Systemic application of the TRPV4 antagonist GSK2193874 induces tail vasodilation in a mouse model of thermoregulation

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Abstract

In humans the skin is a primary thermoregulatory organ, with vasodilation leading to rapid body cooling, whereas in Rodentia the tail performs an analogous function. Many thermodetection mechanisms are likely to be involved including thermosensitive TRPV4 ion channels. Previous studies have shown that TRPV4 can act as a vasodilator by local action in blood vessels, and in this study we investigated whether there was a constitutive role for TRPV4 in *mus muscularis* tail vascular tone and thermoregulation.

We measured tail blood flow and other cardiovascular parameters by pressure plethysmography in lightly sedated CD-1 mice at a range of ambient temperatures, with and without intraperitoneal administration of the blood brain barrier crossing TRPV4 antagonist GSK2193874.

As expected for a thermoregulatory organ, we found that tail blood flow increased with temperature. However, unexpectedly we found that the TRPV4 antagonist GSK2193874 increased tail blood flow.

Since arterial TRPV4 activation is known to cause vasodilation that would increase tail blood flow, these data suggest that increases in tail blood flow resulting from the TRPV4 antagonist must arise from a site other than the blood vessels themselves, perhaps in central cardiovascular control centres such as the hypothalamus.

36 **Introduction**

37 Thermoregulation is one of the key defining homeostatic processes common to mammals.
38 Strict control of temperature is critical to a number of biological processes such as enzyme
39 function and neuronal activity. Core body and brain temperatures are well maintained
40 despite challenges such as changing ambient temperature and exercise to the degree that
41 brain temperature rarely changes outside of a 3 ˚C range [1-3]. Typically, mammals detect
42 temperatures at both central and peripheral sites and responses to changing temperatures
43 can result both from local responses and central, hypothalamus-co-ordinated autonomic
44 responses [4-6]. In turn, a temperature regulating effector system acts to return the core
45 temperature (Tc) towards the set point [7], which changes with time of day [4, 8-10].
46 Typical thermogenic effector mechanisms include liver thermogenesis and skeletal muscle
47 shivering whereas cooling mechanisms include counter-current mechanisms in some
48 species, behavioural changes and redistribution of blood from core to peripheral vessels [4,
49 5, 7]. Brown adipose tissue (BAT) is recognised as the major site of non-shivering
50 thermogenesis during cold exposure and is crucial for maintaining Tc and energy balance
51 [11]. Rodents use basal metabolic rate (BMR) and non-shivering thermogenesis as their
52 principle mechanisms for heat production, mainly because of their small size [12]. In terms
53 of heat loss, transfer of excess heat to the environment is facilitated by so-called heat
54 transfer zones, which are usually found at the body extremities and can be species specific,
55 for example, humans, typically, acute heat loss is mediated by redistributing blood to
56 cutaneous vascular beds [5]. The location of critical heat transfer zones are somewhat
57 species specific however, so for example, the ear for elephants and rabbits [13, 14], head
58 vasculature in large dinosaurs [15, 16], the feet [17] and the tail for rodents [18-20]. The tail
59 of rodents is ideally suited to this role due to its glabrous nature, but unlike the similarly
60 glabrous ear it has the necessary arterio-venous anastomoses [20]. Vasoconstriction and
61 dilatation of the tail arteries are in turn controlled by increases and decreases (respectively)
62 of tail sympathetic nerve activity (SNA) [21]. There is a strong correlation between each of
63 abdominal temperature and tail SNA, skin temperature and tail SNA and brain temperature
64 and tail SNA, but the latter has the steepest correlation slope [21].

65 In this work, we have investigated the role of *mus muscularis* TRPV4 in this homeostatic
66 system using a potent and selective TRPV4 inhibitor, GSK2193874. TRPV4 is one of several
67 temperature sensitive ion channels and expressed in both the hypothalamus and the

