induced inward current at negative voltages and abrogated most of the  
181 difference current at positive voltages. The inward current was further confirmed to be mediated  
182 by  $I_{\text{KACH}}$  in a separate set of experiments where a GIRK channel inhibitor tertiapin-Q (20, 22) at  
183 300 nM significantly attenuated CCh-mediated increase in current density at -100 mV (Fig. 3D).  
184 We next evaluated  $I_{\text{KACH}}$  at -35 mV holding potential, as reported previously (22). Fig. 3E  
185 illustrates representative current recordings, demonstrating prominent activation of  $I_{\text{KACH}}$  within  
186 few seconds upon CCh perfusion in a control cell but negligible activation in a cell from LPS-  
187 injected mouse. Averaged data plotted in Fig. 3F show that CCh significantly increased  $I_{\text{KACH}}$  in  
188 control SAN cells ( $1.24 \pm 0.23$  pA/pF) compared to cells from LPS-injected mice ( $0.35 \pm 0.11$   
189 pA/pF). We further investigated if LPS diminished  $I_{\text{KACH}}$  activation independent of G $\beta\gamma$ -coupled  
190 M2R stimulation by CCh. To do so, we used ML297 and 3hi2one-G4, direct activators of  
191 heteromeric GIRK1/4 and homomeric GIRK4 channels respectively (28, 29), for measuring  $I_{\text{KACH}}$   
192 at -100 mV in ramp recordings. 3  $\mu\text{M}$  ML297 significantly increased the inward current density at -  
193 100 mV in the control SAN cells but not in the cells from LPS-injected mice (Fig. S1G),  
194 suggesting disruption of GIRK1/4 channel function and/or expression. In contrast, 30  $\mu\text{M}$   
195 3hi2one-G4 failed to elicit  $I_{\text{KACH}}$  at -100 mV in the cells from both control and LPS-injected mice  
196 (Fig. S1H), suggesting that in SAN cells, GIRK4 forms functional heterotetramers with GIRK1 but  
197 not GIRK4 homotetramers.

### 198 **LPS reduces expression of M2R and GIRK channels involved in parasympathetic** 199 **regulation of heart rate**

200 GIRK1 and GIRK4 channels are integral components of membrane delimited M2R-induced  
201 activation of  $I_{\text{KACH}}$  (19, 20, 22). So, we investigated whether LPS administration could alter  
202 expression of M2R and/or GIRK channel isoforms accounting for loss of  $I_{\text{KACH}}$  and reduced  
203 response to CCh in SAN pacemaker cells, intact SAN tissues and whole hearts isolated from  
204 LPS-injected mice in the functional assays described above. Western blots of SAN tissues from  
205 LPS-injected mice indeed showed significantly lower expression of GIRK1 ( $0.33 \pm 0.05$  fold),  
206 GIRK4 ( $0.51 \pm 0.09$  fold), and M2R ( $0.49 \pm 0.09$  fold) compared to the SAN tissues from control  
207 mice (Fig. 4A). Next, we investigated if LPS directly impacts the expression of these receptors  
208 and ion channels in intact SAN tissues *in situ* in addition to the above *in vivo* effects. *In vitro*  
209 incubation of the isolated SAN tissues with LPS for 6 h also significantly reduced the expression  
210 of GIRK1 ( $0.65 \pm 0.04$  fold), GIRK4 ( $0.74 \pm 0.05$  fold), and M2R ( $0.69 \pm 0.04$  fold) compared to  
211 the control SAN tissues (Fig. 4B), however, the effect was less pronounced than in the SAN  
212 tissues from LPS-injected mice. Next, we evaluated the expression of M2R and GIRK1/4  
213 channels in the human right atrial appendages (RAA) from septic patients and similarly observed  
214 reduced expression of GIRK1 (0.43 fold), GIRK4 (0.45 fold), and M2R (0.55 fold) compared to the

215 control human tissues (Fig. S2), which corroborates the findings in the SAN tissues from mice  
216 following *in vivo* or *in vitro* LPS treatment.

