Systemic application of the transient receptor potential vanilloid-type 4 antagonist GSK2193874 induces tail vasodilation in a mouse model of thermoregulation

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In humans, 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 transient receptor potential vanilloid-type 4 (TRPV4), an ion channel with thermostensitive properties. Previous studies have shown that TRPV4 is a vasodilator by local action in blood vessels, so here, we investigated whether constitutive TRPV4 activity affects Mus muscularis tail vascular tone and thermoregulation. We measured tail blood flow by pressure plethysmography in lightly sedated M. muscularis (CD1 strain) at a range of ambient temperatures, with and without intraperitoneal administration of the blood–brain barrier crossing TRPV4 antagonist GSK2193874. We also measured heart rate (HR) and blood pressure. As expected for a thermoregulatory organ, we found that tail blood flow increased with temperature. However, unexpectedly, we found that GSK2193874 increased tail blood flow at all temperatures, and we observed changes in HR variability. Since local TRPV4 activation causes vasodilation that would increase tail blood flow, these data suggest that increases in tail blood flow resulting from the TRPV4 antagonist may arise from a site other than the blood vessels themselves, perhaps in central cardiovascular control centres.

1. Introduction
Thermoregulation is one of the defining homeostatic processes common to mammals; core body and brain temperatures are well maintained despite challenges such as changing ambient temperature and exercise to the degree that brain temperature rarely changes outside of a 3°C range [1–3]. Mammals detect temperatures at both central and peripheral sites and responses to changing temperatures can result both from local responses and central, hypothalamus-coordinated autonomic responses [4–6]. Typical thermogenic effector mechanisms include liver thermogenesis and skeletal muscle shivering whereas cooling mechanisms including behavioural changes and redistribution of blood from core to peripheral vessels [4,5,7]. Rodents use basal metabolic rate and non-shivering thermogenesis as their principle mechanisms for heat production, mainly because of their small size [8]. In terms of heat loss, transfer of excess heat to the environment is facilitated by so-called heat transfer zones, which are usually found at the body extremities, for example, in humans, typically, acute
heat loss is mediated by redistributing blood to cutaneous vascular beds [5]. The location of critical heat transfer zones are somewhat species specific, so for example, the ear for elephants and rabbits [9,10], head vasculature in large dinosaurs [11,12] and the feet [13] and tail for rodents [14–16]. The tail of rodents is ideal as a heat transfer zone due to its glabrous nature [16]. It is thought that vasoconstriction rather than counter-current heat exchange provides the major barrier to core-to-tail heat flow [17].

In this work, we have investigated the role of Mus musculus (mouse) transient receptor potential vanilloid-type 4 (TRPV4) in this homeostatic system using a potent and selective TRPV4 inhibitor, GSK2193874. TRPV4 is one of several temperature-sensitive ion channels and expressed in both the hypothalamus and the vasculature, in both smooth muscle and endothelial cells. Recently, there has been considerable interest in the immune, neuromodulatory, cardiovascular and thermoregulatory potential of small molecule TRPV4 modulatory drugs, such as GSK2193874 and HC-067047 [18,19].

TRPV4 is a relatively non-selective Ca\(^{2+}\) channel (PCa/PNa 6–10) that was first characterized as mechanosensory [26,27]; however, it is also activated by temperatures greater than 30°C, and so, at physiological temperatures, it would be expected to be constitutively active under basal conditions [28–30]. Activation of TRPV4 leads to vasodilation [31–34] and logically, therefore, transgenic elimination of TRPV4 (TRPV4 \(-/-\) knock out) would be expected to increase blood pressure, but it does not [18,31].

The precise contribution of TRPV4 to thermosensing and thermoregulation in vivo remains unclear. No changes in escape latency from heat stimuli were observed in the hotplate challenge [35,36]. However, post-subcutaneous injection of capsaicin or carrageenan, TRPV4 \(-/-\) mice showed longer escape latencies from the hot surface compared to wild-type [36]. In another study, it was shown that TRPV4 is required for normal thermal responsiveness in vivo; on a thermal gradient, TRPV4 \(-/-\) mice selected warmer floor temperatures. In addition, TRPV4 \(-/-\) mice also exhibited prolonged withdrawal latencies during acute tail heating [37].

In terms of pharmacological manipulations, the activation of TRPV4 with topological RN1747 decreased the core temperature of Rattus norvegicus and increased tail vasodilation [38]. The effects of a TRPV4 inhibitor (HC067047), in the laboratory animals, was also observed in the control CD1 mice and mice that had received the selective TRPV4 antagonist GSK2193874. Full details of warming methodology and VPR methods are included in the electronic supplementary material. Note, all temperatures reported are ambient temperatures read from the thermocouple.

2. Methods

Extended methods are included in the electronic supplementary material, information, but briefly:

(a) Animals

Fourteen female adult CD1-mice (Charles River, UK) were used. All experimental procedures were ethically approved by the University’s Animal Welfare Committee and performed under a UK Home Office Scientific Procedures licence (70/8746).