68 vasculature; in both smooth muscle and endothelial cells. TRPV4 is a relatively non-
69 selective Ca^{2+} channel (PCa/PNa 6-10) that was first characterised as mechanosensory [22,
70 23]. However, it is also activated by temperatures above approximately 30 ˚C, and so would
71 be expected to be constitutively active, under basal conditions [24]. Nevertheless,
72 activation of TRPV4 leads to vasodilation [25-27]. This is the opposite to what one might
73 expect for activation of a Ca^{2+}/non-specific cation channel in the vasculature, since they may
74 be expected to induce depolarisation which in turn induces contraction of vascular smooth
75 muscle [28]. The apparent anomaly can easily be explained as a relatively conserved
76 intracellular coupling between TRPV4 and Ca^{2+} activated potassium channels, seen in a wide
77 range of cells such as chondrocytes, neurones, endothelial and smooth muscle cells [25, 29-
78 32]. A small influx of Ca^{2+} that is insufficient to cause depolarisation but *is* sufficient to
79 activate nearby Ca^{2+}-activated potassium channels [33] leads to hyperpolarisation and a
80 reduction in over-all Ca^{2+} entry. Additionally, TRPV4 action on endothelial cells has been
81 proposed to induce both direct coupling to vascular smooth muscle cells (and dilatation)
82 and release of endothelially derived relaxant factors such as EDRF/EDHF [25, 26]. There is
83 therefore a consensus amongst several studies that TRPV4 activation is vasodilatory. The
84 logical extension of this body of evidence would be that transgenic elimination of TRPV4
85 (TRPV4-/- knock out) would increase blood pressure, but it does not [25]. Although counter-
86 intuitive data could be explained as resulting from potential compensatory changes of
87 expression in related ion channels, paradoxically, general linear model analyses of the
88 publicly available data from Sand *et al.,* 2015 [10] shows that over a 24hr period mean
89 arterial blood pressure is lower in TRPV4 -/- mice [34]. Furthermore, in terms of
90 temperature regulation, TRPV4 -/- mice do not have altered core temperature. On the
91 other hand, activation of TRPV4 with topological RN1747 decreases core temperature of
92 *rattus norvegicus* and increased tail vasodilatation [35]. The effects of a TRPV4 inhibitor
93 (HC067047) on body temperature, in the same study were mixed.
94 In this study we therefore had a straightforward question; does the small molecule TRPV4
95 inhibitor, GSK2193874, decrease tail vasodilation in response to elevated ambient
96 temperatures. As a surrogate for tail vasodilation we used tail blood flow measured by
97 volume plethysmography [36]. Surprisingly, we found the TRPV4 inhibitor had the opposite
98 effect, raising the possibility that the TRPV4 ion channels expressed outside of the

99 vasculature, for example in the central nervous system, may also be involved with rodent
100 thermoregulation.

Methods

Animals

Fourteen adult CD1-mice (Charles River, UK) were used, kept in our Animal Services unit with *ad libitum* access to food and water. All experimental procedures were performed under a UK Home Office licence (Number 70/8746, approved 09/11/2015) and complied with the UK Animals (Scientific Procedures) Act 1986 and were ethically approved by the University's Animal Welfare Committee (AWERB).

Volume pressure recording (VPR) plethysmography

To investigate the role of TRPV4 in the cardiovascular responses to temperature change, we used the CODA tail volume plethysmography (VPR) system (Kent Scientific, Torrington, CT, USA) on control CD1-mice and mice that had received the selective TRPV4 antagonist GSK2193874. Full details including figures are included in the supplementary materials.

Ambient temperature was increased slowly using heating pads under their housing and insulating the cage lids. Temperature was monitored by thermocouple during the experiment whilst the CODA device was continuously recording.

Statistical Analyses

Blood pressure (MAP), blood flow and heart rate (HR) statistical comparisons were made with the nlme package in R, which incorporates a repeated measures design (using R 4.1.0). For heart rate variability statistical comparions, we used MANOVA in Minitab (version19, Minitab Inc., PA,USA). $p < 0.05$ was taken as significant. The R code and data to repeat these analysis, together with the Minitab workbook are included in the Github repository for this work https://github.com/RichardBJ/TailCuff_Data_Analysis.

Drugs

Midazolam was supplied by our animal service unit, but GSK2193874 (300 µg/kg, *i.p.*) and DMSO were obtained from Sigma-Aldrich. GSK2193874 was dissolved in DMSO at 20 mg/ml stock then diluted 1:100 before i.p. injection (0.2 mg/ml). "Control" therefore includes 1% DMSO, and volume of injection was dependent upon the weight of the mouse.