217 To determine whether LPS-induced reduction of GIRK1/4 expression in the SAN tissues  
218 is reflected at the membrane levels, we performed fluorescence immunolabeling of SAN cells  
219 isolated from control and LPS-injected mice. We also assessed the membrane expression of  
220 other ion channels involved in SAN pacemaking, such as  $Ca_v1.3$  channels (14) and  $I_f$ -conducting  
221 HCN4 (22). Fig. 4C shows the immunolabeled GIRK1, GIRK4,  $Ca_v1.3$ , and HCN4 homogeneously  
222 localized in discrete clusters at the membrane in both conditions, suggesting that LPS does not  
223 alter the overall channel distribution. However, quantitative analysis of fluorescence intensity (Fig.  
224 4D) revealed significant reduction of GIRK1 ( $0.57 \pm 0.04$  fold) and GIRK4 ( $0.86 \pm 0.05$  fold)  
225 membrane levels in SAN cells from LPS-injected mice compared to controls, indicating that LPS  
226 diminishes the amount of functional GIRK channels at the surface. The membrane amount of  
227  $Ca_v1.3$  was also significantly decreased ( $0.55 \pm 0.06$  fold) whereas HCN4 showed strong  
228 variability with no significant change. Low magnification images of the SAN cells from which the  
229 enlarged regions in Fig. 4C were isolated are provided in Fig. S3 and display their typical thin and  
230 elongated morphology. Fig. S4 clarifies the membrane localization of the analyzed channels as a  
231 rim surrounding the intracellular region. Although some intracellular labeling consistent with  
232 organelle localization was observed (arrowheads), this was segregated in restricted regions and  
233 clearly distinguishable from the cell membrane rim (arrows). This differs from the homogeneously  
234 distributed clusters represented in Fig. 4C, consistent with membrane ion channels segregated in  
235 functional domains.

236 Since the intermediate G-protein subunits, namely  $G\alpha_i$  and  $G\beta\gamma$ , mediate cAMP-PKA  
237 pathway inhibition and  $I_{KACH}$  activation respectively upon M2R stimulation by CCh (20), we  
238 checked if LPS affects the expression of  $G\alpha_{i2}$  and  $G\beta$  too. However, we did not observe any  
239 change in expression from western blots of SAN tissues from LPS-injected mice compared to  
240 those from control mice (Fig. S5). We further checked whether the reduced expression in SAN is  
241 mediated by common pro-inflammatory cytokines like tumor necrotic factor-alpha (TNF- $\alpha$ ),  
242 interleukin-1 beta (IL-1 $\beta$ ) or interleukin-6 (IL-6). Elevated level of these cytokines has been  
243 previously shown to promote systemic inflammation (25, 26) and is associated with reduced HRV  
244 in septic rodents (30). We incubated SAN tissues with TNF- $\alpha$  (50 ng/mL), IL-1 $\beta$  (10 ng/mL) or IL-6  
245 (10 ng/mL) for 6 h. These cytokines did not affect expression of M2R and GIRK1/4 channels  
246 compared to the control tissues (Fig. S6A and B), suggesting that cytokine elevation in sepsis  
247 may not directly affect expression of channels involved in  $I_{KACH}$  in the SAN tissues. In addition, we  
248 investigated if  $I_{KACH}$  activation by CCh in SAN cells was affected upon incubation with 10 ng/ml IL-  
249 6 for 2 h. IL-6-incubated cells showed similar increase in CCh-activated  $I_{KACH}$  density at -100 mV  
250 compared to control cells (Fig. S6C), indicating no direct effect of cytokines like IL-6 on GIRK  
251 channel activation in addition to expression.



252 **Discussion**

253 To the best of our knowledge, our study is the first to present evidence of impaired M2R-GIRK  
254 signaling pathway in the SAN tissues of LPS-injected septic mice. As summarized in Fig. 5, LPS-  
255 reduced expression of key ion-channels and receptors (GIRK1, GIRK4, and M2R). This  
256 diminished expression led to diminished parasympathetic regulation of pacemaker activity at  
257 multiple levels namely, (i)  $I_{KACH}$  activation in SAN cells, (ii) reduction in the frequency of  
258 spontaneous  $Ca^{2+}$  transients of SAN tissues, and (iii) lowering of heart rate and increase in HRV  
259 parameters.

260 Our findings are in line with previous studies showing that genetic ablation of GIRK4  
261 channels and hence lack of  $I_{KACH}$  in isolated SAN tissue and atrial myocytes attenuated the effect  
262 of vagal stimulation, namely lowering of heart rate and increase in HRV, in isolated hearts as well  
263 as conscious mice (22, 23). We further revealed substantial reduction in expression of GIRK1,  
264 GIRK4, and M2R in RAA of septic patients, which might in turn contribute to the diminished vagal  
265 response and impaired HRV observed in these patients (5, 6). However, LPS did not affect the  
266 expression of M2R-coupled  $G\beta$  subunit that directly activates  $I_{KACH}$  in mouse SAN tissues but  
267 attenuated  $G\beta\gamma$ -independent and direct GIRK1/4 channel activation by ML297 in mouse SAN  
268 cells, which indicates that LPS impairs GIRK1/4 channel function and expression independent of  
269 regulation of  $G\beta$  expression. Our group has previously reported impairment of  $I_f$  in human RAAs  
270 following LPS incubation without affecting HCN2/4 expression, which further compromises  
271 cardiac pacemaking in response to autonomic inputs (31, 32). Here, we did not observe change  
272 in membrane expression of HCN4 channels in the SAN cells from LPS-injected mice, in line with  
273 our previous report. In addition, our data show that SAN cells most likely conduct  $I_{KACH}$  via  
274 functional heterotetramers of GIRK1/4 but not GIRK4 homotetramers since GIRK4-specific  
275 activator, 3hi2one-G4, failed to elicit  $I_{KACH}$  in the SAN cells from both control and LPS-injected  
276 mice. Moreover, this evidence further supports LPS-induced reduction of  $I_{KACH}$  in SAN cells is due  
277 to concomitant down regulation of GIRK1 and GIRK4.