(b) Volume pressure plethysmography recording

We used the CODA tail volume pressure plethysmography (VPR) system (Kent Scientific, Torrington, CT, USA) on control CD1-mice and mice that had received the selective TRPV4 antagonist GSK2193874. Full details of warming methodology and VPR methods are included in the electronic supplementary material. Note, all temperatures reported are ambient temperatures read from the thermocouple.

(c) Statistical analyses

Blood pressure (MAP), HR and blood flow statistical comparisons were made with the mlmle package in R, which incorporates a repeated-measures design. For HRV statistical comparisons, we used MANOVA in Minitab (PA, USA). \(p \leq 0.05\) was taken as significant.

(d) Drugs

A sedative (midazolam 5 mg kg\(^{-1}\), i.p.) was supplied by our animal service unit and administered prior to recording. GSK2193874 (300 mg kg\(^{-1}\), i.p.) and DMSO were obtained from Sigma-Aldrich. GSK2193874 was dissolved in DMSO at 20 mg ml\(^{-1}\) stock then diluted 1:100 before i.p. injection (0.2 mg ml\(^{-1}\)), following [23,34]. ‘Control’ includes 1% DMSO and volume of injection was dependent upon animal weight.

3. Results

We measured MAP, HR and blood flow (Flow) in 14 animals with and without GSK2193874 over the ambient temperature range of 31°C to 36°C. These are plotted in two-factor (treatment and temperature) format and analysed with a repeated-measures, mixed effects design. There was a statistically significant effect of temperature on all parameters measured, MAP (figure 1a: temperature \(F = 5.34, p \leq 0.05\), drug \(F = 0.38, p > 0.05\), drug \(\times\) temperature \(F = 0.17, p > 0.05\)), HR (figure 1b: temperature \(F = 7.37, p \leq 0.05\), drug \(F = 0.68, p > 0.05\), drug \(\times\) temperature \(F = 0.23, p > 0.05\)) and tail blood flow (figure 1c: temperature \(F = 13.21, p \leq 0.005\), drug \(F = 5.57, p \leq 0.05\), drug \(\times\) temperature \(F = 14.00, p \leq 0.0005\)). In the cases of HR and MAP, there was no

\[ F \leq F_{p,0.05} \]
Since we were able to derive beat-by-beat HR records for several seconds (for example electronic supplementary material, figure S1), we investigated whether HRV could be captured over such short periods. To test whether this was feasible, we simulated mouse HR interval records of decreasing length using a modified version of McSharry et al. [46] and then measured HRV spectral powers using the Lomb–Scargle method [47,48] over 3000 simulations. Figure 2a shows that just a few seconds of ECG are sufficient to obtain a picture of the HRV in a mouse, in so far as, increasing the simulation duration beyond this does not greatly affect the HRV spectra. We therefore measured HRV power in the 0.1 to 1.9 Hz bands in our samples of control and GSK2193874 records (figure 2b,c) and compared these statistically with a MANOVA model, over a range of temperatures. There was no overall statistical difference with temperature; however, there was a statistically different set of spectra between control and GSK2193874-treated spectra. Furthermore, with univariate analyses, there was a significant difference between treatment and control at each individual frequency except the 0.5 Hz banding.

4. Discussion
In this work, we investigate the role of TRPV4 in mouse tail blood flow with a systemic inhibitor of TRPV4, GSK2193874. Surprisingly, we find that tail blood flow is increased by GSK2193874. There was also a detectable effect of GSK2193874 on HRV, but no significant change in blood pressure or HR.

(a) Blood flow, heart rate and blood pressure effects
GSK2193874 is a small lipid-soluble inhibitor of TRPV4 [19] that crosses the blood–brain barrier well (brain: plasma ratio = 0.6, personal communication with Dr David Behm of GSK) and so there are several locations at which TRPV4 could potentially influence the control of blood flow in response to elevated temperatures. A non-exhaustive list of possible sites of action could include the vasculature or cardiovascular control neurons.

TRPV4 is expressed in both vascular smooth muscle and the endothelial cell lining [49]. Activation of these channels leads to vasodilatation. It is difficult to assess the mechanism of this vasodilatation without a full dose–response curve (DRC, see Limitations). However, it is likely to involve both endothelial and smooth muscle cells, potential release of endothelial relaxation or hyperpolarization factors and, ultimately, small local increases of Ca$^{2+}$ activate potassium channels which hyperpolarize the muscle cells and allow relaxation/vasodilatation [31–33]. A TRPV4 inhibitor would therefore be expected to cause vasodilation (or have no effect if there was no constitutive TRPV4 activity) and so it seems unlikely the increase in tail blood flow we report in this study results from direct action on the vasculature. Furthermore, if the effect of GSK2193874 were primarily on blood vessels to cause dilation, we would have expected to see an overall drop in MAP and possibly then a reflex increase in HR since the baroreceptor loop features in established mechanisms of cardiovascular control as well as, specifically, thermoregulation [50,51]. We saw no change in blood pressure or HR, although multivariate analysis detected a small change in short-range HRV analysis. The potential for
us to have missed such a baroreceptor-mediated effect due Type II errors is discussed in the Limitations section below.