130 **Results**

131 Using the VPR equipment we measured blood pressure (MAP), heart rate (HR) and blood
132 flow (Flow) in 14 animals with and without GSK2193874 over the ambient temperature
133 range of 31C to 36C. These are plotted in two-factor (treatment, temperature) format and
134 analysed with a repeated measures design. There was a statistically significant effect of
135 temperature on all parameters measured, MAP, HR and tail blood flow (Figure 1). I the
136 cases of HR and MAP there was no significant effect of treatment with the TRPV4 antagonist
137 (GSK2193874). However, with tail blood flow there was both a significant increase with
138 GSK2193874 treatment and a very highly significant interaction between temperature and
139 GSK2193874 treatment (Figure 1C).

140 Since we were able to derive beat-by-beat heart rate records for several seconds (for
141 example Supplementary Figure 1), we investigated whether heart rate variability (HRV)
142 could be captured over such a short period. To test whether this was feasible, we simulated
143 mouse heart rate interval records of decreasing length using a modified version of McSharry
144 *et al.,* 2003 [37] and then measured HRV spectral powers by the Lomb-Scargle method [38,
145 39] for these over 3000 simulations. Figure 2A shows that just a few seconds of ECG are
146 sufficient to obtain a picture of the heart rate variability in a mouse, in so far as, increasing
147 the simulation duration beyond this does not greatly affect the HRV spectra. We therefore
148 measured HRV power in the 0.1 to 1.9Hz bands in our samples of control and GSK2193874
149 records (Figure 2B, C) and compared these statistically with a MANOVA model, over our full
150 range of temperatures. There was no over-all statistical difference with temperature,
151 however there was a statistically different set of spectra between control and GSK2193874
152 treated spectra. Furthermore, with univariate analyses, there was a significant difference
153 between treatment and control at each individual frequency except the 0.5Hz banding.

154

155 **Discussion**

156 In this work we investigate the role of TRPV4 in rat tail blood flow with a systemic inhibitor
157 of TRPV4, GSK2193874 using the CODA tail volume plethysmography (VPR) system.
158 Surprisingly, we find that tail blood flow is increased by GSK2193874. There was a
159 detectable effect also on HRV, but no significant change in blood pressure or heart rate
160 itself.

161 Using a potent drug inhibitor rather than constitutive transgenic knock down of TRPV4 has
162 the advantage that, realistically, in the time frame of an experiment there will be no
163 compensatory expression of functionally related proteins. The trade-off is selectivity. Whilst
164 GSK2193874 is considered highly selective for TRPV4 compared to the other 200+ proteins it
165 has been assayed against [40], there could still be unknown off target effects. GSK2193874
166 is a small lipid soluble inhibitor of TRPV4 [41] that crosses the blood-brain barrier well
167 (brain:plasma ratio = 0.6, personal communication with Dr David Behm of GSK) and so there
168 are several locations at which TRPV4 could potentially influence the control of blood flow in
169 response to elevated temperatures. A non-exhuastive list of possible sites of action could
170 include the vasculature, sensory neurones or cardiovascular control neurones; we will now
171 discuss each of these in turn.
172 The best studied of these potential sites of action is perhaps the vasculature itself. TRPV4 is
173 expressed in both vascular smooth muscle and the endothelial cell lining [42]. Activation of
174 these channels leads to clear vasodilatation. Whilst the mechanism of this vasodilatation is
175 complex, involving both endothelial cells and smooth muscle cells, potential release of local
176 transmitters and tight junctions, ultimately, small local increases of Ca^{2+} activate potassium
177 channels which hyperpolarize the muscle cells and allow relaxation/vasodilatation [25-27].
178 If there were to be no constitutive activity of TRPV4, an inhibitor would have little effect on
179 tail blood flow via this site of action, if however, there is constitutive activity, an inhibitor
180 would be expected to promote channel closure, vasoconstriction and decreased tail blood
181 flow. It therefore seems unlikely the increase in tail blood flow we report in this study
182 results from direct action on the vasculature. Furthermore, if the effect of GSK2193874
183 were simply on blood vessels to cause dilation, we would have expected to see an over-all
184 drop in blood pressure, and possibly then a reflex increase in heart rate. We saw no change