278 In previous reports, elevated common pro-inflammatory cytokines like TNF- $\alpha$ , IL-1 $\beta$ , and  
279 IL-6 have been associated with blunted anti-inflammatory vagal tone leading to cardiac  
280 inflammation and reduced HRV in LPS-induced sepsis in rodents (26, 30). Among the common  
281 cytokines, IL-6 was previously found to be highly elevated in plasma of LPS-administered mice  
282 within 1 h (30) and IL-6 incubation of atrial-SAN tissues isolated from control mice for 30 minutes  
283 reduced CCh response on beating rate by 4.7 fold (33). However, direct incubation of SAN  
284 tissues isolated from control mice with these common cytokines for 6 h in the present study did  
285 not affect the expression of M2R and GIRK channels. Moreover, isolated control SAN cells in  
286 direct contact with IL-6 for 2 h failed to alter CCh-activated  $I_{KACH}$ . These findings together suggest  
287 that the common cytokines interfere with the vagal response associated with reduced HRV in  
288 sepsis most likely via indirect systemic mechanism(s), rather than directly targeting M2R-GIRK1/4

289 function and expression. However, we cannot exclude that less-known cytokines may contribute  
290 to direct effect of LPS and further mechanistic studies are required to establish the effect of the  
291 other cytokines on HRV during sepsis.

292 Tachycardia and reduced HRV are mostly detected in septic patients (6-8) and reported  
293 in LPS-administered rats *in vivo* though the basal beating rate of SAN-atrial tissue isolated from  
294 these rats was unaffected (24). Besides, conscious GIRK4 knockout mice showed no change in  
295 basal heart rate but reduction in HRV in telemetry (23). Under our *ex vivo* experimental  
296 conditions, we did not observe significant change in basal beating rate and HRV of isolated  
297 hearts and in basal  $\text{Ca}^{2+}$  transient rate of isolated SAN tissues from LPS-injected mice compared  
298 to controls. The impact of LPS-induced endotoxemia on heart rate and HRV as well as GIRK4  
299 knockout on HRV in previous findings in the whole animal likely requires additional factors like  
300 elevated sympathetic inputs and/or eicosanoids (24, 34), which were absent in our experiments  
301 designed to investigate parasympathetic inputs on the isolated SAN cell/tissue and heart. The  
302 parasympathetic inhibition of chronotropic action on SAN tissues and heart isolated from LPS-  
303 injected mice was compromised, as evident from the diminished effect of a muscarinic agonist,  
304 CCh, on the rate of spontaneous  $\text{Ca}^{2+}$  transients in the SAN and heart rate. This functional  
305 impairment of negative chronotropic effect is likely a direct consequence of reduced expression of  
306 M2R and GIRK channels in SAN tissues. This outcome is consistent with previous studies that  
307 have documented the inhibitory role of M2R-GIRK pathway on action potential firing rate in  
308 isolated SAN cells and heart rate (20-23).

309 In addition to slowing action potential firing rate in isolated SAN cells,  $\text{G}\alpha_i$ -coupled M2R  
310 stimulation attenuates SR- $\text{Ca}^{2+}$  release and reuptake via inhibition of cAMP-PKA-mediated  
311 phosphorylation of downstream ion-channels and pumps (20). We observed these effects in SAN  
312 tissues isolated from control mice as reduced peak amplitude and longer  $\text{RT}_{90}$  of  $\text{Ca}^{2+}$ -transients  
313 following CCh administration. However, these effects were absent in the SAN tissues of LPS-  
314 injected mice with lower expression of M2R and  $\text{Ca}_v1.3$  but not  $\text{G}\alpha_{i2}$ , suggesting that cAMP-PKA  
315 downstream signaling could not be inhibited independent of  $\text{G}\alpha_{i2}$  expression. LPS-induced  
316 reduction in membrane expression of  $\text{Ca}_v1.3$  channels in the SAN cells is in line with previous  
317 findings of reduced expression of  $\text{Ca}_v1.3$  in the atrial tissues isolated from LPS-injected guinea  
318 pigs (35). This warrants investigation of LPS effect on cAMP-PKA cascade and L-type  $\text{Ca}_v1.3$   
319 currents in SAN cells/tissues in future studies.