A second location of TRPV4 channels that may be of relevance is the central nervous system, for example the hypothalamus [52]. It is known that other transient receptor potential channels influence the cardiovascular system via changes in sympathetic activity [53,54]. Our previous work shows that TRPV4 channels are located on pre-autonomic neurons of the hypothalamic paraventricular nucleus (PVN) and can influence cardiovascular control in response to osmotic challenge [55,56], and this effect was abolished with a TRPV4 inhibitor [55]. At the neuronal level, we have shown that the action current frequency of parvocellular PVN neurons is dramatically reduced when TRPV4 channels are inhibited [56]. To date, there have been no studies that have explored thermoregulatory roles for TRPV4 in central cardiovascular control neurons.

(b) Heart rate variability effects

HRV analysis is an increasingly common method for cardiovascular assessment. In humans, for example, decreased HRV (i.e. a very steady pulse) is an independent predictor of cardiac mortality [57]. In animals too, it is proving increasingly useful in a range of contexts including phenotyping transgenic animals [58], investigating cardiovascular effects of drugs [59] and predicting arrhythmias [60]. While there are many papers analysing HRV in mice using radiotelemetry [61], we investigated here whether it was possible to do this with VPR and found that it was. It has previously been shown that relatively long photoplethysmography recordings could be used for HRV [44], with high accuracy, but the present study is the first to systematically analyse how long a recording needs to be. The derivation of this short-range HRV from non-invasive apparatus may prove a useful advance in 3Rs. In the electronic supplementary material, information, we compare (qualitatively) data with our previous telemetric study [62]. Since the average mouse HR is approximately eight times that of a human, an 8 s segment would be equivalent to the standard 1 min of recording necessary to detect higher frequency components of human ECG [41]. Here, simulation shows that periodograms from very short segments of ECG are similar to that of conventional 1 min records (figure 2a), and these data themselves and this approach may be of field interest.

In terms of the response to temperature, we did not see an overall effect on HRV, probably because temperature typically affects the low-frequency powers, beyond the scope of ultra-short-range recording [45]; however, GSK2193874 did significantly alter overall frequency power curves.

(c) Limitations

We measured only ambient temperature and not core temperature. We felt that the loss of this important information

Figure 2. Short-range HRV 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 (AU). Below are periodogram surfaces recorded at different temperatures under control (b), or after injection GSK2193874 (c). Power is given in the scale bars. MANOVA (Minitab) analyses show these two distributions are significantly different, Wilks lambda p < 0.05. Overall, n = 14 animals.
was necessary to avoid the disturbance of using a rectal ther-
mcouple on mice in the non-invasive recording equipment.
Also, we used sedation that could influence the whole animal
responses and only female mice, unlike many male only
studies [23,34]. Furthermore, to keep the study manageable,
we opted for a one antagonist dose study rather than a full
in vivo DRC, which would have been useful. It is difficult
to predict accurately the local concentration that an ion ch-
nel will ‘see’ when a drug given systemically will reach, but
if we assume that GSK2193874 has a typical volume of distri-
bution of between 1 and 101 kg⁻¹, our 300 μg kg⁻¹ dose
would translate to approximately 40 to 400 nM, in the order
of the maximal dose for GSK2193874 on TRPV4 channels
[23]. Although GSK2193874 is highly selective for TRPV4
compared to the other 200 + proteins, it has been assayed
against [23], repeating our studies with TRPV4⁻/⁻ lines [31]
would be the only way to confirm with certainty that the
true target was indeed TRPV4.

We encountered technical challenges too, e.g. recording
VPR data below 30°C (ambient) was unreliable, so we
report a relatively limited temperature range rather than
strictly hot versus cold. These limitations could be addressed
by a telemetric study, but large motivation for our current
approach was to use a non-invasive blood pressure design,
for 3Rs ethical reasons. Furthermore, as in many physio-
logical studies, statistical power was an issue. Our initial
design (see electronic supplementary material, information)
included a power analysis for HR and blood pressure,
which made a number of assumptions but passed 80% power
with around eight or nine animals. We then used 14,
however, we were not able to get all conditions for all animals
and so the final statistical power could be below 80%. We
have hypothesized that an increase of blood flow, by
TRPV4 antagonist in the absence of significant changes in
MAP/HR would be compatible with a central mechanism
of vasodilation. However, if we simply missed changes due
to a type II error, the vasodilation could result from baroreflex-mediated mechanisms. This could be addressed
by either increasing animal numbers or by repeating similar
experiments with surgical or pharmacological block of the
baroreceptor reflex [63]. See electronic supplementary
material, information for further discussion.

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 deter-
mined. We also demonstrate non-invasive measurement of
frequency-domain HRV analysis from very short-range data
that may prove useful in future 3Rs friendly research.

Ethics. All experimental procedures were ethically approved by the
University’s Animal Welfare Committee and performed under a
UK Home Office Scientific Procedures licence (70/8746).

Data accessibility. Data are available from the Dryad Digital Repository
at https://doi.org/10.5061/dryad.rn5pk0vq [64].

The data are provided in the electronic supplementary material [65].

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All authors gave final approval for publication and agreed to be
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