185 in blood pressure or heart rate, although multivariate analysis detected a small change in
186 short-range HRV analysis.
187 A second common site of action of TRPV4 channels is sensory neurones [43, 44] and have
188 been implicated in temperature nocifensive responses, but only in situations of heat
189 hyperalgesia [45]. Escape latencies (the time it takes before animals avoid a noxious stimuli)
190 at temperatures of 35-50˚C are unaltered in TRPV4 -/- knock-out mice, but these were
191 delayed following induction of heat hyperalgesia. Together, these data offer no evidence
192 that the TRPV4 inhibitor vasodilating effect we see is likely due to modulation of sensory
193 neurones.
194
195 A third location of TRPV4 channels that may be of relevance is the central nervous system,
196 for example the hypothalamus [46]. It is known that other transient receptor potential
197 channels influence the cardiovascular system via changes in sympathetic activity [8, 47].
198 Our own work shows that TRPV4 channels are located on pre-autonomic neurones of the
199 hypothalamic paraventricular nucleus (PVN) and can influence cardiovascular control in
200 response to osmotic challenge [32, 48]. We have shown that intracerebroventricular (ICV)
201 injection of hypoosmotic solution decreased blood pressure but not heart rate, and this
202 effect was abolished with a TRPV4 inhibitor [32]. At the neuronal level, we have shown that
203 the action current frequency of parvocellular PVN neurones is dramatically reduced when
204 TRPV4 channels are blocked [48]. To date, there have been no studies that have explored
205 thermoregulatory roles for TRPV4 in central cardiovascular control neurones such as those
206 found within the PVN. The changes we observe here in flood flow, HRV and
207 thermoregulatory would be consistent with modulation of central control neurones.
208
209 This is the first recorded use of HRV analysis from such short segments of heart rate
210 recording and its measurement on non-invasive apparatus may prove a useful advance in
211 3Rs. We justify this in two ways; (i) since the average mouse heart rate is some 8 times that
212 of a human, (i) an 8s segment would be equivalent to the standard 1 minute of recording
213 necessary to detect higher frequency components of human ECG [49]. (ii) There is no clear
214 law that states how long a segment should be to faithfully recover a given frequency
215 component, since this is the *opposite* of the Nyquist theorem which sets the minimum
216 sample frequency. The landmark cardiac Electrophysiology Task-Force report stated that

"recording should last for at least 10 times the wavelength of the lower frequency" [49], but without evidence. Here, simulation shows that periodograms from very short segments of ECG are similar to that of conventional 1-minute records (Figure 3A), and these data themselves and this approach may be of field interest.

In conclusion, this whole animal study shows that a TRPV4 antagonist has a significant effect on tail blood flow, in the context of thermoregulation, but its site of action, and the mechanism of such modulation remain to be determined. We also demonstrate non-invasive measurement of frequency domain HRV Lomb-Scargle analysis from very short-range data that may prove useful in future 3Rs friendly research.

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Figure Legends

Figure 1. Effects of Pharmacological inhibition of TRPV4 on blood pressure, heart rate and blood flow.

(A) Mean arterial pressure at a range of ambient temperatures in control and GSK2193874, there was a significant association with temperature, but not drug. There was also no significant association between temperature and drug (Mixed effects model: Temperature $F=5.34$, p-val ≤ 0.05; drug $F=0.38$ p-val > 0.05, Drug x Temperature $F=0.17$, $p>0.05$). **(B)** Mean heart rate across a range of ambient temperature in control and GSK2193874, overall there was a significant change of heart rate with temperature, but no significant difference with drug and no significant association between drug and temperature (Mixed effects model: Temperature $F=7.37$, p-val ≤ 0.05; drug $F=0.68$ p-val > 0.05, Drug x Temperature $F=0.23$, $p>0.05$). **(C)** Tail blood flow across a range of ambient temperature in control and GSK2193874. Overall, there was a statistically significant increase of blood flow with temperature and with GSK2193874 and a significant interaction (Mixed effects model: Temperature $F=13.21$, p-val ≤ 0.005; drug $F=5.57$, p-val ≤ 0.05, Drug x Temperature $F=14.00$, p-val ≤ 0.0005). Overall, $n = 14$ animals.

Figure 2. Short range heart rate variability analysis. (A) Stacked Lomb periodograms for 3000 simulated ECG inter-beat interval datasets. Y-axis is the duration of the simulated ECG record, X-axis is the frequency component of each Lomb-Scargle. The periodograms have been normalized and scaled therefore the power (colour bar on the right) is in arbitrary units. Below are periodogram surfaces recorded at different temperatures under control **(B)**, or after injection GSK2193874 **(C)**. Power given in the scale-bars. MANOVA (Minitab) analyses show these two distributions are significantly different, Wilks lambda $p<0.05$. Overall, $n = 14$ animals.