320 In summary, our findings demonstrate a pivotal role of downregulation of M2R-GIRK  
321 signaling in SAN pacemaking and HRV in a murine sepsis model and likely contribution to the  
322 diminished vagal response and reduced HRV observed in septic patients. Furthermore, our data  
323 support the emerging practice to monitor HRV as an early prognostic marker for sepsis (5-8, 36).  
324 Within 6 h of LPS administration, expression of M2R and GIRK channel is reduced with  
325 consequences on vagal responsiveness and HRV. Hence, HRV monitoring at the onset of sepsis,

326 when clinical symptoms are subtle and can easily be overlooked, is a non-invasive and efficient  
327 tool for timely diagnosis and therapeutic intervention of this fatal disease in patients.

## 328 **Materials and Methods**

### 329 **Animals**

330 The experimental procedure and number of animals used were approved by the ethics committee  
331 of the Medical University of Graz and Federal Ministry of Science, Research, and Economy of the  
332 Republic of Austria (BMWFV-66.010/0101-WF/V/3b/2016), and the University of Montpellier and  
333 the French Ministry of Agriculture (protocol no: 2017010310594939). The experiments were  
334 conducted according to the Directive of the European Parliament and of the Council of  
335 September 22, 2010 (2010/63/EU). C57BL/6 wild-type mice (both sexes, 14-20 weeks old) were  
336 administered with LPS (5 mg/kg, i.p.) to elicit a septic response for 6 h as reported previously (30)  
337 and compared to PBS-injected controls.

### 338 **Human right atrial appendages (RAA)**

339 The use of human tissue was approved by the ethics committee of the Medical University of  
340 Graz, Austria (19–109 ex 07/08) and all patients gave prior informed consent according to the  
341 WMA Declaration of Helsinki, 2013. RAA were obtained from 2 septic and 2 non-septic (control)  
342 patients undergoing cardiac surgery.

### 343 **Reagents**

344 All reagents used were of molecular biology grade and purchased from Sigma-Aldrich or Carl  
345 Roth unless specified otherwise.

### 346 **Langendorff perfusion**

347 Mice were injected with heparin (1000 U/kg, i.p.) and anaesthetized with ketamine (80 mg/kg, i.p.)  
348 and xylazine (10 mg/kg, i.p.). Depth of anesthesia was monitored by toe pinch reflex and mice  
349 were sacrificed by cervical dislocation. Hearts were rapidly excised and arrested in ice-cold  
350 Krebs-Henseleit buffer. After cannulation of the aorta with a 20-gauge needle, retrograde  
351 perfusion was established at a constant flow of 20 ml/min/g wet weight with a modified Krebs-  
352 Henseleit bicarbonate buffer (in mM: 118 NaCl, 25 NaHCO<sub>3</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 4.8 KCl, 1.2 MgSO<sub>4</sub>,  
353 1.25 CaCl<sub>2</sub>, 11 glucose, pH 7.4) using the ISO-HEART perfusion system (Hugo Sachs Elektronik)  
354 as described previously with slight modifications (37). The perfusate was filtered through a 5 µm  
355 filter before reaching the heart and continuously gassed with carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>). Heart  
356 temperature was maintained at 37°C throughout the experiments, monitored with a physitemp  
357 probe (Physitemp Instruments). After removal of the left auricle, a tiny fluid-filled balloon made of  
358 a small square of polyethylene film was inserted into the left ventricle and connected to a  
359 pressure transducer via a 4F biluminal monitoring catheter (Vygon). An epicardial ECG was

360 recorded throughout the experimental period with the use of two stainless steel electrodes  
361 attached directly to the base of the right atrium and left ventricle, respectively. Hearts were  
362 perfused for 30 min to establish stable baseline conditions, followed by 15 min perfusion with 0.1  
363  $\mu\text{M}$  CCh.

364 RR intervals (1000 baseline and 1000 after 10 min of CCh perfusion) were extracted using HRV  
365 module of LabChart 7 Pro software (ADInstruments). Each RR interval was plotted as a function  
366 of time (tachogram) or as a function of the preceding RR (Poincaré plot) to select the normal RR  
367 intervals excluding artifact and ectopic beats, as reported previously (38). Heart rate and time-  
368 domain statistical measures of HRV (SDNN, RMSSD, and pNN6) were calculated from RR  
369 interval using MATLAB<sup>TM</sup>. SDNN, the standard deviation of all normal RR intervals (in ms),  
370 estimates the total HRV arising from autonomic nervous system. RMSSD denotes the square root  
371 of the mean of the sum of the squares of differences between adjacent normal RR intervals (in  
372 ms), quantifying short-term variation between two successive beats as a measure of  
373 parasympathetic regulation. pNN6 is the percentage of consecutive normal RR intervals differing  
374 more than 6 ms, and reflects the relatively abrupt and fast changes in HR, attributed to cardiac  
375 parasympathetic activity.

#### 376 **Isolation of sinoatrial node (SAN) cells**

377 SAN cells were isolated as described previously (22, 39). Briefly, mice were heparinized (250 U,  
378 i.p., Gilvasan Pharma), anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.),  
379 and sacrificed by cervical dislocation. The beating heart was quickly removed and transferred to  
380 the Tyrode's solution (37°C) containing (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5  
381 HEPES-NaOH, and 5.5 d-glucose (adjusted to pH 7.4 with NaOH). The SAN tissue was excised  
382 by cutting along the crista terminalis and the interatrial septum, and transferred into a low-Ca<sup>2+</sup>,  
383 low-Mg<sup>2+</sup> solution containing (in mM): 140 NaCl, 5.4 KCl, 0.5 MgCl<sub>2</sub>, 0.2 CaCl<sub>2</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 50  
384 taurine, 5.5 d-glucose, 5 HEPES, and 1 mg/ml bovine serum albumin (BSA) (adjusted to pH 6.9  
385 with NaOH). The tissue was enzymatically digested by adding 0.15 mg/ml Liberase TH (Roche)  
386 and 0.5 mg/ml elastase (Worthington Biochemical Corporation) for ~24 min at 37°C. The digested  
387 tissue was washed and transferred to a modified Kraftbrühe solution containing (in mM): 100 K<sup>+</sup>-  
388 L-glutamate, 10 K<sup>+</sup>-aspartate, 25 KCl, 10 KH<sub>2</sub>PO<sub>4</sub>, 2 MgSO<sub>4</sub>, 20 taurine, 5 creatine, 0.5 EGTA, 20  
389 glucose, 5 HEPES, and 1 mg/ml BSA (pH 7.2 with KOH). Single cells were manually dissociated  
390 in Kraftbrühe solution at 37°C using a Pasteur pipette. Physiological extracellular Na<sup>+</sup> and Ca<sup>2+</sup>  
391 concentrations were re-established by adding aliquots of solutions containing (in mM): 10 NaCl,  
392 1.8 CaCl<sub>2</sub>, and subsequently, Tyrode's solution containing 1 mg/ml BSA. Cells were used on the  
393 day of isolation.

## 394 **Electrophysiological Recordings**

395 Electrophysiological recordings were performed in the whole-cell configuration of the patch-clamp  
396 technique using Axopatch 200B or Multiclamp amplifiers connected to Digidata 1550B interface  
397 (Molecular Devices) and Clampex v11.1 software. For IL-6 group, control SAN cells were  
398 incubated with 10 ng/ml IL-6 for 2 h before recordings. SAN cells were harvested on a custom-  
399 made glass-bottom dish and mounted on the stage of an inverted microscope (Zeiss Axiovert  
400 135) with a 40x/0.75 objective. Pre-warmed (37 °C) Tyrode's solution was perfused near the cell  
401 using a multi-MPRE8 heating pen and cFlow perfusion controller (Cell MicroControls). Only  
402 elongated, spontaneously beating cells were patched with patch pipettes filled with intracellular  
403 solution containing (in mM): 80 K<sup>+</sup>-aspartate; 50 KCl, 3 Na<sup>+</sup>-ATP, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 HEPES,  
404 and 5 EGTA (adjusted to pH 7.2 with KOH), and a tip resistance of 2-3 MΩ. The current was  
405 recorded from a holding potential of -80 mV using a voltage ramp protocol (1.5 s in duration) from  
406 -100 mV to +40 mV (37) before and after perfusion with 0.1 μM CCh, 300 nm tertiapin-Q  
407 (Alomone Labs) or 3 μM ML297 in Tyrode's solution. For measuring  $I_{KACH}$  (22), the current was  
408 recorded at -35 mV holding potential and after reaching a stable current (baseline), 0.1 μM CCh  
409 was added and the current was recorded till it reached a plateau, usually within a few seconds.  
410  $I_{KACH}$  was calculated as the difference between the baseline and the plateau current. Current  
411 density was calculated by dividing the current amplitudes with cell membrane capacitance to  
412 allow comparison among cells of different size.

## 413 **Ca<sup>2+</sup> imaging of SAN tissue**

414 Intact SAN/atrial tissue was isolated from the excised mouse heart in Tyrode's solution  
415 prewarmed to 37°C as described previously (17, 40). The right and the left atria were pinned flat  
416 to the bottom of 35 mm cell culture dish coated with Sylgard (Sylgard 184 Silicone elastomer kit;  
417 Dow Corning) of ~2 mm thickness. The tissue was loaded with 5 μM Cal-520 AM dye (Biozol) and  
418 0.02% Pluronic F-127 (Thermo Fisher Scientific) in the Tyrode's solution at 20-22°C for 1 h in  
419 dark, washed twice with fresh Tyrode's solution and kept for 15 min to allow de-esterification of  
420 Cal-520 AM. The dish with Cal-520-loaded tissue was then mounted on an upright microscope  
421 (Olympus BX51WI) with a 5x/0.75 objective and perfused with Tyrode's solution at 37°C using  
422 dual automatic temperature controller (Warner Instruments). The SAN area was excited with 470  
423 nm LED of Omicron LEDHUB (Omicron-Laserage Laserprodukte GmbH) and the fluorescent  
424 images were captured at 100 frames/s in Visiview v5.0 software (Visitron Systems) using 530/43  
425 nm emission filter (Semrock) with an ORCA-Flash 4.0 v2 CMOS camera (Hamamatsu). The  
426 fluorescence intensity (F) proportional to the intracellular Ca<sup>2+</sup> concentration in the SAN region  
427 was analyzed after background subtraction and normalized to baseline fluorescence  $F_b$  ( $F/F_b$ ).  
428 The Ca<sup>2+</sup>-transient parameters (transients/min, peak amplitude, RT<sub>90</sub>) were calculated using peak  
429 analysis module of LabChart 7 Pro software (ADInstruments).