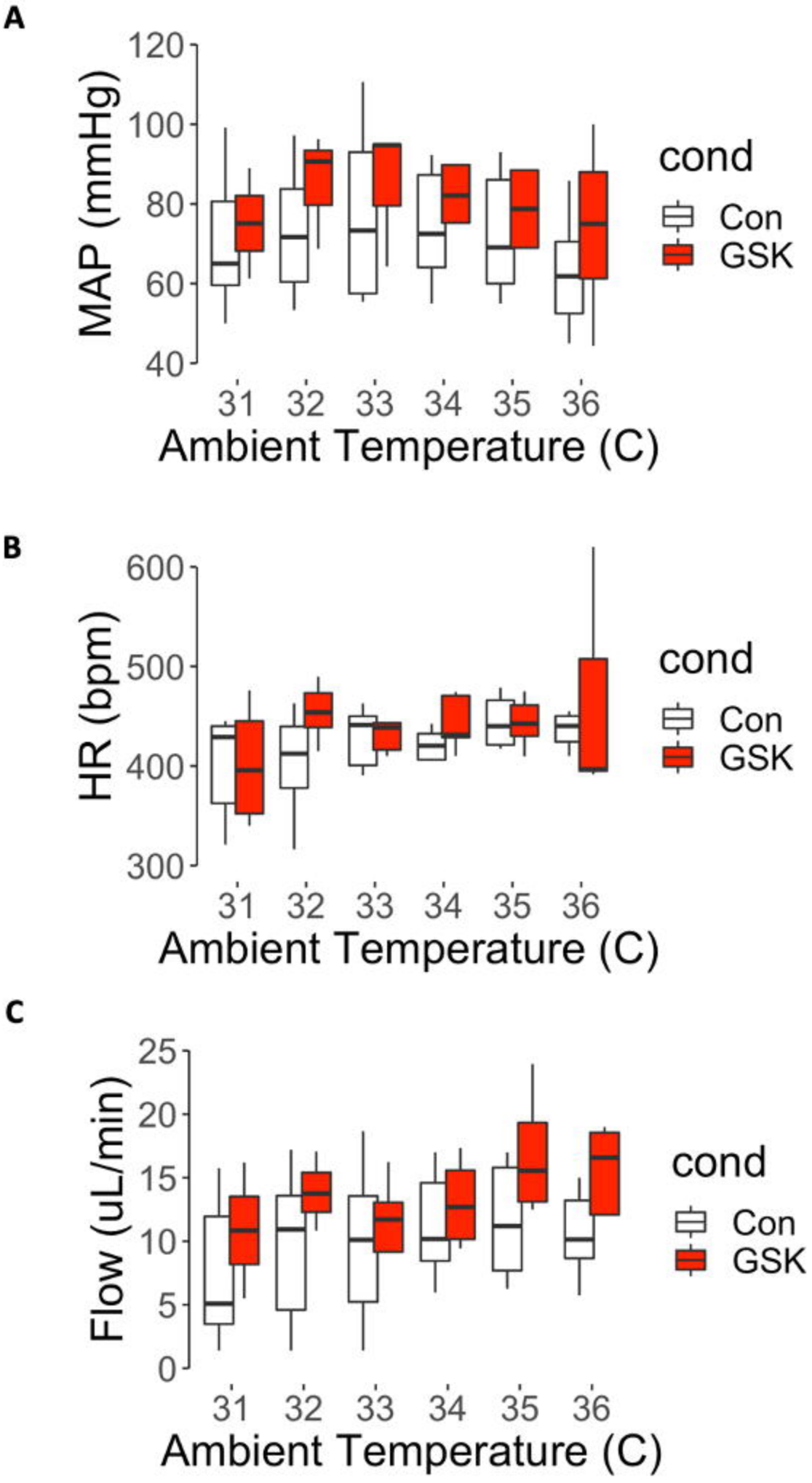
A
MAP (mmHg)
120
100
80
60
40
31
32
33
34
35
36
Ambient Temperature (C)
cond
Con
GSK
B
HR (bpm)
600
500
400
300
31
32
33
34
35
36
Ambient Temperature (C)
cond
Con
GSK
C
Flow (uL/min)
25
20
15
10
5
0
31
32
33
34
35
36
Ambient Temperature (C)
cond
Con
GSK