#### 430 **Western blot**

431 Western blot was performed as described previously (37). SAN tissues were used immediately  
432 after isolation except when incubated with TNF- $\alpha$  (50 ng/mL), IL-1 $\beta$  (10 ng/mL) or IL-6 (10 ng/mL)  
433 (Peprotech) for 6 h. The isolated tissue was homogenized in 50-100  $\mu$ l ice-cold lysis buffer (50  
434 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, pH 7.4 adjusted with  
435 NaOH) containing protease inhibitor cocktail tablet (Roche) with Precellys 24 tissue homogenizer  
436 (2  $\times$  20 s, 6,500 rpm) and then was centrifuged at 13,000 rpm, 4°C for 15 min. Protein estimation  
437 of solubilized tissue lysates was performed using BCA protein assay kit (Thermo Fischer  
438 Scientific). 25-30  $\mu$ g protein was added to 10  $\mu$ l of 4x NuPAGE LDS sample buffer containing 2  $\mu$ l  
439 sample reducing agent (Thermo Fisher Scientific), resolved by electrophoresis in NuPAGE 4–  
440 12% Bis-Tris gel and transferred to nitrocellulose membranes (Thermo Fisher Scientific).  
441 Membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.1%  
442 Tween 20 (TBST, 25°C, 1 h) and cut till 80 KDa protein ladder since all the detected proteins  
443 were below 80 KDa molecular weight and limited amount of working batch of antibodies could be  
444 utilized for incubation. The following primary antibodies (diluted in 5% [w/v] BSA-TBST) were  
445 used for incubation overnight at 4°C: rabbit anti-GIRK1 (1:1,000, Alomone Labs Cat# APC-005,  
446 RRID: AB\_2040113), rabbit anti-GIRK4 (1:500, Alomone Labs Cat# APC-027,RRID:  
447 AB\_2039943), rabbit anti-CHRM2 (1:500, Alomone Labs Cat# AMR-002, RRID: AB\_2039995),  
448 rabbit anti-G $\alpha_{i2}$  (1:2000, GeneTex Cat# GTX101779, RRID: AB\_10721709), and mouse anti-G $\beta$   
449 (1:500, Santa Cruz Biotechnology Cat# sc-166123, RRID: AB\_2109632). For normalization,  
450 membranes were stripped with stripping buffer (58.4 g/L NaCl, 7.5 g/L glycine, pH 2.15 adjusted  
451 with HCl) and incubated with anti-GAPDH (1:10,000, Proteintech Cat# 60004-1-Ig, RRID:  
452 AB\_2107436) as a primary antibody. After washing, the membranes were incubated with HRP-  
453 conjugated goat anti-rabbit IgG (1:20,000, Thermo Fisher Scientific Cat# 31460, RRID:  
454 AB\_228341) or goat anti-mouse IgG (1:10,000, Jackson ImmunoResearch Labs Cat# 115-035-  
455 146, RRID: AB\_2307392) in 4% [w/v] nonfat milk-TBST (25°C, 1 h). Immunoreactive bands were  
456 visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific)  
457 in ChemiDoc MP Imaging System (Bio-Rad) and densitometric evaluation was performed using  
458 Image Lab 6.0 software (Bio-Rad). The relative expression for each protein was calculated by  
459 normalizing to the control samples and GAPDH as a loading control.

#### 460 **Quantitative fluorescence immunolabeling**

461 Fixed cells were immunolabeled for fluorescence imaging as described previously (41). SAN cells  
462 were isolated from 3 control and 3 LPS-injected mice, as described above and plated on poly-l-  
463 lysine-coated glass coverslips with 18 mm diameter at plating density of 25-30 cells/100  $\mu$ l. Two  
464 hours later, the cells were fixed with 4% paraformaldehyde, 4% sucrose in PBS at room  
465 temperature for 10 min. The fixed cells were incubated in 5% normal goat serum in PBS

466 containing 0.1% BSA and 0.2% Triton X-100 (PBS/BSA/Triton) for 30 min. Cells were then  
467 exposed to the primary antibodies in PBS/BSA/Triton at 4°C overnight, washed and incubated  
468 with the fluorochrome-conjugated secondary antibodies at room temperature for 1 h. The primary  
469 antibodies used were rabbit anti-GIRK1 (1:1000, Alomone Labs Cat# APC-005, RRID:  
470 AB\_2040113), rabbit anti-GIRK4 (1:1000, Alomone Labs Cat# APC-027, RRID: AB\_2039943),  
471 guinea-pig anti-Ca<sub>v</sub>1.3 (1:500, Alomone Labs Cat# ACC-005-GP, RRID: AB\_2756614), and  
472 guinea-pig anti-HCN4 (1:500, Alomone Labs Cat# APC-052-GP, RRID: AB\_2340957). The  
473 secondary antibodies goat anti-rabbit Alexa-594 (Thermo Fisher Scientific Cat# A-11012, RRID:  
474 AB\_2534079) and goat anti-guinea-pig Alexa-647 (Thermo Fisher Scientific Cat# A-21450, RRID:  
475 AB\_2735091) were used at 1:4000. Coverslips were mounted in *p*-phenylenediamine glycerol to  
476 retard photo bleaching and observed with a conventional inverted epifluorescence IX3  
477 microscope (Olympus) using a 60x/1.42 apochromat oil-immersion objective lens  
478 (UPLXAPO60XO, Olympus) and SOLA LED solid-state light engine (Lumencor) light source.  
479 Images were acquired with a sCMOS Prime BSI camera (Teledyne Photometrics) using the same  
480 acquisition time for all pictures. Two images were captured for each cell, one at the level of the  
481 cell membrane adhering to the coverslips and another at the longitudinal focal plane passing  
482 through the cell showing the membrane as a rim around the cell.  
483 Quantitative fluorescence analysis was performed with the ImageJ-based open-source software  
484 package Fiji (ImageJ 1.53t) (42). For each SAN cell, membrane and background regions were  
485 selected from the image at the level of the cell membrane adhering to the coverslips using the  
486 polygon selection tool, and mean fluorescence values were measured. Since HCN4 or GIRK1  
487 immunostaining displayed distinct membrane localization, they were used as reference to  
488 observe the membrane and its regions adhering to the coverslip. The membrane regions with the  
489 co-stained GIRK4 and Ca<sub>v</sub>1.3 respectively were co-imaged and analyzed accordingly. Membrane  
490 fluorescence values were background subtracted, obtaining one value for each cell representing  
491 the channel levels at the membrane. Absolute fluorescence values were normalized to the  
492 average fluorescence of control cells. For data presentation, representative membrane regions  
493 were selected and assembled in a composite using Photoshop version 24.1.0.

#### 494 **Statistical analysis**

495 Data were analyzed using IBM SPSS Statistics 27 (IBM Corporation), R version 4.1.0 (2021-05-  
496 18) and RStudio version 1.4.1717. N represents the number of cells or tissues from at least 6  
497 mice unless specified otherwise. The approximate normal distribution of data was assessed by z-  
498 value of skewness and kurtosis, and p-value of Shapiro-Wilk test. If normally distributed and  
499 unless specified otherwise, mixed two-way ANOVA was applied to evaluate interaction and main  
500 effects followed by simple effects. The between-group factor was treatment (Control vs. LPS) and  
501 the within-group, repeated measure factor was baseline (-CCh) vs. CCh response. In case of  
502 pNN6 data, the baseline values in both control and LPS groups were mostly zeroes, thus the

503 difference between baseline and CCh response for control and LPS groups was calculated and  
504 the medians of the delta values were compared by the R function medpb2 as described by Wilcox  
505 in Chapter 7.5.3 (43). This function uses the percentile bootstrap method to account for tied  
506 values. Data from two independent groups were analyzed by unpaired bootstrap t-test. Data from  
507 more than two paired groups were analyzed by repeated measure one-way ANOVA followed by  
508 within group effect analysis with Bonferroni adjustment. Western blot data were analyzed using  
509 one-sample t-test, with reference value for control samples set to one. All tests were two-tailed  
510 and p values < 0.05 were considered significant. Normally distributed data were plotted using  
511 OriginPro 2018 (OriginLab Corporation) as scatter plots along with mean  $\pm$  SEM, otherwise  
512 median with first and third quartile were plotted.

### 513 **Materials and Data Availability**

514 All study data are included in the article and/or supporting information.

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## 627 **Figure legends**

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629 **Fig. 1. LPS attenuates parasympathetic modulation of heart rate and its variability.** Scatter  
630 plots showing mean  $\pm$  SEM for (A) heart rate, (B) SDNN and (C) RMSSD in the absence (-) and  
631 presence (+) of 0.1  $\mu$ M CCh and median with first and third quartile for (D) difference in pNN6  
632 following 0.1  $\mu$ M CCh perfusion in the isolated heart from LPS-injected mice vs controls. N = 6;  
633 \*\*p < 0.01, \*\*\*p < 0.001, <sup>ns</sup>p > 0.05; mixed two-way ANOVA followed by simple effect analysis in  
634 A, B, C; percentile bootstrap based test of equal medians in D.