Figure 1

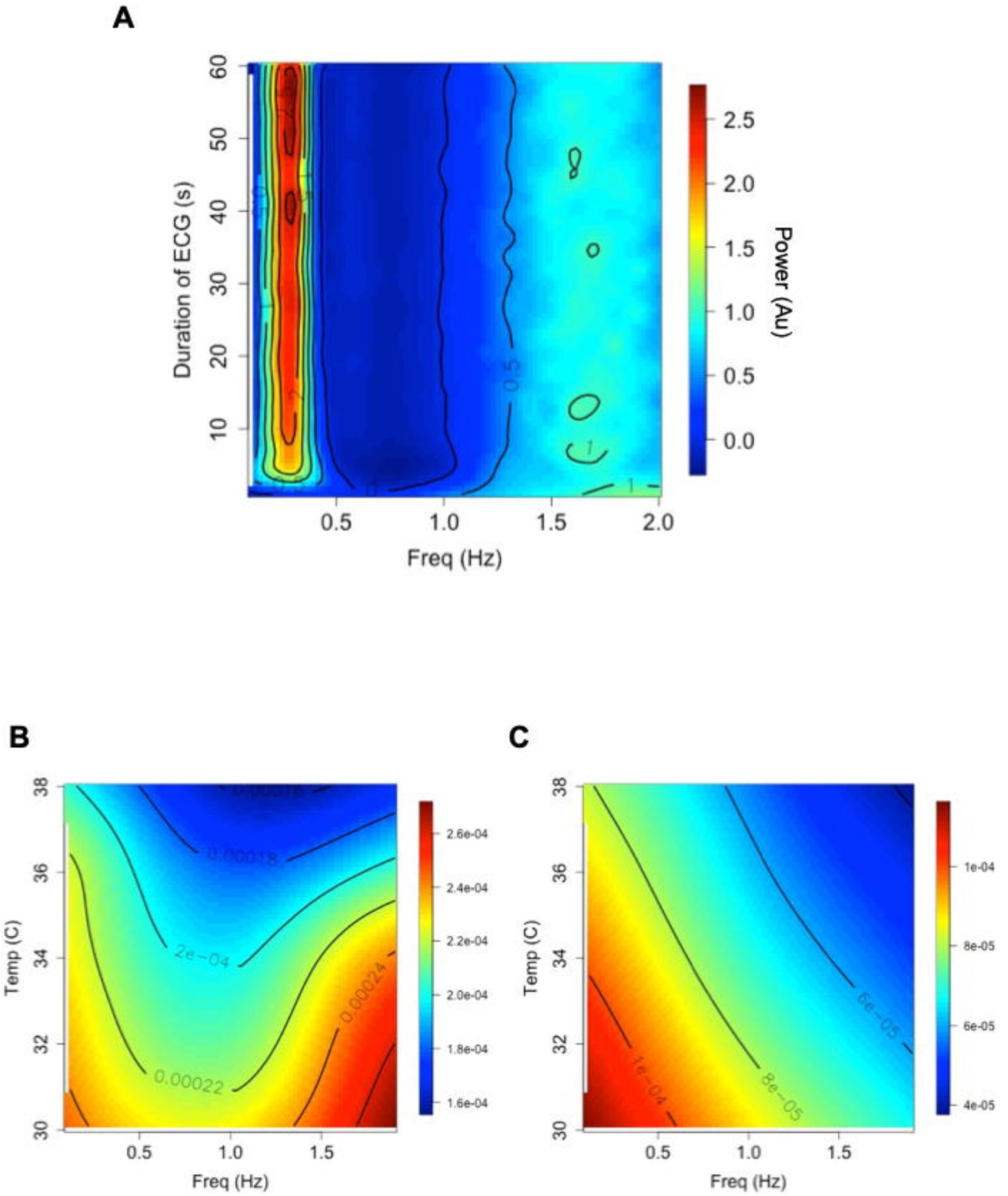
A
Duration of ECG (s)
Freq (Hz)
Power (Au)
B
Temp (C)
Freq (Hz)
C
Temp (C)
Freq (Hz)

Figure 2