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636 **Fig. 2. LPS dampens CCh-mediated negative chronotropic effect on Ca<sup>2+</sup> dynamics in SAN**  
637 **tissue.** (A) Representative traces of Ca<sup>2+</sup>-sensitive Cal-520 fluorescence (F/F<sub>b</sub>) depicting  
638 baseline-corrected intracellular Ca<sup>2+</sup>-transients over time in the SAN tissue isolated from control  
639 (left) and LPS-injected (right) mice in the absence (-) and presence (+) of 0.1  $\mu$ M CCh. Scatter  
640 plots showing mean  $\pm$  SEM for (B) Ca<sup>2+</sup>-transients/min, (C) peak amplitude and (D) RT<sub>90</sub> in the  
641 absence (-) and presence (+) of 0.1  $\mu$ M CCh in the isolated SAN tissues from LPS-injected mice

642 vs controls. N = 8; \*p < 0.05, \*\*\*p < 0.001, <sup>ns</sup>p > 0.05; mixed two-way ANOVA followed by simple  
643 effect analysis.

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645 **Fig. 3. LPS inhibits CCh-mediated increase in steady-state current density and  $I_{KACH}$  in**  
646 **isolated SAN cells.** (A, B) Traces showing mean  $\pm$  SEM of current density from -100 to +40 mV  
647 ramp over 1.5 s and (C) scatter plot with mean  $\pm$  SEM for current density at -100 mV in the  
648 absence (-) and presence (+) of 0.1  $\mu$ M CCh in the isolated SAN cells from (B) LPS-injected mice  
649 (N = 6) vs (A) controls (N = 12). (D) Scatter plot with mean  $\pm$  SEM for current density at -100 mV  
650 in isolated control SAN cells (N = 8) perfused with 0.1  $\mu$ M CCh  $\pm$  300 nM tertiapin-Q. (E)  
651 Representative traces and (F) scatter plot with mean  $\pm$  SEM representing change in the  $I_{KACH}$   
652 outward current density at -35 mV holding potential following 0.1  $\mu$ M CCh perfusion in SAN cells  
653 isolated from LPS-injected mice (N = 5) vs control mice (N = 6). \*\*p < 0.01, \*\*\*p < 0.001, <sup>ns</sup>p >  
654 0.05; (C) mixed two-way ANOVA followed by simple effect analysis, (D) repeated measure one-  
655 way ANOVA followed by within group effect analysis with Bonferroni adjustment, (F) independent  
656 samples t-test.

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658 **Fig. 4. LPS reduces expression of ion-channels involved in parasympathetic modulation.**  
659 Representative western blots (left) and bar graphs with mean  $\pm$  SEM of densitometric evaluation  
660 of immunoreactive bands (right) showing reduced expression of GIRK1, GIRK4 and M2R in  
661 mouse SAN tissue after 6 h of (A) LPS i.p. injection in mice (*in vivo*) or (B) LPS incubation (*in*  
662 *vitro*). GAPDH is used as a loading control. (C) Representative immunofluorescence images of  
663 SAN cells isolated from control (above) and LPS-injected (below) mice showing the distribution of  
664 GIRK1, GIRK4,  $Ca_v1.3$  and HCN4 in discrete clusters at the membrane. Scale bar = 5  $\mu$ m. (D)  
665 Bar graphs with mean  $\pm$  SEM showing the fluorescence intensities of membrane-expressed  
666 GIRK1, GIRK4,  $Ca_v1.3$  and HCN4. Data are expressed as symbols in panels B (N = 6  
667 independent experiments) and D (N=31 control cells and 30 LPS cells for co-labeled pair GIRK1,  
668  $Ca_v1.3$ ; 28 control cells and 25 LPS cells for GIRK4, HCN4 pair); \*p < 0.05, \*\*p < 0.01, \*\*\*p <  
669 0.001; <sup>ns</sup>p > 0.05; one-sample t-test in B; unpaired bootstrap t-test in D.

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671 **Fig. 5. Schematic diagram illustrating the impact of LPS-induced sepsis on M2R-GIRK**  
672 **signaling in SAN tissue pacemaking and HRV.** Upon M2R stimulation by a muscarinic agonist,  
673 CCh in SAN cells, the  $\beta\gamma$  subunit of the coupled G-protein directly activates outward  $K^+$  current  
674 ( $I_{KACH}$ ) via inwardly-rectifying potassium (GIRK) channels, consisting of GIRK1 and GIRK4  
675 heteromers, which results in  $K^+$  efflux and membrane hyperpolarization. Further downstream  
676 signaling leads to reduction in  $Ca^{2+}$  mobilization and contractility of SAN tissues and decrease in

677 heart rate and increase in HRV. LPS-induced sepsis reduces expression of M2R, GIRK1 and  
678 GIRK4 channels, which diminishes the above mentioned effects of CCh stimulation.











